Development of spirulina for the manufacture and oral delivery of protein therapeutics

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The use of the edible photosynthetic cyanobacterium Arthrospira platensis (spirulina) as a biomanufacturing platform has been limited by a lack of genetic tools. Here we report genetic engineering methods for stable, high-level expression of bioactive proteins in spirulina, including large-scale, indoor cultivation and downstream processing methods. Following targeted integration of exogenous genes into the spirulina chromosome (chr), encoded protein biopharmaceuticals can represent as much as 15% of total biomass, require no purification before oral delivery and are stable without refrigeration and protected during gastric transit when encapsulated within dry spirulina. Oral delivery of a spirulina-expressed antibody targeting campylobacter—a major cause of infant mortality in the developing world—prevents disease in mice, and a phase 1 clinical trial demonstrated safety for human administration. Spirulina provides an advantageous system for the manufacture of orally delivered therapeutic proteins by combining the safety of a food-based production host with the accessible genetic manipulation and high productivity of microbial platforms.

Modern biotechnology relies on the domestication of cells as biological factories through genetic engineering. Expression platforms include Escherichia coli, used to manufacture relatively small and simple therapeutic proteins, and yeasts and mammalian cells for more complex molecules. Adoption of new expression platforms depends on the availability of methods for genetic manipulation of the organism to achieve stable, high expression of exogenous proteins, and on whether the organism possesses biological traits compatible with large-scale manufacturing and commercialization. Genetically engineered plants have performance characteristics different from cultured cells, such as photosynthetic growth and easy scalability. However, their promise has not been realized for reasons including cumbersome genetic methods, slow growth rates, low product yields and regulatory constraints. Algae have been considered as alternatives to plants for biotechnology applications, but are difficult to engineer genetically and expression levels of exogenous protein are low and often unstable. To date, no biologic therapeutic has been commercialized using an algal platform.

Photosynthetic spirulina is the only microorganism that is commercially farmed worldwide as a food. Its protein content exceeds that of all other food crops, making it a strong candidate for the expression of therapeutic proteins at high levels. Spirulina’s asexual reproduction mitigates the risk of gene escape into the food chain and the associated food security concerns and regulatory burden. Spirulina therefore promises the benefits of plant-based biopharmaceuticals and may overcome the challenges and limitations of other crop- and algal-based platforms.

Here we report our discovery of versatile genetic engineering methods for spirulina that include integration of exogenous genes into the spirulina chromosome by markerless homologous recombination, and stable, high-level expression of therapeutic proteins including bioactive peptides, single-chain antibodies, enzymes, signaling proteins and vaccine antigens. We describe the development of indoor cultivation technology for the large-scale manufacturing of biopharmaceuticals in spirulina under current good manufacturing practices (cGMP). We further report the development of an edible, antibody-based therapeutic targeting gastrointestinal infection by Campylobacter jejuni to illustrate application of the spirulina platform to an important unmet medical need, including validation in animal models of campylobacteriosis and demonstration of its safety and pharmacokinetics in a human phase 1 clinical trial.

Results

Spirulina is naturally competent for transformation. Our experimental analyses showed that spirulina is naturally competent
for transformation, despite being viewed as refractory to genetic manipulation\textsuperscript{18,19}. Transformation competence was achieved by cocultivation of spirulina with companion microorganisms (below). Competent spirulina (UTEX LB1926 and NIES-39) were exposed in liquid culture to an integrating DNA vector containing a selectable marker and a gene of interest, flanked on both sides by sequences homologous to the spirulina chromosome. Targeted integration of the transforming DNA by homologous recombination was demonstrated by sequencing of chromosomal DNA using primers flanking the insertion site (Fig. 1a,b). This transformation method yielded a pool of approximately 100 independent transformants, as determined by next-generation sequencing of a culture that had been exposed to a library of barcoded integrating DNA vectors. Consistent with these observations, the 15 genes associated with the pathway for natural competence in cyanobacteria are present as complete open reading frames in most available Arthrospira genomes (Extended Data Fig. 1).

Segregation of the transgene to homozygosity in polyploid spirulina occurred 8–10 weeks after transformation under continuous selection (Fig. 1c). Once homozygous, PCR and DNA sequencing showed that the transgene was genetically stable. No changes in transgene DNA sequence were found in nine of nine strains continuously propagated for at least 1 year, and two of two grown for 3 years (>800 cell generations) (Fig. 1d and Methods). Clonal derivatives were recovered by microisolation of individual spirulina filaments and were verified as containing a single insertion per chromosome by comparison to an endogenous gene using quantitative PCR (qPCR) (Extended Data Fig. 2).

**Induction of spirulina competence.** Spirulina UTEX LB1926 (UTEX culture collection) was not axenic, but microisolation of single filaments was used to prepare axenic strains. Eight of 11 single filaments produced axenic cultures, as verified by microscopic examination and confirmed by cultivation on lysogeny
proteins present in spirulina extracts (SP1464 and SP1477, respectively). The concentration of VHH in the spirulina extracts was determined by CeIA.

Purified monomeric (PP622) and dimeric (PP661) forms of RBD-binding VHH were assayed with RBD and compared with the binding activity of identical apparent binding activity by dimerization of VHHs, as measured by eLISA with purified VHH (top) and spirulina extract (bottom).

E. coli, and its deletion is associated with the presence of other microorganisms.

Two microorganisms in the original xenic UTEX LB1926 culture were clonally isolated and identified as belonging to the genera Sphingomonas and Microcella (Supplementary Table 1). Twelve individual colonies were cocultured in liquid medium with axenic UTEX LB1926. The axenic spirulina strain remained nontransformable, suggesting that competence for transformation is associated with the presence of other microorganisms.

A markerless method for engineering of spirulina. The site of integration was determined by 1.0–1.5-kb homology arms flanking the transforming genes. A single homology arm was ineffective, indicating that integration occurs by double crossover, as is typical for natural transformation. Six of the 11 tested sites of integration accommodated an insertion with no associated growth deficit.

One insertion site corresponded to NCBI reference sequence NIES39_RS07765, which codes for a protein homologous to kanamycin aminoglycoside acetyltransferases (hereafter referred to as KmR). It conferred kanamycin resistance in E. coli, and its deletion rendered spirulina kanamycin sensitive. This allowed development of a markerless engineering strategy by replacement of the KmR gene with an exogenous streptomycin resistance gene (aadA), followed by replacement of the exogenous aadA gene with a tandem gene cassette comprising the KmR gene and the gene of interest. This strain contained the gene of interest integrated adjacent to the KmR gene and no other exogenous DNA (Fig. 1e).

We introduced a variety of exogenous DNA vectors into the spirulina genome, including single genes, tandem genes, operons and sequential engineering of different insertion sites (Supplementary Table 2). The largest transforming DNA cassette was 6.0 kb and contained a seven-gene C-phycocyanin operon. Exogenous proteins were expressed uniformly in spirulina filaments (Extended Data Fig. 3). We demonstrated stable intracellular expression of diverse exogenous proteins, including bioactive peptides, antigen-binding domains (VHHs), protein pigments and enzymes (Extended Data Fig. 3). Proteins expressed using a strong, constitutive promoter from the C-phycocyanin locus (Pcpc600) accumulated to represent up to 29% of total soluble protein (Extended Data Fig. 3e).

Expression of single-chain antibody fragments in spirulina. Antigen-binding domains (VHHs) from camelid single-chain antibodies are expressible in prokaryotes like spirulina. Intracellular VHHs were constitutively expressed in various formats, including monomers, dimers, trimers and heptamers (Fig. 2a and Extended Data Fig. 3).
Fig. 3 | Characterization of spirulina-expressed, anti-campylobacter VHH. 

**a.** Epitope mapping of VHH interaction with FlaA. Peptides derived from the D2/D3/D4 region of FlaA (amino acids 177–482) were panned by phage display. Enriched clones were sequenced after two or three rounds of panning. Results represent average positional frequency observed in two independent panning experiments. 

**b.** CEIA quantification of aa682 expressed in SP1182. Clarified lysate from SP1182 was displayed on a Jess system, with an anti-His-tag antibody used for detection. A single peak was observed at the predicted MW of 54.8 kDa. Using a standard curve of purified protein (Extended Data Fig. 7), the amount of soluble aa682 was ~3% of total biomass. Result is representative of dozens of independent experiments. 

**c.** Binding kinetics of spirulina-expressed aa682 with recombinant FlaA measured by BLI. The D3 domain of FlaA is surface accessible for VHH binding. 

**d.** Positively charged amines were added as a fusion protein with a chaperone, such as the maltose-binding protein (MBP), to increase expression levels. The D2/D3/D4 region of FlaA (amino acids 177–482) were panned by phage display. Enriched clones were sequenced after two or three rounds of panning. Results represent average positional frequency observed in two independent panning experiments. 

**e.** Mouse models were used to test whether orally delivered spirulina containing an anti-campylobacter VHH could prevent enteric campylobacter infection. 

**Prevention of campylobacter disease.** Enteric infectious diseases are designated as high-priority antimicrobial resistance threats by the Centers for Disease Control and Prevention, adding urgency to the search for new therapeutic tools. Diarrhea accounts for 10% of the 7.6 million annual deaths in children under the age of 5 years. Campylobacter jejuni is among the most common causes of bacterial gastroenteritis and is a leading cause of infant mortality in the developing world. 

The VHH FlagV6 binds to flagellin (FlaA), a subunit of C. jejuni flagella. Its binding site was mapped to the D3 domain of FlaA by phage display of peptides tiled across the FlaA protein. Spiralina strain SP526 expressed a monovalent fusion of FlagV6 VHH and MBP, which bound to recombinant FlaA with a $K_D$ of 53 nM and was constitutively expressed in the cell cytoplasm at 3% of dry biomass (equivalent to 8% of soluble protein).

Specific binding of FlagV6-MBP to intact C. jejuni flagella was demonstrated by flow cytometry. Aqueous extracts of SP526 were incubated with live C. jejuni 81–176 and stained with a fluorescently labelled anti-His-tag antibody. Fluorescence was measured in the allophycocyanin channel (APC-A) by flow cytometry. 

Inhibition of C. jejuni motility by aa682. Two strains of C. jejuni (81-176 and CBG421) were grown on soft agar plates in the presence of aa682 or an irrelevant VHH control (PP496). Halo areas (mean ± s.d.) were measured for triplicate samples at either 40 h (81-176) or 66 h (CBG421) after plating.
and on days 1 and 2. Control groups were either untreated or treated with 10 mg of spirulina containing either an irrelevant recombinant protein or an irrelevant VHH. Treatment with SP526 reduced campylobacter fecal shedding by three to four orders of magnitude compared with 10 mg of spirulina strain SP227 (no VHH, $n = 4$), strain SP526 (analog of aa682, $n = 7$) or vehicle ($n = 8$) on days -1, 0, 1, 2 and 3 relative to challenge. Bacterial shedding in stool (CFU 10$^{10}$ g$^{-1}$ feces) was measured 7 days after challenge. Uninfected mice were treated with vehicle ($n = 4$). C. jejuni challenge dose of 2 mg of dry SP526 biomass (60 $\mu$g of FlagV6-MBP; final concentration of approximately 10$^{10}$ mol l$^{-1}$ mouse small intestine) prevented campylobacter disease, as measured by stool LCN-2 and myeloid cell infiltration of the cecum. Furthermore, this accelerated campylobacter expulsion from the gut 24 h after challenge and reduced campylobacter shedding at 72 h (Fig. 4a,b).

A second experiment used a challenge dose of 10$^8$ CFU of C. jejuni 81–176. Dose-ranging experiments showed that a single prophylactic oral dose of 2 mg of dry SP526 biomass (60 $\mu$g of FlagV6-MBP; final concentration of approximately 10$^{10}$ mol l$^{-1}$ mouse small intestine) prevented campylobacter disease, as measured by stool LCN-2 and myeloid cell infiltration of the cecum. Furthermore, this accelerated campylobacter expulsion from the gut 24 h after challenge and reduced campylobacter shedding at 72 h (Fig. 4c,d).

**Large-scale continuous growth.** Spirulina is cultivated in open ponds at commercial scale, but uncontrolled exposure to environmental contaminants makes this unsuitable for the manufacture of biopharmaceuticals under FDA cGMP. Therefore, we developed modular, indoor, 160–2,000-l, vertical, flat-panel photobioreactors that were pH controlled and air-mixed. Commercial-scale manufacturing is accomplished by constructing arrays of these reactors rather than by constructing larger reactors.

An advantage of this platform is the simplicity of large-scale growth compared with traditional fermentation platforms. Spirulina thrives at pH $> 10$ and high total salinity, and grows without a carbon-based source of energy. These allowed the use of unsealed reactors under sanitary, but not aseptic, conditions. Single-use polyethylene (mylar) bags contained the spirulina culture, eliminating sterilization downtime, which is one of the bigger bottlenecks in biopharmaceutical processes.

Energy for illumination with full-spectrum, adjustable-intensity light-emitting diodes (LEDs) was the major component of production cost (Extended Data Fig. 8a). The complex relationship between the capital and operational costs of biomass growth was evaluated as a function of light intensity, and an optimum that achieved the greatest productivity per unit energy cost was identified (Extended Data Fig. 8b).

Cultures were maintained without antibiotic selection for sequential 1-week growth cycles. Growth rates of photosynthetic microbes differ from those of heterotrophic microbes, because growth is light limited. At low densities spirulina can undergo exponential growth with a doubling time of 2–3 h. At densities greater than $\sim 0.5$ g l$^{-1}$, growth becomes light limited and growth rates are linear (that is, a constant amount of biomass l$^{-1}$ d$^{-1}$). On a weekly basis, cell densities reached $\sim 4$ g l$^{-1}$ and the biomass was harvested by passage over a series of stainless steel screens. A portion of the resulting slurry was used to reinoculate the reactors, the remainder being processed into drug product.

**Simple downstream processing.** The spirulina slurry was rinsed with a trehalose solution and then spray-dried. A large parameter
aa682 is a version of FlagV6-MBP containing two alterations in the N-terminal residues of the framework region that increase resistance to chymotrypsin. Purified aa682 was fully degraded within 2 min of incubation under simulated gastric conditions (Fig. 6a). However, when delivered within dry spirulina biomass, >70% of aa682 remained intact after 2 h of incubation under gastric conditions (Fig. 6b). Therefore, bioencapsulation of therapeutic proteins within dry spirulina biomass provides protection during gastric transit. Transition of biomass to the higher pH of a simulated duodenal environment was sufficient to extract >90% of encapsulated aa682 from spirulina within 60 min (Fig. 6c). The binding activity of aa682 extracted under duodenal conditions was minimally affected by previous incubation of the biomass under the gastric environment (Fig. 6d).

In vivo efficacy is also dependent upon the sensitivities of therapeutic proteins to intestinal proteases, especially trypsin and chymotrypsin. Protein aa682 was resistant to constitutive intestinal levels of both trypsin and chymotrypsin. Some sensitivity of aa682 to chymotrypsin, but not trypsin, was evident when it was exposed to the higher protease levels that are present immediately after a meal11 (Fig. 6e).

**First-in-human clinical safety trial.** SP1182, a markerless strain expressing aa682, was constructed for human clinical testing. The molecular weight and amino acid sequence of purified aa682 were confirmed by liquid chromatography–mass spectrometry (LC–MS). The measured intact mass matched the theoretical mass of full-length protein, minus post-translational removal of the N-terminal methionine. The amino sequence of aa682 was confirmed by proteolyzed peptide fragment analysis, with 98% coverage of the expected protein sequence (Extended Data Fig. 10). Strains in human clinical testing, including SP1182, were banked as frozen vials, and the DNA sequence of the transgene was confirmed each time a vial was taken from the bank and used to manufacture drug product.

SP1182 and wild-type strain SP3 were cultured in large-scale bioreactors under cGMP conditions and used to formulate the drug product. Each time a vial was taken from the bank and used to manufacture drug product.

**Discussion**

The efficacy of oral biologics in the treatment of human disease was first demonstrated for antibody therapy of *E. coli* infection in human infants33. Additional successful human clinical trials against rotavirus and Clostridium difficile have been reported35–38. While these studies established that orally delivered antibodies can be effective, commercial development has suffered from reliance on production systems—such as the milk of hyperimmunized cows—that have proven difficult to scale cost effectively while maintaining FDA-grade lot-to-lot consistency.

Food-based systems for the manufacture of biologics would be ideally suited for this purpose, and some progress has been made with rice-based expression of antimicrobial therapeutics39. Here we report our discovery of methods that allow constitutive and stable expression of protein therapeutics in spirulina, with productivities (g therapeutic g–1 biomass d–1) and potencies (gthera-
Fig. 6 | Protease sensitivity of aa682. a, SDS-PAGE analysis of purified aa682 incubated with simulated gastric fluid supplemented with 2,000 U ml⁻¹ pepsin. Results are representative of two independent experiments. b, CEIA of pepsin-digested spirulina biomass resuspension. Dried spirulina biomass of SP1182 was resuspended in simulated gastric buffer and incubated with pepsin for 0–120 min or overnight (O/N). Whole biomass samples were denatured and analyzed on a Jess system. Recombinant aa682 was detected with an anti-His-tag antibody, and data are representative of four independent experiments. c, CEIA of spirulina biomass extracted under different conditions. Dried spirulina biomass of SP1182 was resuspended in simulated gastric buffer (pH 3.0, no pepsin) and the presence of aa682 in buffer was analyzed (A). The biomass was first incubated (A), brought to intestinal pH (pH >5.0) (B) then compared with aa682 extraction by direct biomass resuspension in bicarbonate buffer (pH >7.0) (C). Data are representative of two independent experiments. d, ELISA-based, antigen-binding analysis of spirulina lysates prepared as in c assayed for binding to recombinant FlaA. Samples B and C yielded approximate EC₅₀ binding values of 85.8 and 29.2 µg ml⁻¹ biomass, respectively. Data are average of two replicates. e, ELISA binding activity of aa682 after in vitro exposure to intestinal proteases. Lysates from SP1182 were incubated with trypsin or chymotrypsin for 1h. After protease neutralization, aa682 binding activity to recombinant FlaA was measured by ELISA. Data are average of two replicates. Unless noted otherwise, experiments were performed once.

peutic g⁻¹ biomass) that surpass, by tens to hundreds of fold, what can be achieved in other food-based platforms⁴⁰,⁴¹. Isolation of seed, where plant recombinant proteins are usually expressed, increases apparent potencies but these still fall short of potencies achievable in whole, unfractionated spirulina biomass. Low productivity and potency in plants not only amplify production costs but may also necessitate expensive downstream processing, which is reported to be a weakness of plant-based production systems⁴¹.

Based on the observations reported here, the repertoire of therapeutics expressible in the spirulina platform would include bioactive peptides, antibody fragments (for example, VHHs), enzymes, antioxidants, signaling proteins (for example, hormones, cytokines) and vaccine antigens, as well as metabolic products of biosynthetic pathways. Like other prokaryotes, however, spirulina can neither recapitulate human protein glycosylation patterns nor efficiently express proteins containing multiple disulfide bonds. Therefore, the subset of therapeutics requiring these for activity would be excluded. It is therefore useful to compare the productivity of the spirulina platform for expression of antibody fragments (VHHs) with that of Chinese hamster ovary (CHO) cells for full-length antibodies. VHH volumetric productivity in spirulina is approximately 25–50 versus 500 mg antibody l⁻¹ d⁻¹ in CHO cells⁴². Process development of spirulina VHH production is in its infancy, and current volumetric productivity is similar to antibody production in CHO when that was introduced as a production platform⁴³. Nevertheless, the materials required for spirulina cultivation—open-topped, LED-illuminated, steel-framed reactors containing single-use disposable bags with a minimal salt growth medium—are considerably simpler than the aseptic fermentation reactors and complex growth media required for production of biologics in CHO. Moreover, the safety of spirulina as an expression host further simplifies the drug manufacturing process. Spirulina hold FDA Generally Recognized as Safe status⁴⁴. The toxicology profile has been positively reviewed by the FDA, and clinical trials have established its safety in both adult⁴⁵,⁴⁶ and pediatric populations⁴⁷. Consequently, therapeutics produced in spirulina do not need to be purified before oral delivery. Because the manufacturing pipeline is comprised solely of growing and drying spirulina biomass, the technical challenges, operational expenses, in-process controls and safety tests associated with downstream drug processing are either reduced or eliminated. Especially for oral delivery, the simplification of both equipment and upstream and downstream processes can more than offset the benefit apparent from a comparison of volumetric productivities.

These considerations suggest that the spirulina platform may present an opportunity to use biologics not just for treatment but also for prevention of prevalent diseases. Biologics are traditionally...
produced in small quantities and priced at amounts per dose that make their widespread prophylactic administration almost inconceivable. In contrast, prophylaxis using the spirulina platform could be affordable, even in the developing world. Moreover, refrigerated distribution and intravenous infusion requirements impose further constraints on the deployment of traditional biologics. VHVs and other biologics in dry spirulina powder are shelf stable without refrigeration, facilitating distribution, especially into regions lacking high-quality infrastructure.

Targeted delivery of a therapeutic to its site of action is a key consideration for efficacy. Systemic therapeutics have off-target toxicities, especially at higher doses, and have low partitioning coefficients into mucosal tissues that reduce the amount of active product at the site of action. These are often reasons for clinical failure and a major driver of the high cost of new drug development. Direct delivery of a nonabsorbed therapeutic to the intestine reduces these concerns.

Beyond the example of infectious diseases, current programs in inflammatory bowel diseases, metabolic diseases and oral vaccines illustrate the broad applicability of the spirulina platform; its advantages may also apply to other topical and mucosal surfaces, including upper airway delivery. Perhaps the greatest impact may be with multicomponent biologic cocktails. Cocktails comprised of ten or more therapeutic proteins are in development, whereas therapeutics comprising more than two biologics are generally impractical using conventional platforms due to the complexity of cell line development, manufacturing costs and compounding toxicities of systemic therapeutics. Therapeutic cocktails may be transformative for treatment of complex diseases with multiple underlying pathological processes, and for treatment of pathogens commonly exhibiting therapy-evading genetic variation.

Online content
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Methods

Small-scale spirulina culture. Spirulina strains were grown in liquid culture using SOT medium. For antibiotic selection, medium was supplemented with 70–100 μg·ml⁻¹ kanamycin or 2.3–5.0 μg·ml⁻¹ streptomycin. Culture volumes ranged from 3 ml to 100 ml. Transformation of strains for transfection or downstream processing, cultures were grown in Multitron incubators at 35 °C, 0.5% CO₂, 110–150 μl of light and shaking at 120–270 r.p.m., depending on culture volume. Long-term cultures were maintained by incubation in Innova incubators at 30°C, atmospheric CO₂, 50–110 μl of light and shaking at 120 r.p.m.

Design of integrating vectors for spirulina transformation. For each genomic locus targeted for integration, PCR primers with a 18–20 base-pair (bp) overlapping sequence and a vector backbone were designed to amplify 1.0–1.5-kb DNA fragments from the 5’- and 3’-regions flanking the locus. These regions represented the left homology arm (LHA) and right homology arm (RHA), respectively. Gel-purified fragments were assembled with the linearized backbone vector, which contained a p15 origin and an E. coli ampicillin resistance marker, by Gibson assembly. Markers for antibiotic resistance in spirulina were cloned between the two homology arms of the plasmid.

Transformation of spirulina. Spirulina cultures were grown for 3 days in Innova to optical density (OD₇₀₀ nm) 0.5–1.0. A cell volume of 50 ml was harvested by centrifugation for 10 min at 1,600× central centrifugal force (RCF). Cells were washed once with SOT medium at room temperature then resuspended in 2 ml of SOT. A 30-μl aliquot of cells was mixed with 300 ng of plasmid DNA and incubated at room temperature for 3 h. Samples were transferred to 0.6 ml of SOT medium in 14 ml round-bottom tubes and incubated overnight at 25–30°C under 70–100 μl of fluorescent light on a light rack. Each tube received 2.4 ml of SOT with appropriate antibiotics and was incubated under Multitron conditions to start selection. For the first 20–30 days, culture medium was changed every 3–5 days. After 30 days, when the transformations were robustly growing, cells were diluted every 3–5 days to facilitate segregation.

Genotyping of transformed spirulina. Genomic DNA was prepared from spirulina cells by digestion with proteinase K. In brief, 0.2–0.5 OD₅₀₀ ml of cells was washed once with sterilized water. A 30-μl sample of cell pellet was mixed with 120 μl of buffer EB (10 mM Tris·Cl pH 8.5). Proteinase K was added to samples at a final concentration of 0.2 mg·ml⁻¹. Samples were incubated at 56°C for 1 h followed by 95°C for 10 min, to deactivate proteinase K. Samples were centrifuged briefly in a microfuge to pellet cell debris. A 1-μl sample of the supernatant was used per genotyping PCR reaction. Specific integration of the transgenic cassette was determined by separate PCRs for each homology arm. For each PCR, one primer annealed to a genomic sequence outside the homology arm and the other to a region within the transgene. Segregation of the chromosome was assessed using a primer pair annealing to regions within the RHA and LHA. Segregation PCR yielded fragments of two different sizes: one from the wild-type allele and the other from the transgenic allele. Strains were considered fully segregated when no wild-type allele amplification was observed.

To verify the sequence of the transgene, PCR was performed with genomic DNA to amplify the fragments, which includes the transgene, the homology arms and 300 bp flanking each homology arm. PCR products were separated by electrophoresis on an agarose gel, and amplified bands were gel extracted using the QIAquick Gel Extraction Kit. Purified PCR products were sequenced to verify the integrated gene and surrounding sequences.

To exclude the possibility of cross-contamination with other strains, PCR was performed to check other loci used for integration of exogenous genes. PCR of genomic DNA using locus-specific primers was performed, and fragment size was analyzed by agarose gel electrophoresis. DNA fragments were gel extracted and characterized by Sanger sequencing. A strain was considered free of other spirulina strains if only wild-type loci were observed. Once strains were homozygous, the DNA sequence of the transgene was periodically reassessed by Sanger sequencing of PCR products. No DNA sequence variation of the transgene was observed, even after 3 years of continuous cultivation.

Isolation of single spirulina filaments. From an actively growing spirulina culture, 200–500 filaments were spread on a SOT agar plate. Cells were allowed to settle on the plate for 1–2 h and examined under a dissection microscope.

Well-separated single filaments were picked with a 1-ml pipette tip and transferred in 3 ml of SOT with appropriate antibiotics in a round-bottom tube. Typically, 10–20 single filaments were cultured in Innova for 15 days for propagation.

Determination of transgene copy number. To assess the copy number of an integrated transgene, three sets of primer/tagmane probe pairs were designed to target three regions: an endogenous spirulina gene present at a single locus (spch), a promoter region present at both an endogenous and transgenic locus (that is, two chromosomal copies) and an exogenous region unique to the transgene. A synthetic g-block containing the three target loci plus flanking sequences was purchased from IDT as a calibrator. Real-time PCR was performed with the above primer/probes using genomic DNA from the transgenic strain as the g-block as templates. As controls, the parental spirulina strain and a second transgenic strain lacking template for the transgene-specific probes were tested. The relative copy number of the integrated transgene was calculated as the fold difference between transgene and endogenous genes using the ΔΔCt method. The experiment was repeated five times with three separate preparations of genomic DNA. The expected abundance ratio for the endogenous gene, promoter and exogenous gene was 1, 2 and 1 respectively.

Axein strain isolation. To establish axenic spirulina strains, cells were washed with SOT medium on 10-μm filters to exclude small, unicellular bacteria, and single spores were isolated from cells captured on the filter. Cells were grown to a density of 0.5–1.0 OD₅₀₀ ml⁻¹ in an Innova incubator with appropriate antibiotics. Cells were pelleted from 5 ml cultures by centrifugation for 10 min at 1,600×RCF. To maintain sterility, the following steps were performed in a laminar-flow hood. The cell pellet was resuspended in 1 ml of SOT and transferred to a 10-μm filter prior to SOT medium, which was removed by gentle filtration. Cells were washed with successive 1-μl aliquots of SOT medium until at least 200 μl of total medium had passed through the filter. The remaining cells were resuspended with 0.5 ml of SOT and transferred to a sterile Eppendorf tube. Filaments were counted under a microscope as above, and 200–500 were spread on a SOT agar plate. Single filaments were isolated as described. After 15–days, 10 μl of culture was spread on LB plates without antibiotics. Plates were incubated for 3–5 days in an incubator at 37°C. Filament cultures free of contaminants on the LB agar plates were then sequenced in 10 ml of SOT with 2.5 g l⁻¹ dextrose at a density of 0.1 OD₅₀₀ ml⁻¹. Cultures were grown in Multitron for 3 days. A 100-μl sample of the culture was plated on LB agar plates without antibiotics and incubated at 37°C for 5 days. Cultures with no contaminants observed on either set of LB plates were considered axenic.

Cultivation of non-spirulina microbes. To cultivate non-spirulina microbes, a flask of spirulina culture was placed on bench for 3–5 h to allow cells to settle at the bottom of the flask. A 100-μl sample of supernatant was carefully pipetted and transferred to LB/ SOT agar plates. Plates were incubated at 37°C for 3–5 days on a light rack (60–70 μlE) for 5–7 days. Single colonies were streaked on fresh plates for between five and ten rounds. Cells from single colonies were spread on fresh plates to propagate for further experiments.

Identification of non-spirulina bacteria from spirulina cultures. To culture non-spirulina microbes, a flask of spirulina culture was placed on the bench for 3–5 h to allow cells to settle at the bottom of the flask. A 100-μl sample of supernatant was carefully pipetted and transferred to LB agar plates. Plates were incubated at 37°C for 3–5 days on a light rack (60–70 μlE) for 5–7 days. Genomic DNA was extracted from bacterial samples following the extraction method described above. Highly conserved and degenerate 16S and 23S ribosomal DNA PCR primers (Supplementary Table 1) were used to amplify genomic DNA, following published protocols46,47 from samples derived from both LB and LB/SOT plates. PCR product libraries were subcloned and sequenced. The probable species of origin was identified by BLAST query for similar sequences in the NCBI database.

Markerless strain engineering. To create a platform for markerless integration, a parental strain containing a recombinant, non-native antibiotic marker was first generated. An integrating plasmid bearing homology arms for the D01030 (kmR) locus flanking an aadA gene for streptomycin resistance was transformed into wild-type spirulina. The integrating vector was designed to precisely replace the open reading frame (ORF) of D01030 with the sequence for aadA. This vector was transformed into spirulina strains UTEx (SP3) and NIES (SP7), generating strains SP205 and SP207, respectively. After transformation, strains were propagated for 2 months and confirmed to be fully segregated by genotyping. The strains were also challenged with kanamycin to demonstrate loss of native kanamycin resistance.

Verification of markerless spirulina strains. Clonal isolates of fully segregated strains were verified as follows: (1) qPCR to demonstrate a single transgene per genome (above); (2) sequencing of chromosomal DNA to verify the absence of mutations in the homology arms and inserted gene(s) (above); (3) PCR to
analyzed was performed using the Protein Simple Compass software. To exclude the possibility of nonspecific integration of the vector backbone DNA, PCR was performed with primer pairs targeting the ampicillin resistance gene and E. coli origin of replication. At no point were these fragments observed in spirulina, suggesting that there is no integration of the vector outside of the homology arms.

**Construction of markerless transgenes for spirulina integration.** To ease cloning of transgenes into spirulina, a standardized vector was built for markerless integration. This ‘destination’ vector included integrating homology arms for the _kmR_ locus flanking an ORF for the native _kmR_ gene and a terminator. The antibiotic marker was followed by a recombinant promoter–terminator pair for transgene expression. The promoter–terminator pair consisted of a constitutively active, _native_ _A. platensis_ promoter (600 bp upstream of the _cpcl_ gene, named _Pcpcl600_) and the terminator of the _E. coli_ ribosomal RNA gene _rnbR_ (named _TrnR_). A pair of _Battarachytium salamandriovarans_ restriction endonuclease sites was added to the terminator pair to assist in Golden Gate cloning of protein coding sequences for transgenic expression. Protein coding sequences with compatible _B. salamandriovarans_ sites were purchased from IDT and cloned into the destination vector using a Golden Gate Assembly Kit (NEB). Plasmid DNA was purified from _E. coli_ by the QIAprep Spin Miniprep Kit (Qiagen) and transformed into electrocompetent TG1 cells. The _destination_ vector backbone DNA was sequenced to confirm the absence of non-native antibiotic markers are present.

**Purification of recombinant protein from spirulina.** Recombinant aa682 was purified from spirulina by immobilized metal affinity chromatography (IMAC). In brief, a 10-μl pellet of spirulina cells from strain SP1182 was collected from 21 of culture by centrifugation. The pellet was resuspended in a total volume of 35 ml with lysis buffer (50 mM sodium phosphate buffer pH 8.0, 500 mM NaCl, 20 mM imidazole) supplemented with Pierce Protease Inhibitor MiniTABS (Thermo Scientific) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The resuspension was passed through a French pressure cell twice to lyse the cells. Samples were kept on ice throughout. The insoluble fraction was pelleted by centrifugation at 5,000×_g_ for 30 min. The partially clarified lysate was mixed with 2 ml of washed HisPur Ni-NTA Resin (Thermo Scientific) and incubated at 4 °C with gentle rocking for 2 h. The resin was gelled gently by centrifugation at 500×_g_ for 1 min, supernatant discarded and the resin resuspended in fresh lysis buffer. This process was repeated until the supernatant was clear. The resin was collected in a small column by gravity filtration, washed with 20 ml of lysis buffer and spirulina-expressed aa682 was eluted with lysis buffer containing 200 mM imidazole. Purified aa682 was further polished by separation on a Superdex 75 Prep Grade column on an ÄKTA Pure, yielding a single band by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis.

**Preparation of spirulina lysates for analysis of soluble protein.** Soluble lysates from spray-dried spirulina samples were prepared using a freeze–thaw protocol. Dried spirulina biomass was resuspended in PBS containing Pierce Protease Inhibitor miniTABS and 1 mM PMSF at a biomass concentration of 10–40 mg ml–1. Samples were mixed to resuspend biomass powder and flash-frozen in liquid nitrogen for 2–5 min. Resuspensions were transferred to a water bath at 37 °C for 2–10 min. Samples were well mixed by inversion once thawed. The freeze–thaw procedure was repeated an additional two times. Biomass samples were then centrifuged at 15,000×_g_ at 4 °C for 15–30 min, and the soluble fraction was transferred to a separate tube for downstream applications.

**Expression analysis of recombinant proteins in spirulina.** Recombinant protein expression in spirulina was measured by capillary electrophoresis immunoassay (CEIA) using a Jess system (ProteinSimple), which was run as recommended by the manufacturer. In brief, dried biomass samples were diluted to a concentration of 0.2 mM _E. coli_ cells (as measured from an Eppendorf Pack 1, in either reducing or nonreducing format (Bio-Techne). Purified protein controls used to generate standard curves were typically loaded at a range of concentrations from 0.5 to 20 μg ml–1. A 12–230-kDa Jess/Wes Separation Module (ProteinSimple) was used and 3 μl of each sample was loaded for 9s. A mouse anti-His-tag antibody (SDS–PAGE) was used as the primary detection antibody. An anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (ProteinSimple) was primarily used for chemiluminescent detection; fluorescently labeled anti-mouse antibodies (ProteinSimple) for near infrared detection were used for some experiments. Data analysis was performed using the Protein Simple Compass software.

**Expression, purification and biotinylation of _E. coli_–expressed proteins.** Recombinant _C. jejuni_ flagellin was expressed and purified from _E. coli_. A region of _FlaA_ (sequence ID: WP_17888893.1) predicted to be soluble and exposed on the surface of flagella (amino acids 177–482) was cloned with _B. salamandriovarans_ DNA integration with other engineered spirulina strains (above); (6) PCR to demonstrate absence of the integrating DNA vector backbone, which should be eliminated during integration by homologous recombination (below); and (7) verification of streptomycin sensitivity and kanamycin resistance by antibiotic challenge.

Inhibitor minitablets and 1 mM PMSF at a biomass concentration of 10–40 mg ml–1 _E. coli_ cells were grown overnight with shaking at 225 rpm and 37 °C, back diluted to _OD_600 = 0.05 and grown at 37 °C until cells reached mid-log growth phase ( _OD_600 = 0.4–0.6). Cells were cultured with 5% d-thiogalactoside and incubated with shaking at 16 °C overnight. The following day, cells were pelleted by centrifugation at 3,500×_g_ for 20 min at 4 °C, resuspended in 30 ml of lysis buffer containing protease inhibitors and lysed in a Q700 Sonicator (Qsonica). The MBP–FlaA fusion was purified from the clarified lysate using Amylose Resin (Novagen) according to the manufacturer’s recommendations, and purified protein was aliquoted and stored at –80 °C. Biotinylated MBP–FlaA protein was prepared using an EZ-Link NGS-PEG4 Biotin kit (Thermo Scientific) following the manufacturer’s guidelines.

VHIs expressed in _E. coli_ used similar expression vectors and bacterial cell lines. Culturing, induction and lysis of _E. coli_ expressing VHIs followed the same protocol as for _FlaA_ expression. Purification of VHIs from lysates was performed by IMAC, following the purification protocol described for aa682.

The RBD antigen used with VH1–72 was kindly provided by R. Strong (Fred Hutchinson Cancer Institute).

**ELISA binding assays.** The half-maximal effective concentration (EC50) binding activity of VHIs as a purified protein, and in spirulina lysate, was measured by ELISA. High-binding, 96-well plates (Greiner Bio-one or NUNC MaxiSorp) were coated with antigen by the addition of 100 μl of 1–5 μg ml–1 recombinant protein (FlaA or RBD antigen) in carbonate-bicarbonate buffer (Sigma) to each well and incubated overnight at 4 °C. Plates were washed three times with 300 μl of PBS-T supplemented with 0.05% Tween-20 (PBS-T). Washed plates were blocked with 250 μl of PBS-T supplemented with 5% nonfat dry milk (PBS-TM) for 2 h at room temperature. Blocking solution was discarded, and 100 μl of sample containing VH1 was added to each well. VH1 samples were prepared by dilution of purified protein or spirulina extracts with PBS-TM, and samples in a dilution series were serially diluted with PBS-TM. Samples were incubated at room temperature for 1 h to allow binding of VH1 to antigen. After incubation, plates were washed three times with 300 μl of PBS-T. Wash was discarded, 100 μl of primary antibody diluted with PBS-TM was added to each well and plates were incubated at room temperature for 1 h. A 1:10,000 dilution of either a mouse anti-His-tag antibody (GenScript) or rabbit anti-camellia VH1 antibody cocktail (GenScript) was used as the primary antibody. After incubation, plates were washed three times with 300 μl of PBS-T, and 100 μl of a secondary antibody was added to each well. A 1:10,000 dilution of either HRP-conjugated goat anti-mouse antibody or HRP-conjugated donkey anti-rabbit antibody was used as the secondary antibody. Plates were incubated at room temperature for 30–45 min, then washed twice with PBS-T and once with PBS. Plates were developed using either a SeraCare KTL TMB Microwell Peroxidase Substrate System (Sera Care Life Sciences) or 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Scientific) following the manufacturer’s recommendations. Peroxidase activity was quenched after 5–10 min with 50 μl of either 30% H2O2 or 2 M sulfuric acid. Absorbance at 450 nm (A450) was measured on an M2 plate reader ( Molecular Devices, SoftMax Pro software). All samples were tested in duplicate. Data analysis was performed using Prism (GraphPad Software).

**Kinetics binding analysis of VHIs.** Kinetics binding measurements were performed by biolayer interferometry (BLI) using an Octet Red96e (ForteBio). Biotinylated MBP–FlaA was loaded onto streptavidin biosensors at a loading concentration of 100 nM and loading time of 4 min. After loading, probes were allowed to reach a baseline equilibrium in kinetics buffer (PBS with 1% bovine serum albumin and 0.05% Tween-20) for 2 min. Association and dissociation were monitored for 20 and 140 s, respectively. Purified aa682 diluted in kinetics buffer was assayed at concentrations ranging from 1 μM to 10 nM; the 10-nM sample was excluded from analysis due to a weak signal. Two biosensors were used as references: a 0-nM aa682 control and a no-ligand control. Kinetic binding values were determined using Octet Data Analysis HT software (ForteBio). Curve fits were performed using a global fit across all concentrations of aa682 assuming a 1:1 binding model.

**Epitope mapping of VH1–antigen interaction.** Epitope mapping of the interaction between FlagV6 and flagellin was performed using phage–displayed peptide fragments derived from a ~300-amino-acid-soluble fragment of _C. jejuni_ FlaA. The phage was displayed on the surface of _C. jejuni_ FlaA–hydrophobic phage (Bacillus subtilis) and used as a bait. The library was aliquoted and stored in 2 M sulfuric acid. The phage library was cloned into the FlagG site of the phagemid by Gibson Assembly and transformed into DH5α _E. coli_, yielding 6 × 104 transformants. The phage library was cleaned up with QiaPrep Spin Minikit columns and transformed into electrocompetent T7i cells.
2–4-week-old C57BL/6 male mice were fed a zinc-deficient diet before challenge. Animal experiments at the University in independent mouse models were used to test the efficacy of spirulina-expressed transgene and the absence of contaminating sequences. CEIA and ELISA were performed for two additional rounds. After the third round of panning, all phagemid-containing colonies were observed to contain the same peptide fragment by Sanger sequencing. Two independent replicates of the experiment yielded overlapping fragments that mapped to the D3 domain of FlaA.

Flow cytometry of VH1 binding to C. jejuni. Binding of spirulina-expressed VH1s to a pure culture of C. jejuni was measured by flow cytometry. An aliquot of lysate prepared from spray-dried spirulina biomass was incubated with an equivalent volume of 10^6 CFU ml⁻¹ C. jejuni 81–176 for 1 h at 4 °C. After washing with PBS containing 2% fetal bovine serum (FBS), bacteria were incubated for 30 min with the His-tag ELISA antibodies (Abcam) washed with PBS containing 2% FBS, resuspended in 2% paraformaldehyde and acquired on an LSR Fortessa flow cytometer (BD Biosciences) using forward and side scatter parameters in logarithmic mode. Data were analyzed using either FlowJo (TreeStar) or FACS Diva software (BD Biosciences).

Motility inhibition assay. The motility-inhibitory activity of spirulina-expressed aat682 was measured by the motility of C. jejuni through soft agar. All C. jejuni cultures were performed in a tri-gas incubator at 40 °C under microaerobic conditions (5% O₂, 10% CO₂) unless otherwise stated. Glycerol stocks of C. jejuni were first streaked on Campy Blood Agar Blaser plates (Thermo Scientific) and grown for 48 h. Bacteria were then inoculated 0.4% soft agar Mueller-Hinton (MH) plates by stab and incubated for 48 h. A slice of agar from the leading edge of motility halos was used to inoculate 10 ml of MH broth. Liquid cultures were incubated under standard conditions for 72 h. A 20-μl spot of 5 ng/ml purified aat682 in PBS was added to the center of soft agar MH plates and allowed to fully adsorb into the agar. VH1 spots were inoculated with 1 μl of OD₅₆₀=0.03 of C. jejuni from the liquid culture. Samples and controls were set up in triplicate. Plates were incubated under standard conditions. The diameter of motility halos was periodically measured and used to calculate area.

Midscale production of spirulina biomass for preclinical trials. To prepare biomass for preclinical mouse trials, the scale of spirulina cultivation was increased and harvested biomass was spray-dried. Spirulina cultures were initially propagated in shake flasks in medium based on the standard cyanobacterial SOT medium under Multitron conditions. Shake flask cultures were inoculated to airlift reactors, with medium modified by partial replacement of sodium bicarbonate with solid carbonates. Cultures were grown at light levels of 500–2,500 μmol m⁻² s⁻¹, with temperature maintained at 35 °C. As the culture utilizes CO₂ and grows, pH rises and thus CO₂ is added to the airlift stream to maintain pH between 9.8 and 10. Cultures were inoculated at a concentration of 0.1–0.5 g l⁻¹ biomass by ash-free dry weight, and harvested by filtration at 2–4 g l⁻¹.

To prepare for spray-drying, the harvested biomass was rinsed with a dilute (0.1%) trichloroacetic acid solution to remove excess media salts, concentrated again by filtration and then spray-dried in a centrifugal nozzle spray-dryer. Feed rate, air flow and inlet air temperature were controlled to maintain an outlet air temperature of 68–72 °C at the powder-separation hydrocyclone. Once collected from the hydrocyclone, the powder was sealed and stored in airtight, opaque mylar bags to prevent exposure to moisture or light. The powder was stored at room temperature.

Before use in animal trials, spirulina biomass was analyzed to confirm strain identity. Dried biomass was genotyped to confirm the presence of the correct transgene and the absence of contaminating sequences (above). CEIA and ELISA binding assays (above) were also performed to confirm expression and binding activity of spirulina-expressed VH1.

Prophylactic treatment of C. jejuni infection in two mouse models. Two independent mouse models were used to test the efficacy of spirulina-expressed VH1s in the treatment of C. jejuni infection. Animal experiments at the University of Virginia were performed according to institutional (IRB) protocols. Animal experiments performed at the IRB were in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the Cantonal Veterinary Office. In a pilot experiment with the first model of C. jejuni infection, 2–4-week-old C57BL/6 male mice were fed a zinc-deficient diet before challenge. Animals were maintained according to institutional protocols and fed a regular diet with ad libitum water for 3 days. Animals were then started on the diet study for 10 days, after which water was replaced by water containing an antibiotic cocktail for 3 days to condition gut flora for C. jejuni colonization. Water was replaced with water containing 10 pfu ml⁻¹ of an ELISA-positive plasmid-coated overnight with 100 μl of 1 μg ml⁻¹ FlagV6 VH1 in carbonate-bicarbonate buffer, washed with PBS-T and blocked with PBS-TM. The phage library was diluted with PBS-TM to a concentration of 10¹³ phages ml⁻¹ and incubated at room temperature for 30 min. Phages were then panned for VH1 binders by the addition of 100 μl of blocked phage to wells of the ELISA plate and incubation on a vibrating platform for 2 h at room temperature. Unbound phages were washed from wells with 6,300 μl of PBS-T. Bound phages were eluted at low pH by the addition of 100 μl of 100 mM glycine pH 2.0 and incubation for 5 min with shaking. The elution buffer was neutralized with 40 μl of 2 M Tris pH 7.5 and used to reinfect phage–competent TG1 cells (Antibody Design Labo). Library amplification and panning were performed for two additional rounds. After the third round of panning, all phagemid-containing colonies were observed to contain the same peptide fragment by Sanger sequencing. Two independent replicates of the experiment yielded overlapping fragments that mapped to the D3 domain of FlaA.

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Large-scale, continuous culture of spirulina. Spirulina cultures were grown at large scale (250 l) in airlift reactors following protocols similar to the midscale reactors described above. Cultures were inoculated into the same media described above for midscale cultures, at a concentration of 0.1–0.5 g l⁻¹ biomass by ash-free dry weight, grown under identical temperature and pH controls and harvested by filtration over stainless steel screens at 2–4 g l⁻¹. A portion of the harvested culture was freeze-dried with the addition of 100 μl 1 M NaCl and 100 μl 25% (v/v) glycerol and stored in airtight, opaque mylar bags at −20 °C until used.

Post collection, quality control of powder lots included determination of concentration of the 6x-His-tagged protein using CEIA performed on a Jena (ProteinSimple). Specific ligand binding activity was determined on an Octet Red96e biosayer interferometry instrument (Fortis Bio) using recombinant, biotinylated C. jejuni FlAa protein attached to streptavidin-coated biosensors. In
addition, microbial characterization was performed with USP <61> and <62> and elemental impurities determined by USP <233>.

Long-term stability of dried spirulina biomass. Batches of SP1182 spray-dried biomass were stored at room temperature and collectively assessed for binding activity by ELISA. Duplicate biomass samples from each batch were resuspended in PBS, lysed by freeze–thaw extraction and clarified by centrifugation. The binding activity of aa682 present in lysates was determined by ELISA with a recombinant FlAA antigen as described above. Purified aa682 was used to generate a standard curve for binding activity by linear regression using Excel (Microsoft software). The standard curve was used to calculate the concentration of aa682 in SP1182 lysates. The percentage of expected VHH activity was determined by normalization of aa682 concentration in each lysate to an assumed concentration of 3% aa682 per unit biomass.

In vitro gastric protease digests of dried spirulina biomass. Spray-dried SP1182 biomass was exposed to simulated gastric fluid (SGF) to determine the stability of the aa682 present in spray-dried spirulina. A sample of spray-dried SP1182 biomass was resuspended in PBS at 30 mg ml⁻¹. This resuspension was diluted 1:30 with prechilled SGF (50 mM citrate-phosphate buffer pH 3.0, 94 mM NaCl, 13 mM KCl pH 3.5 with 2,000 U ml⁻¹ trypsin (MP Biomedicals)) and incubated in a water bath at 37 °C. Protease activity was neutralized by the addition of 50 mM NaOH and 1 mM PMSE. Samples were pelleted by centrifugation at 17,000g for 5 min. Biomass pellets were solubilized utilizing 1× NuPAGE LDS sample buffer to a final biomass concentration of 1 mg ml⁻¹ and heated at 90 °C on a heat block for 10 min. A similar process was used to assess the stability of purified aa682, omitting the centrifugation step.

The stability and activity of biomass-encapsulated aa682 after exposure to low-pH, simulated gastric buffer was assessed by CEIA and ELISA binding assay. Spray-dried SP1182 biomass was resuspended in either 50 mM bicarbonate buffer or citrate-phosphate buffer pH 3.1 with 1 mM PMSE. Samples were incubated in a water bath at 37 °C for 60 min. After incubation, biomass suspensions were pelleted at 10,000 rpm for 5 min. The supernatant was transferred to fresh tubes and stored at 4 °C. Pellets were resuspended in 1 ml of 50 mM bicarbonate buffer to a final biomass concentration of 30 mg ml⁻¹ and incubated in a water bath at 37 °C for 30 min. Resuspensions were treated to three cycles of flash-freezing in liquid nitrogen, followed by thawing at 37 °C for extraction of soluble protein. After the final thawing, samples were pelleted utilizing a refrigerated tabletop centrifuge for 30 min at 17,000g to separate soluble protein from insoluble cellular debris. The supernatant was used to measure the expression level and binding activity of aa682 by CEIA and ELISA, respectively.

In vitro protease digests with intestinal proteases. To measure intestinal protease resistance, SP1182 lysates were digested with trypsin and chymotrypsin, and VHH binding activity was assessed by ELISA. Total soluble extract was prepared from a resuspension containing 40 mg of dried SP1182 biomass per 1 ml of pH 7.0 buffer (200 mM bis-tris pH 6.6) followed by the freeze–thaw protocol described above. Two volumes of soluble extract were mixed with one volume of protease in bis-tris buffer and one volume of PBS, to yield a final digest concentration of 0.1 or 0.01 mg ml⁻¹ of trypsin or chymotrypsin with a reaction pH of 6.5. Digests were performed at 37 °C for 1 h with shaking at 900 rpm on an Eppendorf Thermomixer. Protease activity was neutralized by the addition of an equivalent volume of 2 mM PMSF and 2 μM TOF (Agilent) using an ACQUITY UPLC Protein BEH C4 VanGuard pre-column. The mass of purified aa682 was analyzed with a 6230 Intact mass spectrometry.

First-in-human clinical trial. A phase 1 clinical trial was designed and conducted to assess the safety and tolerability of LMM-101. The study protocol and all its amendments were reviewed and approved by the Alfred Hospital Ethics Committee. Eligible, healthy volunteers aged 18–50 years were enrolled following informed consent. The study was performed in accordance with ICH guidelines and in compliance with all local and international requirements. Details of the study can be found at clinicaltrials.gov (ID: NCT0099263).

Data availability
Source data for the findings of this study will be made available on FigShare. Additional information on the strains reported here can be found in Supplementary Table 2. Due to some results containing unpublished proprietary information, these will be available from the corresponding author upon reasonable request.

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Author contributions
B.W.J., H.Z. and M.G. designed and performed experiments, analyzed data and wrote the manuscript. T.A., D.T.B., J.A., N.K., R.K., B.W.J., H.Z. and M.G. designed and performed experiments, analyzed data and wrote the manuscript. K.C., R.L., D.C., N.S., E.A., T.N., R.G. performed mouse experiments. J.X. and D.B.V. performed mass spectrometry experiments. J.R. directed overall research and wrote the manuscript. B. Stoddard, G. McDonald, L. Tonkovich, M. Spigarelli, M. Heinnickel, C. Sherr, F. Cross, B. Kerwin, K. Stein, W. Chen, D. Wattendorf and O. Vandal for help, discussions and advice. This work was supported in part by funding from The Bill and Melinda Gates Foundation (nos. OPP12111977 and OPP118364, to J.R.).

Competing interests
J.R. and B.F. are the founders and current employees of Lumen Bioscience, Inc. (Lumen) and own stock/stock options in Lumen. B.W.J., H.Z., M.G., T.A., D.T.B., J.A., N.K., R.K., C.G., J.E., T.P., C. Brady, S.E., M.Z., A.P., J.L., M.T., T.S., D.D. and C.D. designed and performed experiments and analyzed data. L.G., J.D., N.S., D.F., A.M., B.K. and K.S. designed and performed experiments. E.A., T.N., R.A., A.T., A.K. and B.B. performed experiments. J.A. designed and analyzed human clinical trials. J.M. collected and managed information. L.P., D.T.B., F.G. and R.G. performed mouse experiments. I.J. and D.B.V. performed mass spectrometry experiments. R.T. contributed foundational research. B.F. wrote the manuscript. C. Behnke designed experiments and wrote the manuscript. J.R. directed overall research and wrote the manuscript.
A.R., A.T., A.K. and B.R. were employees of Lumen at the time of data generation. Lumen has issued patents (US no. 10,131,870) and a pending patent application (International Application no. PCT/US2020/040794) relating to certain research described in this article. Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41587-022-01249-7.

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Extended Data Fig. 1 | Table of competence genes. The presence of competence genes in sequenced Arthrospira/Limnospira genomes was determined using reciprocal best hits against the A. platensis NIES-39 competence genes previously identified\(^1\). Genomes were retrieved on GenBank and BLASTp was used to identify reciprocal hits with e-values <1\(^{-5}\) and query coverage >50%. Cell labels indicate the percent identity relative to the respective NIES-39 gene; cells in grey indicate that no homolog was identified. Lyngbya aestuarii BL J was included as an outgroup.
**Extended Data Fig. 2 | Determination of transgene copy number by qPCR.** Specific primer pairs were used in qPCR reactions to detect for the presence of three different sequences. Genomic DNA from spirulina strains SP205 (parental), SP985 (Ppc600-driven expression of anti-C. difficile VHH 5D), and SP1182 (Ppc600-driven expression of aa682) were used as templates. The gene for cpcB was used as an endogenous reference gene. The cpc600 promoter was expected to be present as a single copy in SP205, while both SP985 and SP1182 contained a second copy for transgene expression. The gene for aa682 should only be present in SP1182. Gene copy number ratio was calculated using the \( \Delta \Delta C_t \) method for a. cpc600 promoter and cpcB and b. VHH transgene for aa682 and cpcB. Bars in both figures represents the mean ± SD of four independent replicates. Statistical significance was assessed by a two-tailed Mann-Whitney t-test.
Extended Data Fig. 3 | Expression of representative transgenic proteins in spirulina. Uniform expression of GFP in a population of spirulina transformed with a transgene (SP699). Shown are a bright field image (top), and fluorescence images of chlorophyll (middle) and GFP (bottom). b. Western blot of spirulina expressing a 12 kDa orally active and antibacterial peptide, MAA (mammary associated amyloid) (SP13); peptide represents 4-5% of cell protein. c. Western blot of spirulina expressing a vaccine antigen, a repeat domain of malaria P. yoelii circumsporozoite (CSP) protein fused to with core protein of the woodchuck hepadnavirus (WHcAg) (SP82), which self-assembles into a multivalent virus-like particle; antigen represents 4% of cell protein. d. CEIA of spirulina expressing a single-domain antibody, an anti-SARS-CoV-2 VHH fused to a self-dimerizing scaffold (SP1741); VHH represents 9.3% of cell protein. e. CEIA of spirulina expressing a single-domain antibody, an anti-SARS-CoV-2 VHH fused to a self-dimerizing scaffold (SP1825); VHH represents 29.8% of cell protein. f. CEIA of spirulina expressing an enzyme, the catalytic domain from a C. difficile-specific, bacteriophage-derived endolysin (SP1287); enzyme represents 0.54% of cell protein. g. Spectroscopic analysis of spirulina lysates containing a transgenic pigment protein (SP84); pigment protein represents 10% of cell protein. Phycocyanin, a natural blue pigment protein present in wild type spirulina, was overexpressed with a transgenic copy of the native gene. Absorbance spectra for wild-type (black) and transgenic (red) spirulina are shown. Each experiment was performed once.
Extended Data Fig. 4 | Model representations of heterologous proteins designed for expression in spirulina. a. Ribbon representation of a monomeric VHH (orange; PDB ID: 6WAQ)\(^5\) with the solubility enhancer, MBP (green; PDB ID: 5M13)\(^5\). The mature, folded protein results in a monomeric VHH as a fusion to MBP and a C-termini 6X-his affinity tag. b. Ribbon representation of a VHH (orange) with a dimerization motif (blue; PDB ID: 5HVZ)\(^6\) and the solubility enhancer, MBP (green). The mature, folded protein results in a dimeric VHH where dimerization is facilitated by the disulfide-linked dimerization motif. The single polypeptide also contains the solubility enhancer MBP and C-terminal 6X-his affinity tag. c. Ribbon representation of a trimeric VHH (orange). The mature, folded protein results in trimeric VHH (orange) where trimerization is facilitated by the self-assembling homotrimer t-cTRP9\(_{X_3}\) (blue)\(^7\). The single polypeptide also contains a C-terminal 6X-his affinity tag. d. Ribbon representation of heptameric VHH (orange) with the heptamerization motif (blue; PDB ID: 4B0F)\(^8\). The mature, folded protein results in a heptameric VHH where heptamerization is due to intrachain disulfide bond between individual protomers. The polypeptide also contains an N-terminal solubility enhancer MBP fusion and C-terminal 6X-his affinity tag. All structures generated using Pymol (Schrodinger).
Extended Data Fig. 5 | Effect of MBP fusion on VHH expression in spirulina. CEIA was used to evaluate the relative expression level of fusions of two norovirus-binding VHVs in spirulina: a) M6 (ref. 59) and b) Nano26 (ref. 60). The VHH M6 was expressed with no fusion (SP1785), MBP fused to the C-terminus (SP1786), or MBP fused to the N-terminus (SP1790). The VHH Nano26 was expressed with no fusion (SP1772) or MBP fused to the C-terminus (SP1773). All proteins contained a C-terminal His-tag. Clarified lysates from the indicated spirulina strains were normalized by total soluble protein, run on a Jess system, and the protein of interest (POI) detected with an anti-His-tag antibody. The POI was quantified with a purified His-tagged reference standard and the percentage in lysate calculated relative to total soluble protein. Results represents a single measurement for each strain.
Extended Data Fig. 6 | CEIA analysis of the oxidation state of disulfides in dimeric proteins expressed in spirulina strain SP1313. Strain SP1313 expresses the anti-tcdB VHH 7F6 fused to MBP through the dimerization domain 5HVZ. The dimeric form of the protein should have one disulfide within each VHH and two disulfides to maintain association between 5HVZ subunits. Relative percentage of monomer and dimer peak areas observed under reducing and non-reducing conditions is shown. Protein was detected with an anti-His primary antibody followed by an anti-mouse secondary NIR antibody. SP1313 lysate was run in reducing conditions with DTT for 6 independent experiments with 3 lysates assessed in each run, and in non-reducing conditions for 5 independent experiments with 2 lysates assessed in each run. Each data point represents a peak area as quantified by Protein Simple’s SW Compass Software. Bars indicate mean ± SD. Four representative spirulina strains expressing different VHVs on the 5HVZ scaffold have been analyzed in this manner, and the portion of scaffolded VHH in the dimeric state in non-reduced samples ranged from 50 to 100%.
Extended Data Fig. 7 | Linear regression analysis of CEIA standards for aa682 quantification in SP1182. A reference standard curve of purified aa682 protein measured on a Jess system by anti-His-tag detection is shown. Clarified lysate from spray dried SP1182 was loaded at a concentration of 0.2 mg biomass/mL. Using the standard curve, soluble recombinant aa682 was measured at ~3% of total dried biomass.
Extended Data Fig. 8 | Cost optimization. a. Cost components of cGMP biomass production. b. Spirulina productivity is a function of light intensity and is empirically determined in the described system with SPII82 as the production organism and with current operating parameters. Cost per unit biomass includes labor, capitalized cost of operating lighting system (varies by light intensity), and capitalized costs of other upstream components (independent of light intensity). Minimal cost per unit biomass was achieved at a light intensity of approximately 100 μmol/m²/sec.
Extended Data Fig. 9 | VHH stability during spray drying. FlaA binding activity of aa682 in biomass versus drying temperature. Biomass of strain SP1182 was dried across a range of temperatures, extracted at 10 mg/ml biomass, and the extracts were diluted to a constant 0.039 mg/ml assay concentration. Binding activity of the extracts to FlaA was measured by ELISA. Binding activity was unaffected by drying temperatures <73 °C.
Extended Data Fig. 10 | Mass spectrometry analysis of purified aa682. **a.** Intact mass spectrum of aa682 measured by LC-MS. The theoretical mass was 54913 Da, with a measured mass of 54783 Da. The difference in mass would be consistent with loss of the N-terminal methionine. **b.** LC-MS peptide mapping of aa682. Denatured aa682 was digested with trypsin and chymotrypsin, with the resulting peptide fragments characterized by LC-MS. Tryptic and chymotryptic peptide fragments mapping to aa682 are indicated below the sequence of aa682 in blue and red respectively. Tryptic peptides could be mapped to 52% of the full-length, while chymotryptic peptides mapping to 94% of aa682. Together, the peptide mapping covered 98% of the expected sequence for aa682.
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Software and code

Policy information about availability of computer code

Data collection: Compass for Simple Western (V5.0.10), SoftMax Pro (V2.1.28), ForteBio Data Acquisition (V11.1.2.24)

Data analysis: Microsoft Excel (V16.49), Prism (V9.1.1), ForteBio Data Analysis HT (V11.1.2.48), PepFinder (V2.0), FlowJo (V10.7.1), FACS Diva (V6.2), PyMol (V2.1)

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For in vitro protein characterization experiments were performed in duplicate or triplicate. This sample size was considered sufficient because the methods used tend to be highly reproducible. No statistical test was used to calculate in vitro sample size. In vivo animal experiments were performed with cohorts of either 4 or 5 mice. This group size was dependent on the number of animals permitted per cage at the institution performing the experiment. The total number of mice used for each experiment was limited by the resources of the researchers. |
| Data exclusions | No data were excluded. |
| Replication | For most in vitro protein and biomass characterization, individual proteins and spirulina strains were evaluated in one to two independent experiments. Because the methods used for this characterization [PCR, ELISA, SDS-PAGE, CEIA, etc.] are standard and well-developed, a single replicate was considered sufficient for high confidence in the results. Animal and human trial experiments have not been replicated due to the high cost of these experiments. |
| Randomization | Animal’s purchased for pre-clinical studies were randomly assigned to treatment or control groups. Human study participants were randomly assigned to active or placebo groups. |
| Blinding | Investigators managing the animal trials were provided with spirulina strain numbers but were blinded to the identity of the strain used for each treatment or control group. Strain identity was unblinded after data collection and analysis. Human clinical trial staff were blinded to drug assignment (active or placebo). |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| | Antibodies |
| | Eukaryotic cell lines |
| | Palaeontology and archaeology |
| | Animals and other organisms |
| | Human research participants |
| | Clinical data |
| | Dual use research of concern |

### Methods

| n/a | Involved in the study |
| --- | --- |
| | ChiP-seq |
| | Flow cytometry |
| | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- THE™ His Tag Antibody mAb, Mouse (GenScript Cat. No. A00186; clone id: 6G2A9)
- THE™ His Tag Antibody [8Fluor 647] mAb, Mouse [GenScript Cat. No. A01802; clone id: 6G2A9]
- MonoRab™ Rabbit Anti- Camelid VHH Cocktail [GenScript Cat. No. A02014]
- Goat anti-mouse IgG (H+L)-HRP [Bio-Rad Cat No. 170-6516]
- Donkey anti- rabbit igG HRP [Santa Cruz Bio Cat. No. sc 2305]
- Anti-mouse CD11B, APC-conjugated [Biolegend Cat No. 101212; clone id: M1/70]
- Anti-mouse Gr1, PE conjugated [TONBO Bioscience Cat. No. 5D-5931-U100; clone id: RB6-8C5]

### Validation

Primary and secondary antibodies were validated for use in ELISA and western blot by testing with appropriate negative and positive controls.

- His Tag Antibody - manufacturer describes antibody as high affinity, with specificity for 6xHis, as well as 5xHis and 4xHis
- Anti-Camelid VHH antibody - manufacturer describes antibody as a mixture of several monoclonal antibodies, and according to the manufacturer, “it has no cross-reactivity with mouse, rat, rabbit, goat or human immunoglobulins.”
- Goat anti-mouse - manufacturer notes antibody “is prepared from antisera raised in goats immunized with purified mouse IgG.
- Immunofluorescence chromatography procedures are used to isolate antibodies and to remove antibodies which cross react with human immunoglobulin.”
Donkey anti-rabbit - this antibody has been discontinued, but www.citeab.com denotes that this antibody has been reported in 56 citations (https://www.citeab.com/antibodies/3244172-sc-2305-donkey-antirabbit-igg-hrp)

Anti-mouse CD11B, APC-conjugated - manufacturer notes quality control testing by immunofluorescent staining with flow cytometric analysis and that antibody has been verified for immunocytochemistry

Anti-mouse Gr1, PE-conjugated - manufacturer notes application use for flow cytometry; antibody may be cross-reactive with Ly-6G (le Gr1) and Ly-6C

Animals and other organisms

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Labratory animals
In the first set of animal experiments, 2-4 week old C57BL/6 male mice were used. In the second set of experiments, 3 week old C57BL/6 female mice were used.

Wild animals
Study did not involve wild animals.

Field-collected samples
Study did not involve samples collected in the field.

Ethics oversight
Animal experiments at University of Virginia were performed per IRB protocols. Animal experiments performed at IRB were in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the Cantonal Veterinary Office.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
Human research participants were male or female between the ages of 18 and 50 years, of general good health without significant medical illness.

Recruitment
Healthy volunteers were recruited from the community by media and from the existing clinical research center population of healthy volunteers, following informed consent.

Ethics oversight
The study protocol and all its amendments were reviewed and approved by the Alfred Hospital Ethics Committee.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

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All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
NCT04098263

Study protocol
The full study protocol is available from the corresponding author upon reasonable request.

Data collection
This study was conducted November 20, 2019 to April 21, 2020 at Q-Pharm Pty Ltd (Herston QLD, Australia).

Outcomes
Primary outcome measures were the rate of adverse events in LMN-101 subjects and tolerability of LMN-101 compared to placebo. Adverse events were graded according to severity and rates were compared between LMN-101 subjects and placebo subjects. Tolerability was assessed by the proportion of subjects completing study drug and remaining on study and free from possibly drug-related and dose-limiting serious adverse events. Secondary outcome measures were peak serum concentration, area under the curve in serum, and anti-drug antibodies in LMN-101 subjects. The secondary outcomes were assessed by sandwich ELISA.