

ENGINEERED DYSBIOSIS-SENSING PROBIOTIC FOR *CLOSTRIDIUM DIFFICILE* INFECTIONS AND RECURRING INFECTIONS MANAGEMENT

FIELD OF THE INVENTION

5 The present invention relates to methods of metabolic engineering cells to produce bile salt hydrolase to inhibit the germination of *C. difficile* endospores and colonisation within the human gastrointestinal tract, a probiotic, and methods of prophylaxis or treatment of *C. difficile* infection.

BACKGROUND OF THE INVENTION

10 *Clostridium difficile* (also classified as *Clostridioides difficile*) are pathogens responsible for causing *C. difficile* infections (CDIs) and recurring CDIs (rCDIs). CDI is one of the common hospital-acquired infections worldwide. However, treatment of CDIs is difficult due to formation of bacterial endospores which evade antibiotic treatment. Recurrence of CDI occurs in 20.9% of the patients and mortality rate due to these infections is at 9.3%. The
15 germination of dormant endospores that follows disruption of the native microbiome, or dysbiosis, is postulated to lead to the infections as well as the recurrences (Figure 1). The germination of endospores is predominantly facilitated by bile salt taurocholate in the human gastrointestinal tracts. Dysbiosis of the microbiome in the human gastrointestinal tracts are postulated to lead to an increase in amount of free taurocholate and facilitate the
20 germination of the endospores.

 Fecal microbiota transplantation (FMT) is an experimental treatment for CDIs. In FMT, liquid stool suspension extracted from a healthy donor is infused to patient suffering from CDI. It aims to restore the microbiota balance in the gastrointestinal tract. While disease prognosis generally improved, adaptation of the treatment is limited. This is in part due to
25 safety concerns with the use of fecal matters. Furthermore, this strategy is a form of black box engineering that does not identify the specific species of the microbiome necessary to inhibit the infection. The mechanism behind the improvement in prognosis is largely unknown aside from being assumed to be bulk replacement of the disrupted microbiome.

SUMMARY OF THE INVENTION

30 This invention takes the form of an engineered probiotic strain that can inhibit the germination of *C. difficile* endospores within the human gastrointestinal tract. The probiotic expresses bile salt hydrolase that deconjugates *C. difficile* endospore germinant taurocholate into cholate. In contrast to taurocholate, cholate has a lower endospore germination efficiency. Furthermore, cholate exhibits growth inhibition on vegetative *C.*
35 *difficile*. These result in an inhibition of endospore germination as well as a retardation of the

onset of *C. difficile* proliferation and colonisation (Figure 2). Recovery of microbiome during this delay can prevent CDIs and rCDIs. The probiotic is further augmented to respond to dysbiosis of the microbiome. Dysbiosis was reported to cause elevated free sialic acid level in the gastrointestinal tract. The expression of bile salt hydrolase is placed under the control of sialic acid-responsive element. This enables timely expression of bile salt hydrolase upon changes in the microbiome. In addition, the expression of the enzyme through probiotic delivery chassis enables long-term and robust expression through colonisation within the human gastrointestinal tract. This invention was shown to inhibit the germination of *C. difficile* by 97.8% *in vitro* compared to untreated control.

This invention is of clinical relevance. It addresses the prophylactic needs against CDIs and rCDIs. Two groups of patients will especially benefit from this invention. Patients who are at risk of CDI, such as those who are currently on antibiotic regimes in hospital, will find it helpful as prevention against CDIs onset. It can also be administrated to current CDIs patients as prevention to rCDIs.

In a first aspect the present invention relates to an expression cassette comprising;

i) a bile salt hydrolase gene, and

ii) a sialic acid-responsive promoter operably linked to the bile salt hydrolase gene.

In some embodiments the bile salt hydrolase gene is a Cbh protein-encoding polynucleotide sequence from *Clostridium perfringens*, preferably encoding the amino acid sequence set forth in SEQ ID NO: 13 or a functional variant thereof.

In some embodiments the bile salt hydrolase polynucleotide sequence comprises a nucleic acid sequence that has at least 80%, at least 85%, at least 90%, at least 95% sequence identity or 100% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 8 or SEQ ID NO: 9.

It would be understood that due to the redundancy in the genetic code, a polynucleotide sequence may have less than 100% identity and still encode the same amino acid sequence, such as the amino acid sequence of bile salt hydrolase set forth in SEQ ID NO: 13.

In some embodiments the sialic acid-responsive promoter is pNanA from *E.coli*, preferably comprising the nucleic acid sequence set forth in SEQ ID NO: 4 or a functional variant thereof.

In some embodiments a repressor of pNanA is positioned upstream of pNanA when there is expression of pNanA in the absence of sialic acid, wherein preferably the repressor is a NanR protein-encoding polynucleotide sequence, preferably encoding the amino acid sequence set forth in SEQ ID NO: 11 or a functional variant thereof.

5 In some embodiments the NanR protein-encoding polynucleotide sequence comprises a nucleic acid sequence that has at least 80%, at least 85%, at least 90%, at least 95% sequence identity or 100% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 5.

10 In some embodiments, the cassette further comprises a constitutive promoter operably linked to NanR, wherein the promoter is selected from the group comprising pBad with AraC; J23108 with rbs2, rbs3 or rbs5; and J23113 with rbs4.

In some embodiments the constitutive promoter operably linked to NanR is J23113-rbs4.

15 In some embodiments the cassette comprises J23113-rbs4-NanR, preferably comprising the nucleic acid sequence set forth in SEQ ID NO: 6 or a functional variant thereof.

20 In some embodiments, the cassette further comprises an activator and promoter to increase the expression of Cbh, such as the transcription activator CadC protein-encoding sequence and promoter pCadBA, wherein CadC is positioned downstream and under the control of pNanA and pCadBA is positioned downstream of CadC and operably linked to the bile salt hydrolase Cbh protein-encoding sequence.

In some embodiments, the CadC amino acid sequence is set forth in SEQ ID NO: 12 or a functional variant thereof. In some embodiments, the CadC nucleic acid sequence is set forth in SEQ ID NO: 14 or a functional variant thereof.

25 In some embodiments, the activator and promoter nucleic acid sequence comprises the nucleic acid sequence set forth in SEQ ID NO: 7 or a functional variant thereof.

In some embodiments, the cassette is comprised in one or more plasmid vectors.

30 In some embodiments, the plasmid vector is pEaat, preferably comprising the nucleic acid sequence set forth in SEQ ID NO: 1. This vector comprises an origin of replication, an selection marker (SEQ ID NO: 2 and 3), kanamycin resistance marker, and multiple cloning sites.

In some embodiments, the cassette further comprises an antibiotic resistance gene flanked by FRT sites to enable its removal.

In some embodiments, the gene polynucleotide sequence for *cbh* is codon-optimised for expression in a probiotic cell. An example of a codon-optimised gene sequence for *cbh* is the nucleic acid sequence set forth in SEQ ID NO: 9.

In some embodiments, the gene polynucleotide sequence for *cbh* is codon-optimised for expression in a probiotic cell selected from the group comprising *E. coli sp.*, *Bacteroides sp.*, *Clostridium sp.*, *Faecalibacterium sp.*, *Lactococcus lactis*, and *Lactobacillus sp.*

In another aspect of the invention there is provided a use of an expression cassette according to any aspect of the invention for the recombinant production of bile salt hydrolase proteins.

In another aspect of the invention there is provided a composition comprising:

a) a probiotic bacteria; and

b) an expression cassette according to any aspect of the invention,

wherein the probiotic bacteria comprises the expression cassette for production of bile salt hydrolase.

The probiotic bacteria may be selected from any suitable genera of probiotic bacteria.

In some embodiments, the probiotic bacteria is selected from the group comprising *E. coli sp.*, *Bacteroides sp.*, *Clostridium sp.*, *Faecalibacterium sp.*, *Lactococcus lactis*, and *Lactobacillus sp.*

In some embodiments, the probiotic bacteria is auxotrophic.

In some embodiments, the auxotrophic bacteria has had Alanine racemase genes deleted and cannot divide in the absence of D-Alanine.

In another aspect of the invention there is provided a composition according to any aspect of the invention for use in a method of treating *C. difficile* infections (CDIs) and/or recurring CDIs (rCDIs).

In some embodiments, the CDIs and/or rCDIs are caused by dysbiosis.

In another aspect of the invention there is provided a method of treatment or prophylaxis comprising administering to a subject in need of such treatment or prophylaxis an efficacious amount of a composition according to any aspect of the invention.

In some embodiments, the subject has a *C. difficile* infection (CDI) or recurring CDI.

In another aspect of the invention there is provided a composition according to any aspect of the invention for the manufacture of a medicament for the treatment or prophylaxis of CDI and/or rCDI.

5 In some embodiments, the *C. difficile* infection (CDI) and/or recurring CDI is due to dysbiosis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a schematic of dysbiosis-induced infections and recurrent infections of *C. difficile*. 1) *C. difficile* (CD) endospores acquired from the environment can exist as dormant member of microbiome. 2) Disruption of normal microbiome produces ecological niches for *C. difficile* colonisation. 3) Endospores germinate into vegetative cells and further expand into ecological niches within the microbiome leading to infection of the host. 4) At later stage of infection, additional endospores will be produce by the vegetative cells. 5) These endospores are able to evade antibiotics treatment. The continued dysbiosis of microbiome provides window of vulnerability for recurrent infection as endospores can germinate once treatment ceases. 6) Even with recovery from infection, endospores may still persist and trigger the infection when dysbiosis occurs.

Figure 2 shows a schematic of a strategy employed by the invention to achieve inhibition of *C. difficile* endospores. Dysbiosis of the microbiome changes metabolite profile in the gastrointestinal tract. This includes elevated level of free sialic acids. Engineered probiotics are activated by sialic acids to express bile salt hydrolases. The enzymes are able to deconjugate *C. difficile* endospore germinant taurocholate into a weaker germinant cholate. Inhibition of *C. difficile* endospores germination is hence achieved, and transition from avirulent to virulent form of *C. difficile* prevented.

Figure 3 shows a proposed model for the mechanisms of dysbiosis-induced *C. difficile* infections. Two groups of microorganisms in the microbiome (top) are disrupted in event of dysbiosis (bottom). i) The first group mediates the deconjugation of bile salts from small intestine (SI). The loss of this group of microorganism during dysbiosis results in conjugated bile salts in colon where *C. difficile* (CD) endospores reside. Conjugated bile salts trigger the germination of endospores into vegetative *C. difficile*. ii) The second group comprising of sialic acid utilising species and sialidase-expressing species regulate free sialic acid level within the gastrointestinal tract. The upset in balance of the species during dysbiosis results in elevation of sialic acids. Germinated *C. difficile* are able to utilise the sialic acids as carbon source for their expansion leading to infection of the host.

Figure 4 shows that conjugated bile salt taurocholate is a more efficiency germinant of *C. difficile* endospores and deconjugated bile salt cholate inhibits the growth of *C. difficile*. A) Conjugated bile salt taurocholate is deconjugated by bile salt hydrolase into primary bile salt cholate and taurine. B) Germination from endospores extracted from *C. difficile* strains i) CD630, ii) VPI10463, iii) BAA-1870, and iv) 9689 by incubation with taurocholate (filled circle) and cholate (blank circle) at different concentration. Error bars represent S.E.M of duplicates. C) Growth of *C. difficile* strains i) CD630, ii) VPI10463, iii) BAA-1870, and iv) 9689 with taurocholate (filled circle) and cholate (blank circle).

Figure 5 shows a plan for engineering of an antibiotic selection-free probiotic chassis. A) Schematic diagram of D-alanine auxotrophic antibiotic selection-free chassis. Disruption to alanine racemase gene results in inability to produce D-alanine endogenously. Deficiency of D-alanine inhibits cell wall synthesis and cell proliferation. This phenotype can be rescued by expression of alanine racemase from plasmid. This enables selection for the plasmid and, with it, the synthetic genetic circuit. B) Plasmid map for pEaat vector (SEQ ID NO: 1). *alr* (SEQ ID NO: 3) encodes for alanine racemase (SEQ ID NO: 10) under control of native promoter (SEQ ID NO: 2); *NeoR* encodes for antibiotic resistance gene neomycin phosphotransferase. It is flanked by a pair of FRT sequences for excision via FLP; *ori* encoded for origin of replication ColE1; nine unique restriction sites were added by design. The primary insertion site is between BglII and BamHI in accord to the BglBrick standard. C) Growth assay of ECN without (blank square) or with (blank circle) the D-alanine supplement, and phenotype rescue with pEaat plasmid (grey circle). Wild type EcN (black circle) included as control. D) Percentage of viable cells expressing neoR plasmids co-expressed on the plasmids of EcN WT expressing pBbE8K-J23108-*lasR*-pLas-*gfp* (square) or EcN expressing pEaat-J23108-*lasR*-pLas-*gfp* (triangle), with (filled) and without (blank) D-alanine supplement. Cells were subcultured daily without antibiotic selection. Viability of cell indicates presence of plasmids.

Figure 6 shows optimisation of a NanR-dependent sialic acid inducible promoter. A) Plasmid design (pEaat-pNanA-*gfp*) for initial characterisation of pNanA. SA represents sialic acid induction. B) Histogram of flow cytometry fluorescence reading of 10,000 samples for pEaat-pNanA-*gfp* characterisation in T10 (left) and EcN (right) at various concentration of sialic acid induction. C) Plasmid design of co-expressed plasmid for characterisation of NanR (SEQ ID NO: 11) and pNanA (pSC101-pBad-*nanR*). LA represents L-arabinose induction; SA represents sialic acid induction. D) Matrix data of median GFP fluorescence reading of 10,000 samples on flow cytometry for each combination of sialic acid (SA) and L-arabinose (LA) induction. Colour of each cell is graded to scale. E) Plasmid design for co-

expression of *nanR* in pEaat-pNanA-*gfp*. F) Optimisation of pNanA expression by modulating *nanR* expression. Relative GFP fluorescence expression of constructs expressing *nanR* under different promoter and ribosome binding site. Cells were induced with 0.2% sialic acid.

5 **Figure 7** shows the characterisation of an engineered sialic acid biosensor under gastrointestinal-specific conditions. Responses of A) sialic acid inducible construct (pEaat-J2113r4-*nanR*-pNanA-*gfp*) and B) sialic acid repressible construct (pEaat-pNanA-*gfp*) to induction by sialic acid and glucose combinations. Logic gate diagrams depict the response to sialic acid and glucose as inputs. C) Plasmid design of sialic acid inducible-amplifier
10 construct (pEaat-J2113r4-*nanR*-pNanA-*cadC*-pCadBA-*gfp*). Characterisation of sialic acid inducible construct and sialic acid inducible-amplifier construct in D) LB and E) M9 minimal medium with glycerol. Relative fluorescence expression after 3 and 6 hours of induction shown. F) Characterisation of sialic acid inducible-amplifier constructs under different pH. Relative fluorescence expression after 3 hours of induction shown. G) Growth of EcN with
15 and without sialic acid in M9 minimal medium without carbon source.

Figure 8 shows purified Cbh-his6 deconjugates taurocholate into cholate and inhibit *C. difficile* endospore germination. A) 12% SDS-PAGE resolution of purified Cbh-his6 after IMAC and size exclusion chromatography. Expected size of Cbh-his6 is 38 kDa. L represents protein ladder. Standard curves of spectra area against B) taurocholate and C)
20 cholate concentrations from HPLC analysis. D) Concentrations of taurocholate and cholate with and without 3 hours treatment of 10 μ M purified Cbh-his6. Student's *t*-test was performed on taurocholate concentration between treated and untreated. * $P < 0.005$. HPLC spectra of peaks corresponding to elution of E) taurocholate and F) cholate at retention time 12.2-minute and 19.6-minute respectively. G) Germination efficiency of bile salt after
25 treatment with Cbh-his6 based on the CFU enumeration of *C. difficile*. Negative controls of no taurocholate and positive control of no enzyme were performed. Student's *t*-test was performed between treatment and positive control. * $P < 0.05$. Error bars represent S.E.M of duplicates.

Figure 9 shows Cbh expression in probiotics inhibits the germination of *C. difficile* endospores. A) Germination efficiency of bile salt after treatment with Cbh-expressing EcN based on the CFU enumeration of *C. difficile*. Two sets of constructs were used, one without (pNanA; blank columns) and one with amplifier module (pNanA-*cadC*-pCadBA; filled columns). Each set of experiment was performed with *gfp* expression control, non-induced control, and no-taurocholate negative control. A cell-free positive control (grey column) was
30 performed as well. One-way ANOVA and Student's *t*-test were performed on amplifier
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constructs. * $P < 0.005$. Error bars represent S.E.M of duplicates. B) Immunoblot of Cbh-his6 expressed under different promoters from EcN. pBad construct (pEaat-*araC*-pBad) was induced with 0.2% L-arabinose, pNanA (pEaat-J23113r4-*nanR*-pNanA) and pNanA-*cadC*-pCadBA amplifier (pEaat-J23113r4-*nanR*-pNanA-*cadC*-pCadBA) constructs were induced by 0.2% sialic acid. Cell densities were adjusted by OD₆₀₀ before protein extraction. L represents protein ladder; S represents soluble fraction; IS represents insoluble fraction. Expected size of Cbh-his6 is 38 kDa. C) Concentrations of taurocholate and cholate after treatment with Cbh-expressing EcN from amplifier construct. Cell-free positive control, *gfp* expression control, non-induced control, and no-taurocholate negative control were performed. Student's *t*-test was performed on taurocholate concentration between treated and *gfp* expression control. * $P < 0.001$.

Figure 10 shows Cbh-treated *C. difficile* endospores exhibit reduced exotoxin secretion and improve infection prognosis of Caco-2 cells. A) Immunoblot of TcdA from 10-times concentrated supernatants collected at respectively time points of germinating *C. difficile* culture. Germinant taurocholate was treated under different conditions before incubation with *C. difficile* endospores. From left, no-cell positive control, *gfp* expression control, *cbh* expression treatment, and no-taurocholate negative control. L represents protein ladder. Expected size of TcdA is 308 kDa. Relative cell viability of Caco-2 treated with supernatants collected from germinating *C. difficile* culture under respective conditions, namely B) no-cell positive control, C) *gfp* expression control, D) *cbh* expression treatment, and E) no-taurocholate negative control. Supernatants were diluted to final concentration of 1- (filled), 0.1- (grey), and 0.01-fold (blank). Relative cell viability was given by MTT assay normalised to untreated Caco-2 control. Numeric relative cell viability data is shown in matrix. F) Inverted light microscopy photo of Caco-2 cells after 24 hours treatment with respective supernatants to final concentration of 1-fold. tau represents taurocholate; SA represents sialic acid.

Figure 11 shows efficacy of Cbh-expressing EcN in treatment of murine CDI models infected with *C. difficile*. A) Experimental timeline of infection assay showing point of administration for treatment, probiotics, and *C. difficile*. B) Table of experimental groups and expression constructs of engineered probiotics given. C) Survival curve and D) mean weight of respective groups following infection with *C. difficile* cells over nine days. Gehan-Breslow-Wilcoxon test was performed between treatment and control groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. E) Maximum clinical sickness score (CSS) exhibited by respective mice within each group following infection with *C. difficile* cells over six days.

Unpaired student's *t*-test was performed between treatment and control groups. ** $P < 0.01$, *** $P < 0.001$.

Definitions

Certain terms employed in the specification, examples and appended claims are collected here for convenience.

The terms "amino acid" or "amino acid sequence," as used herein, refer to an oligopeptide, peptide, polypeptide, or protein sequence, or a fragment of any of these, and to naturally occurring or synthetic molecules. Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native amino acid sequence associated with the recited protein molecule.

The term "functional variant" or "variant" as used herein, refers to an amino acid sequence that is altered by one or more amino acids, but retains the same function as the non-variant reference sequence, for example bile salt hydrolase. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "non-conservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR® software (DNASTAR, Inc. Madison, Wisconsin, USA).

As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

The term "probiotic", as used herein, refers to a viable microbial supplement, which has a beneficial influence on a patient through its effects in the intestinal tract, urinary tract or the vaginal tract.

The term "prophylaxis", as used herein refers to treatment given or action taken to prevent disease, such as prevention of CDI-linked disease.

The term "treatment", as used in the context of the invention refers to ameliorating, therapeutic or curative treatment.

The term "subject" is herein defined as vertebrate, particularly mammal, more particularly human. For purposes of research, the subject may particularly be at least one animal model, e.g., a mouse, rat and the like. In particular, for treatment or prophylaxis of dysbiosis, more particularly CDI-linked diseases, the subject may be a human.

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

The general consensus on the mechanisms of CDI and rCDI involves the dysbiosis of microbiome and subsequent germination of dormant endospores as a result (Figure 1) [Petrosillo, N. *Med. Mal. Infect.* 48(1): 18-22 (2018)]. In normal healthy microbiome, *C. difficile* may remain as commensal dormant endospores in the human gastrointestinal tracts without causing infection due to lack of ecological niche. Paradoxically, administration of broad-spectrum antibiotics has been established to be a risk factor that promotes CDI [Petrosillo, N. *Med. Mal. Infect.* 48(1): 18-22 (2018); Roberts, A. P., & Mullany, P. *Clostridium difficile: methods and protocols*. Ed: P.Mullany, A. Roberts. Springer US, New York. 3-8 (2010)]. Administration of antibiotics is postulated to upset the balance of existing microbiome and lead to dysbiosis [Gebhart, D., et al. *mBio*. 6(2): doi: 10.1128/mBio.02368-14 (2015)]. *C. difficile*, either from the germinated dormant endospores or newly acquired from the environment, can exploit this opportunity to rapidly expand to virulence load leading to infection [Gebhart, D., et al. *mBio*. 6(2): doi: 10.1128/mBio.02368-14 (2015); Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008)]. Compounding the problem, in addition to antimicrobial resistance that arises due to selection pressure from antibiotic treatments, *C. difficile* endospores are naturally resistant to antibiotics [Petrosillo, N. *Med. Mal. Infect.* 48(1): 18-22 (2018); Roberts, A. P., & Mullany, P. *Clostridium difficile: methods and protocols*. Ed: P.Mullany, A. Roberts. Springer US, New York. 3-8 (2010)]. This enables the endospores to evade eradication treatment, and is suspected to be a cause of rCDI through relapse following the window of vulnerability until the microbiome homeostasis recovers [Petrosillo, N. *Med. Mal. Infect.* 48(1): 18-22 (2018)].

Evidence suggests that bile salt metabolising species, such as *C. scindens*, within the microbiome confer colonisation resistance against *C. difficile* [Chilton, C. H., Pickering, D. S., & Freeman, J. *Clin. Microbiol. Infect.* Published ahead of print. doi: 10.1016/j.cmi.2017.11.017 (2018); Buffie, C. G., et al. *Nat. Lett.* 517: 205-8 (2015)]. Bile

salts are known germinant of *C. difficile* [Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008)] and disruption of bile salt metabolism due to disrupted intestinal microbiome, could result in germination leading to CDI.

In humans, bile salts are synthesised in liver and secreted into the gastrointestinal tract at the duodenum of small intestine through the gall bladder [Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008)]. Bile salts exist in different molecular forms depending on their conjugates and functional groups. Discharged bile salts from the human liver exist as primary bile salt conjugated to taurine or glycine, forming taurocholate or glycocholate respectively. Taurocholate is a known germinant of *C. difficile* and is routinely utilised to induce endospores germination in laboratory manipulation of the bacteria [Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008)]. It is understood that bile salts undergo modification by the microbiome as they proceed down toward lower gastrointestinal tract [Ridlon, J. M., Kang, D., & Hylemon, P. B. *J. Lipid. Res.* 47: 241-59 (2006); Begley, M., Gahan, C. G. M., & Hill, C. *FEMS. Microbiol. Rev.* 25: 625-51 (2005)]. Notably, the deconjugation of conjugated bile salts into primary unconjugated bile salts, and the 7 α -dehydroxylation of primary bile salts into secondary bile salts. The latter reaction can be mediated by the previously mentioned *C. scindens* [Ridlon, J. M., & Hylemon, P. B. *J. Lipid. Res.* 53: 66-76 (2012)]. Furthermore, secondary bile salt, deoxycholate, was shown to inhibit *C. difficile* colonization [Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008)]. As a result of these modifications, bile salts in colon exist predominantly as unconjugated primary or secondary forms [Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008)].

A model of the mechanisms leading to CDI onset due to dysbiosis is proposed here (Figure 3). Two events due to dysbiosis are suggested to induce CDI. It is hypothesised that dysbiosis disrupted these species in the microbiome that are involved in bile salt modification. This resulted in a dysregulation of bile salt composition in the lower gastrointestinal tract. The increased concentration of conjugated bile salt, particularly taurocholate, acts as a trigger for germination and to signify vacant ecological niches within the microbiome for *C. difficile* colonisation. The second event involves the elevation of free sialic acids in the gastrointestinal tract. Dysbiosis disruption of the microbiota leading to loss of sialic acid utilising strains and/or proliferation of sialidase-expressing strains [Ng, K. M., et al. *Nat. Lett.* 502: 96-9910 (2013)]. This causes an elevation of free sialic acids within the gastrointestinal tract. Germinated vegetative *C. difficile* utilise the free sialic acid as carbon source for further proliferation and expansion leading to CDI.

Here, we sought to invent a prophylactic antimicrobial strategy for the prevention of CDI. Through the understanding of the pathogenesis of *C. difficile*, germination of *C. difficile* endospores was identified as a potential intervention point to prevent progression of the infection. The mechanisms for the onset of infections can be exploited by engineered probiotics to modulate the germination of endospores.

In the proposed model of mechanisms in dysbiosis-induced CDI, conjugated bile salts are hypothesised to be the driving germinant for *C. difficile* endospores germination. During homeostasis state of the microbiome, conjugated bile salts are metabolised by microbiota before reaching the colon where *C. difficile* colonise during CDI. This is disrupted during dysbiosis. As a proof-of-principle to the model, the germination efficiencies of conjugated and deconjugated bile salts were assayed. Taurocholate is deconjugated into cholate and taurine by the enzyme bile salt hydrolase (Enzyme Commission number: EC3.5.1.24) [Coleman, J. P., & Hudson, L. L. *Appl. Environ. Microbiol.* 61(7): 2514-20 (1995)] (Figure 4A). The deconjugated bile salt displayed significantly reduced germination efficiency compared to taurocholate (Figure 4B). Higher concentration of cholate further inhibits the germination in consistent with existing report [Sorg, J. A., & Soenenshein, A. L. *J. Bacteriol.* 190(7): 2505-12 (2008); Begley, M., et al., *FEMS. Microbiol. Rev.* 25: 625-51 (2005)]. Furthermore, cholate was shown to inhibit the growth of vegetative *C. difficile* cells (Figure 4C). These results suggest that modulation of bile salt deconjugation to prevent the germination of *C. difficile* endospores and inhibit their subsequent growth can function as a viable prophylactic antimicrobial strategy against *C. difficile*.

We envision a prophylactic antimicrobial strategy that utilises engineered probiotic strain EcN (D-alanine auxotrophic *Escherichia coli* Nissle) [Hwang, I, Y., et al., *Nat. Commun.* 8: 15028. doi: 10.1038/ncomms15028 (2017)] to modulate the bile salt deconjugation through *in vivo* expression of bile salt hydrolase. This expression of bile salt hydrolase can reduce local taurocholate concentration, which in turn inhibits the germination of *C. difficile* endospores. The delay of *C. difficile* germination from endospores and growth inhibition can prevent excessive expansion leading to CDIs or rCDIs. Bile salt hydrolase, Cbh, from *Clostridium perfringens* was selected for application [Coleman, J. P., & Hudson, L. L. *Appl. Environ. Microbiol.* 61(7): 2514-20 (1995)]. The expression of the enzyme is coupled to sialic acid-responsive promoter, pNanA (SEQ ID NO: 4). Sialic acid was shown to upregulate during dysbiosis of microbiome (10). By coupling pNanA to Cbh expression, pNanA can regulate the expression of Cbh in the event dysbiosis *in vivo*. Under this design, when free sialic acid level is elevated during dysbiosis, pNanA will respond and express Cbh. This enables autonomous *in vivo* response to the onset of dysbiosis.

EcN are utilised as chassis for the delivery of the designed strategy. As gram-negative bacteria, EcN are less susceptible to gram-positive-targeting vancomycin that is commonly administrated for CDI treatment [Nelson, R. Cochrane. Database. Syst. Rev. 18(3): CD004610 (2007)]. This will permit the concurrent administration of antibiotic for treatment and probiotic for preventing rCDIs. Furthermore, EcN are able to utilise sialic acid for metabolism. As probiotic strain, they colonise as part of the microbiome. Hence, they are able to compete against the *C. difficile* for nutrient as well as vacant ecological niches within the gastrointestinal tract. In addition, the auxotrophic characteristic of EcN will enable design of plasmid that can be maintained without antibiotics. The strain has been engineered to display auxotrophic phenotype for D-alanine through the deletion of alanine racemase genes from the genome [Hwang, I, Y., et al., *Nat. Commun.* 8: 15028. doi: 10.1038/ncomms15028 (2017)]. The essential alanine racemase gene is used as a selection marker in plasmid carrying the engineered circuit. The resulting engineered strain can stably maintain designed plasmid for extended period without additional selection pressure. Taken together, the engineered EcN will confer long-term prophylactic effect against CDI in the gastrointestinal tract.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (2001).

EXAMPLE 1

Engineering of antibiotic selection-free probiotic chassis

One of the challenges in implementing engineered probiotics *in vivo* is to ensure long-term stability of genetic circuits in the chassis. Engineered genetic circuits are commonly engineered on plasmids and maintained by antibiotics selection, but such techniques are not feasible for *in vivo* applications. Maintenance of plasmid stability by continuous antibiotics selection cannot be conveniently implemented for colonised probiotics in the gastrointestinal tract.

In order to enable a plasmid-chassis system that does not require antibiotic maintenance, an auxotrophic phenotype was generated in the *E. coli* Nissle wild type strain. Alanine racemase genes, which are essential for D-alanine biosynthesis were deleted from the *E. coli* Nissle genome to generate the strain EcN (Figure 5A). D-alanine is required for the biosynthesis of bacterial cell wall. The absence of D-alanine will prevent the bacteria from further cell division, thereby creating an auxotrophic phenotype.

A plasmid was designed to rescue the auxotrophic phenotype in the EcN chassis (Figure 5B). This plasmid contained a copy of alanine racemase to function as selection gene. It also contained a kanamycin resistance gene for characterisation and amplification purpose. This kanamycin resistance gene was flanked by FRT sites which enable the gene to be removed through the expression of Flp recombinase. This design enables the easy removal of the antibiotic resistance gene to generate a final probiotic strain that contains a self-selecting plasmid. This works to prevent potential horizontal gene transfer of resistance gene from the engineered strain into the microbiome during applications.

The alanine-deleted EcN strain successfully displayed alanine auxotrophic phenotype (Figure 5C). The phenotype was rescued by the expression of designed plasmid pEaat. The strain was also rescued by exogenous supplementation of D-alanine. This enables the strain to be maintained in lab conditions. Furthermore, the plasmid was stably maintained in EcN for a month without the presence of any antibiotic selection (Figure 5D). Plasmids with the antibiotic resistance gene were transformed into EcN or the wild type strain. The cells were grown in the absence of antibiotic. Cells were then tested at regular interval for the presence of plasmids. It was found that only in the engineered EcN were the plasmids maintained consistently at close to 100%. Together, these results show the applicability of EcN to function as chassis for long-term delivery of engineered genetic circuit *in vivo*.

EXAMPLE 2

Optimisation of NanR-dependent sialic acid-inducible promoter

Sequence of sialic acid-inducible promoter pNanA (SEQ ID NO: 4) from MG1655 *E. coli* genome was obtained from EcoCyc database [Keseler, I. M. et al. *Nuc. Acids. Res.* 41: 605-12 (2013)]. The promoter was subcloned upstream to *gfp* gene in pEaat plasmid (pEaat-pNanA-*gfp*) and then transformed into T10 and EcN for expression characterisation (Figure 6A). Surprisingly, the two strains of *E. coli* displayed different responses to sialic acid induction (Figure 6B). Induction of sialic acid resulted in forward activation of T10 pNanA promoter leading to GFP expression. On the other hand, *gfp* under pNanA promoter in EcN was strongly expressed in the absence of sialic acid and was instead repressed with high concentration of sialic acid induction.

The difference in response suggests pNanA is regulated differently between T10 and EcN. NanR (SEQ ID NO: 5) was previously identified as a transcriptional regulator of sialic acid catabolism in *E. coli* [Kalivoda, K. A., et al., *J. Bacteriol.* 185(16): 4806-15]. The sequence of *nanR* in MG1655 genome (GenBank accession CP012868.1) was identified.

The gene sequence along with flanking sequence 500 bp upstream and 500 bp downstream of the gene was isolated (*nanR*±500). BLASTn search with the *nanR*±500 as the query and T10 (identified as DH10β; GenBank accession CP000948.1) or Nissle (GenBank accession CP007799.1) genome sequence as the subject was performed. T10 genome sequence yielded a 100% match with the MG1655 genome. Nissle genome sequence returned a strong match of 96% against *nanR* sequence and 500 bp downstream. However, there is no similarity for 500 bp upstream of *nanR*. Regulatory element of *nanR* was found within 200 bp upstream of the gene. This suggests that although Nissle carries *nanR* sequence, its genetic regulation is disrupted in Nissle in contrast to *E. coli* of K-12 descent.

NanR was hypothesised to function as a transcriptional repressor in *nan* operon expression and the disrupted genomic expression of *nanR* in Nissle resulted in the activity of pNanA promoter observed. To test the hypothesis, *nanR* was subcloned from MG1655 genome into pSC101 vector under pBad promoter (pSC101-pBad-*nanR*) (Figure 6C). The plasmid was co-transformed with pEaat-pNanA-*gfp* into EcN. The promoter pBad is regulated by a co-expressed AraC and can be induced by L-arabinose. The co-induction of L-arabinose and sialic acid in different combinations can determine the expression level of *nanR* required to elicit an ideal response from pNanA. The induced cells were quantified by flow cytometry for the median GFP fluorescence reading of 10,000 size-gated samples. From the results, even in the absence of L-arabinose induction, basal expression of NanR represses pNanA basal activity significantly (Figure 6D). It also enabled pNanA promoter to be induced for expression by sialic acid. Enhanced expression of NanR reduced both pNanA basal and induced activity. This suggests that NanR is a regulator upstream of pNanA expression and only a low level of NanR is required for the regulation.

Since NanR expression is required to achieve a sialic acid-inducible response of pNanA in EcN, the genetic construct pEaat-pNanA-*gfp* was redesigned to co-express *nanR* under constitutive expression (Figure 6E). The gene *nanR* was subcloned under constitutive promoter J23108 with ribosome binding site rbs5, rbs3, or rbs2. The three ribosome binding sites have different translation initiation rate, with rbs5 being the strongest and rbs2 being the weakest. The plasmids were transformed into EcN for characterisation of GFP expression with sialic acid induction. Consistent with the flow cytometry results, it was found that weak constitutive expression of NanR under ribosome binding site rbs2 showed better inductibility with sialic acid (Figure 6F). The expression of NanR was further reduced in constructs whereby the constitutive promoter was replaced with J23113 and the ribosome binding site with rbs4 (J23113-rbs4-NanR; SEQ ID NO: 6). Characterisation of GFP

expression upon sialic acid induction in these constructs maintained similar expression levels compared to previous constructs despite the weaker expression of NanR.

Through the co-expression of NanR transcriptional regulator, the activity of promoter pNanA was successfully reversed from that of a repressible promoter to that of an inducible promoter of sialic acid. Further, the basal expression of pNanA was reduced. These resulted in a versatile dual-functional promoter for EcN that can respond differently to sialic acid depending on the engineered circuitry. The promoter pNanA can function as an inducible promoter or a repressed promoter of sialic acid depending on the presence of NanR. Moreover, *nanR* can be placed under further inducible or repressible expression to enable an additional layer of control. The construct pEaat-J23113r4-*nanR*-pNanA-*gfp* was selected for further characterisation as a sialic acid-based dysbiosis biosensor.

EXAMPLE 3

Characterisation of engineered sialic acid biosensor under gastrointestinal-specific conditions

Parental strain of EcN preferentially colonises in the lower gastrointestinal tract specifically the colon and rectum [Sonnenborn, U., & Schulze, J. *Micob. Ecol. Health Dis.* 21: 122-58 (2009); Schultz, M. *Inflamm Bowel Dis.* 14(7): 1012-8 (2008)]. This makes it ideal as a dysbiosis biosensor for the lower gastrointestinal tract. The selected construct was characterised under a series of conditions specific to the lower gastrointestinal tract to assess its suitability.

It was noted that pNanA sequence contains a catabolite activator protein (CAP) binding sites at position 4 → 8. CAP is a transcriptional activator protein that initiates the transcriptional process when bound to cyclic AMP (cAMP). cAMP level is elevated in the absence of glucose, thereby effectively functions as a transcriptional activator for low glucose response. EcN harbouring the inducible (pEaat-J23113r4-*nanR*-pNanA-*gfp*) or repressible (pEaat-pNanA-*gfp*) constructs were subjected to GFP expression characterisation with sialic acid and/or glucose induction. Expectedly, EcN expressing the inducible construct presented a response similar to that of a classical *lac* operon. GFP expression was repressed in the presence of glucose even when sialic acid was present (Figure 7A & 7B). This suggests that NanR might be functionally similar to LacI. Interestingly, EcN expressing the repressible construct responded similarly to glucose, whereby GFP expression is silenced in presence of glucose. These results suggest that transcription of pNanA is driven by cAMP-CAP. The repression of the promoter in the presence of glucose makes it ideal for use in the lower gastrointestinal tract where the level of glucose is

expected to be significantly lower. The constructs, both inducible and repressible, are likely to be silenced and non-responsive to sialic acid in upper gastrointestinal tract, and hence their effects will be minimised during the passage through the upper gastrointestinal tract.

Although the trend of pNanA activity in EcN was reversed from a sialic acid repressible promoter to an inducible promoter, it was noted that the induced signal of the inducible construct was significantly lower than even the repressed signal of the repressible construct. The low induced expression level was an issue for the sufficient Cbh expression to deconjugate taurocholate. To overcome this issue, an amplifier module was designed and added to the inducible construct. The gene that encodes for transcriptional activator CadC (SEQ ID NO: 12) was subcloned under pNanA promoter. The promoter pCadBA regulated by CadC was subcloned upstream of *gfp* (J23113r4-*nanR*-pNanA-*cadC*-pCadBA-*gfp*). Under this design, upon sialic acid induction, expression of CadC under pNanA will in turn activate pCadBA promoter for stronger expression of GFP (Figure 7C). Here, CadC and pCadBA acts as intermediate transduction module to amplify the signal from pNanA and lead to stronger expression of GFP. Furthermore, CadC was selected due to its responsiveness to external pH as evident from later characterisation (Figure 7F). The construct was characterised and the induced GFP fluorescence signal was shown to improve significantly. The addition of *cadC*-pCadBA (SEQ ID NO: 7) into the circuit successfully enabled amplification of signal from the inducible construct (Figure 7D). However, a temporal lag in GFP expression compared to non-amplifier construct was observed. This could be attributed to the need for intermediate CadC expression, thereby delaying the response, as well as increasing the basal expression level.

The targeted site for the biosensor colonisation is in the lower gastrointestinal tract, where nutrient level of the environment is expected to be poor. All prior characterisation assays were performed in nutrient-rich LB. It is likely that the construct may behave differently in the gut environment where overall nutrition level is different. To determine if the biosensor can function as intended *in gastro*, the sialic acid inducible (J23113r4-*nanR*-pNanA-*gfp*) and sialic acid inducible-amplifier (J23113r4-*nanR*-pNanA-*cadC*-pCadBA-*gfp*) constructs were characterised in M9 minimal medium which functioned as a closer approximation to the gut environment. M9 minimal medium typically contain a carbon source in the form of glucose. As shown previously where glucose interfered with pNanA activity, glycerol was used as the carbon source for M9 minimal medium. Despite the nutrient-poor condition, EcN expressing sialic acid inducible constructs were able to respond as intended to sialic acid induction (Figure 7E). Further, the temporal response lag previously observed was not as prominent during the characterisation in M9 minimal medium. As previously

theorised, pNanA expression could be driven by cAMP-CAP. The lower nutrient content of M9 minimal medium could have resulted in a high level of cAMP, and therefore led to a faster response to sialic acid induction. However, the current result is still inconclusive to verify this proposition. Nonetheless, the results demonstrate that the lower nutrient condition
5 does not adversely affect activity of the inducible constructs.

Another gut environment condition simulated was pH level. The pH level in the gastrointestinal tract is dynamic and differs based on factors including health conditions. The range of pH in healthy subjects was reported to be in the range of 1.6 to 4.2 in the gastric, 6.7 to 7.3 in the small intestine, 5.4 to 6.5 in the cecum, and 6.0 to 7.2 in the colon [Maurer,
10 et al. *PLoS. One.* 10(7): e0129076 doi: 10.1371/journal.pone.0129076 (2015)]. The sialic acid inducible-amplifier construct was characterised in M9 with glycerol medium at pH ranging from 3 to 9. The pH level was observed to influence the activity of the construct (Figure 7F). No induction activity was observed at low pH from 3 to 5. Surprisingly, instead the expression of GFP was repressed in the presence of sialic acid. The basal expression
15 level increased at pH 6 and subsequently decreased. The *cad* operon was reported to be pH sensitive and was activated at pH 5.8 [Watson, N., et al., *J. Bacteriol.* 174(2): 530-40(1992)]. This could have resulted in the basal activity at pH 6 which subsequently decreased at higher pH. The construct was mildly induced from the basal level at pH 6 but strongly induced at high pH from 7 to 9. From the data, the biosensor can be expected to be inactive
20 in gastric due to the low pH environment. Its activity is expected to be higher in the small intestines and colon where pH is between 6.0 and 7.3. Further, it was reported that pH of colon and rectum shift towards basic during *C. difficile* infections with 87% of the CDI patients having stool of more than pH 7 [Gupta, P., et al., *South. Med. J.* 109(2): 91-6 (2016)]. Consistent with the report, a lower pH is associated with a protective effect against
25 *C. difficile* infections [May, T., et al., *Scand. J. Gastroenterol.* 29(10): 916-22 (1994)]. It is unclear whether the pH shift is a result of dysbiosis of the microbiome or a result of pathogen expansion. Nonetheless, the change in pH may enable the biosensor to elicit a stronger response.

It was observed that EcN was able to utilise sialic acid as a carbon source for growth
30 in M9 minimal medium without glycerol (Figure 7G). The difference in growth rate was not observed in M9 minimal medium containing glycerol. Sialic acid was reported to be utilised by *C. difficile* and pathogenic *E. coli* for expansion during infections [Ng, K. M., et al., *Nat. Lett.* 502: 96-99 (2013); Huang, Y. L., et al., *Nat. Commun.* 6: 8141. doi: 10.1038/ncomms9141 (2015)]. This can enable EcN to double as a nutrient competitor

against pathogens for sialic acid in addition to being a biosensor or delivery chassis. Further, the supplementation to EcN growth can amplify the expression of Cbh (SEQ ID NO: 13).

EXAMPLE 4

Purified bile salt hydrolase Cbh deconjugates taurocholate into cholate and inhibits *C. difficile* endospore germination

The native gene sequence for *cbh* (SEQ ID NO: 8) was codon-optimised for expression in *E. coli* as well as for compatibility to the BglBrick standard. The codon-optimised sequence is set forth in SEQ ID NO: 9. A C-terminus his6-tag sequence was added and the final gene sequence was subcloned under pBad promoter in pEaat-araC vector. The plasmid was then transformed into *E. coli* strain BL21 for protein expression. Cbh-his6 was induced for expression by L-arabinose and then purified first by IMAC, followed by size exclusion chromatography. Fractions containing Cbh-his6 were then concentrated to 2 mL and yielded a final concentration of 30.66 μ M. Proteins with size corresponding to Cbh-his6 can be observed on SDS-PAGE assay of the purified proteins (Figure 8A).

The activity of Cbh was determined by taurocholate to cholate conversion. HPLC was utilised to determine the concentration of bile salts after enzymatic treatment. HPLC analysis was first run against known concentrations of taurocholate and cholate to determine the retention times and obtain standard curves. Detection of bile salts was performed at 205 nm. Taurocholate was eluted in approximately 12.2-minute runtime, while cholate was eluted in approximately 19.6-minute runtime. The standard curves of taurocholate and cholate were constructed (Figures 8B and 8C).

The activity of Cbh was tested against taurocholate by incubating 10 μ M of purified Cbh-his6 with 10 mM of taurocholate. Bile salt was extracted and analysed with HPLC. The concentrations of both taurocholate and cholate were determined for the experiment and negative control without Cbh-his6 (Figures 8D-8F). No cholate was detected in the negative control. On the other hand, incubation with the protein resulted in a 100-fold decrease in the concentration of taurocholate. Conversely, a high concentration of cholate was detected. A peak corresponding to taurine was not detected within the scope limited by HPLC operating conditions. It can be inferred that heterologous expressed Cbh-his6 retained its native activity to deconjugate taurocholate into cholate. Further, the result showed that approximately 99% of taurocholate was deconjugated within 3 hours of incubation with purified Cbh-his6.

Further reactions of Cbh-his6 with taurocholate were set up with appropriate controls and aliquots were collected. The aliquots were then incubated with purified *C. difficile* endospores. Germination of the endospores were enumerated by CFU counting. Since taurocholate composition was reduced when incubated with Cbh-his6 due to deconjugation into cholate, the germination efficiency from that of the aliquots was expected to reduce. Expectedly, taurocholate incubated with Cbh-his6 showed a 12-fold reduction in endospore germination compared the positive control (Figure 8G). This result is consistent with the proof-of principle performed on taurocholate and cholate germination efficiency (Figure 4B). These results supported the proposed strategy for CDI prevention through bile salt composition modulation.

EXAMPLE 5

Bile salt hydrolase Cbh expression in probiotics inhibits the germination of *C. difficile* endospores

The gene *cbh-his6* was subcloned under pNanA promoter in the sialic acid inducible construct pEaat-J23113r4-*nanR*-pNanA. The construct expressing *gfp* in place of *cbh* functioned as an expression control. The resulting plasmids pEaat-J23113r4-*nanR*-pNanA-*cbh-his6* and pEaat-J23113r4-*nanR*-pNanA-*gfp* were transformed into EcN chassis. The strains were incubated with taurocholate and sialic acid. Cell-free positive control, non-induced *cbh-his6* expressing strain control, and no-taurocholate *cbh-his6* expressing strain negative control were set up as well. Filtered culture medium from each experiment was then collected and tested for *C. difficile* endospore germination efficiency. The EcN strain expressing Cbh-his6 reduced *C. difficile* endospore germination by approximately 1-fold after sialic acid induction compared to the GFP expression negative control (Figure 9A; blank columns). Although this is a promising result, the fold change is significantly lower in relation to those obtained from purified Cbh-his6. The difference was due to the low expression level of sialic acid-inducible construct. An immunoblot with anti-his antibody was performed and the expression level of Cbh-his6 under pEaat-J23113r4-*nanR*-pNanA construct was found to be significantly lower than under pEaat-*araC*-pBad construct (Figure 9B; lane 2-5). This was in agreement with our dysbiosis sensor characterisation data. To resolve this issue, the CadC amplifier module was designed into the genetic circuit to amplify the inducible expression as previously outlined.

The gene *cbh-his6* was subcloned under the amplifier construct pEaat-J23113r4-*nanR*-pNanA-*cadC*-pCadBA. Expression of Cbh-his6 in EcN strain was verified by immunoblot (Figure 9B; lane 6-9). The expression level was amplified, however, it also resulted in a high basal expression level. We then repeated *C. difficile* endospore

germination assay with EcN expressing Cbh-his6 or GFP under the new amplifier circuitry. A stark improvement in *C. difficile* endospore germination reduction was observed (Figure 9A; filled columns). The EcN strain expressing Cbh-his6 was able to reduce *C. difficile* endospore germination by approximately 40-fold after sialic acid induction compared to the expression control. In the absence of sialic acid induction, an approximately 10-fold reduction in endospore germination was achieved. This could be attributed to the strong basal expression of Cbh-his6.

Filtered culture medium from the experiment and controls were also collected for HPLC analysis (Figure 9C). Taurocholate and cholate concentrations were detected based on previously identified retention time. No cholate was detected in cell-free positive control and GFP expression control, while no taurocholate was detected in induced Cbh-his6 expressing strain. Uninduced Cbh-his6 expressing strain only showed approximately 45% deconjugation of taurocholate in spite of a 10-fold reduction in endospore germination. This suggests that a high accumulation of taurocholate is necessary to achieve effective *C. difficile* endospore germination. Conversely a slight disruption to the build-up of taurocholate can strongly reduce the germination of endospores. These results are consistent with the proof-of-principle germination efficiency assay performed (Figure 4B). The amplifier construct exhibited a strong basal expression level of Cbh-his6. This basal expression of Cbh-his6 provides a ready-response against any accumulation of taurocholate even in the absence of sialic acid induction.

EXAMPLE 6

Bile salt hydrolase Cbh-treated *C. difficile* endospores exhibit reduced exotoxin secretion and improve infection prognosis of Caco-2 cells

Expression of Cbh in EcN cells was shown to inhibit *C. difficile* endospore germination by modulation of the bile salt conjugation state. The inhibition of endospore germination will in turn delay expansion of vegetative *C. difficile*. In order to determine whether the delayed expansion will represent a difference in pathology of CDI, germinated *C. difficile* were tested against Caco-2 cells, a human colon epithelial cell line isolated from colorectal adenocarcinoma.

As *C. difficile* and Caco-2 were unable to grow in the same laboratory condition, a staggered coculture was performed. This was made possible due to the etiology of CDI being secreted exotoxins [Carter, G. P., et al., *mBio*. 6(3): e00551. doi: 10.1128/mBio.00551-15 (2015)]. Germinated *C. difficile* were first grown in permissible conditions with culture medium collected at regular intervals. The filtered culture medium was then concentrated

and buffer-exchanged before incubation with Caco-2 cells. Although this method does not take into account direct interaction between *C. difficile* and Caco-2 cells, it allows secreted toxins from *C. difficile* to be tested against Caco-2 cells.

Experiment and controls similar to previous *in vitro* assay were set up. *C. difficile* endospores were germinated with taurocholate treated by Cbh-expressing EcN (Figure 10A, lanes 8-10; 10D; 10F bottom left panel), along with cell-free taurocholate positive control (Figure 10A, lanes 2-4; 10B; 10F top middle panel), GFP-expressing EcN expression control (Figure 10A, lanes 5-7; 10C; 10F top right panel), or taurocholate-free negative control (Figure 10A, lanes 11-13; 10E; 10F bottom right panel). Immunoblot was performed on 10-times concentrated filtered supernatants from the experiments collected at regular intervals, and probed for toxin TcdA. Results indicated a reduction in the amount of TcdA secreted into supernatants when treated with Cbh-expressing EcN (Figure 10A). A time-dependent accumulation of toxins can also be consistently observed. It can be suggested that the inhibition of *C. difficile* endospore germination delayed the growth of vegetative cells and reduced the toxin load secreted into the medium.

Supernatants were collected at regular intervals following *C. difficile* induction for germination. The supernatants were concentrated and then serial diluted to 1- to 0.01-fold when incubated with Caco-2 cells. The cell viabilities of Caco-2 were assayed with MTT following 48 hours incubation. Expectedly, Caco-2 incubated with supernatant collected from Cbh-expressing EcN treated *C. difficile* showed higher final cell viability compared to those of expression control (Figures 10B-10E). This is consistent with the amount of TcdA in the supernatants. Further, the cell morphology of Caco-2 was observed to be different between treatment with the supernatants from Cbh-expressing EcN and expression control (Figure 10F). Caco-2 treated with supernatant from Cbh-expressing EcN showed morphology close to untreated Caco-2. On the other hand, treatment with supernatant from expression control result in similar morphology as treatment with supernatant from positive control. The Caco-2 cells showed detachment from the culture plates and shrivelled cell shape associated with cell death.

Overall, the assays showed that Cbh-expressing EcN can improve prognosis of *ex vivo* Caco-2 cell culture. Cbh expressed from EcN can deconjugate taurocholate into cholate, resulting in reduced germination of *C. difficile* endospores. The delay in germination affected the secretion of exotoxins into culture medium, and this in turn resulted in improvement in Caco-2 infection prognosis. It was noted TcdA continued to be secreted into supernatant from Cbh treatment and not entirely inhibited. This is consistent with the *in vitro* assay, a small *C. difficile* germination even after treatment with engineered EcN (Figure 9A). These

vegetative cells proliferated in the culture medium leading to secretion of TcdA. It is possible that the exotoxins may accumulate even after treatment with engineered EcN. However, the nutrient-poor environment *in vivo* may limit the proliferation of *C. difficile*, and initial germination of cells has a greater effect on the overall virulence load than proliferation that follows. The extent of these dynamics will need to be further elucidated *in vivo* to determine the effectiveness of the treatment. Nonetheless, it is conclusive that the treatment of *C. difficile* endospores with engineered EcN results in a reduced amount of exotoxins secreted. This in turn improved Caco-2 cell viability compared to the expression control.

Example 7

Pre-treatment with engineered probiotics expressing dysbiosis sensor-controlled bile salt hydrolase Cbh provided protection against infection from *C. difficile* in murine model

Murine model of CDI had previously been demonstrated [Chen, X., et al., *Gastroenterology*. 135: 1984-1992 (2008)]. This model was adapted and modified in this study to test the efficacy of the engineered EcN (Figure 11A). Briefly, the mice were given a dose of engineered probiotics (treatment or control groups) or blank (infection control group) 3 days prior to infection (day -3). Infection by *C. difficile* (10^7 CFU) was performed on day 0. Mortality, weight, and clinical symptoms of the mice were then recorded over the course of 9 days. The clinical symptoms were then scored and tabulated according to previously established standards [Shelby, R. D., et al., *Int. J. Surg.* doi: 10.1080/08941939.2019.1571129 (2019)].

The treatment group ('EcN-*cbh*') was given probiotics harbouring the Cbh-expressing construct (pEaat-J23113r4-*nanR*-pNanA-*cadC*-pCadBA-*cbh*). This construct consists of multiple genetic modules namely, sensor, amplifier, and actuator. In order to adequately demonstrate the efficacy of the fully engineered probiotics, various control probiotics were generated to comprise constructs that lack one of each genetic module. The probiotics generated for these control groups are summarised in Figure 11B. No-sensor control group ('EcN-pCon-*cbh*') was given engineered probiotics that constitutively express Cbh without dysbiosis sensor. No-amplifier control group ('EcN-pNanA-*cbh*') was given engineered probiotics that lack amplifier module. No-actuator control group ('EcN-*gfp*') was given engineered probiotics that express GFP in place of Cbh. All probiotics were administered in a single dose of 10^9 CFU on day -3, and infection control was given sucrose in place of engineered probiotics.

Mice were infected with 10^7 CFU of *C. difficile* on day 0 of the assay (Figure 11A). Survival of the treatment group performed significantly better than all control groups. 100% of mice survived the infection (Figure 11C) compared to infection control (70.0%; $P = 0.0461$), no-sensor control (14.3%; $P < 0.0001$), no-amplifier control (50.0%; $P = 0.0063$), and no-actuator control (25.0%; $P = 0.0005$). Infected mice displayed severe symptoms between day 2 to day 4. The relative weight of treatment group during this period was comparatively more stable to all control groups which showed relative weight loss of more than 10% (Figure 11D). Each mouse was also assigned a clinical sickness score (CSS) ranging from 0 to 12 daily from day 0 to 6. The CSS is assigned according to three criteria; stool, behaviour, and weight loss [Shelby, R. D., et al., *Int. J. Surg.* doi: 10.1080/08941939.2019.1571129 (2019)]. Based on CSS, the severity of the infection can be described as normal (0 to 2), mild (3 to 5), moderate (6 to 8) and severe (9 to 12). The CSS scored by each mouse was then used to tabulate the mean score of the group (Figure 11D). Treatment group scored the lowest CSS compared to all groups with a mean of 3.3. This score is significantly lower compared to infection control group (7.0; $P = 0.0075$), no-sensor control group (8.7; $P = 0.0005$), no-amplifier control group (7.5; $P = 0.0026$), and no-actuator control group (8.5; $P = 0.0011$). Taken together, these results demonstrate that the administration of engineered probiotics prior to *C. difficile* infection was able to confer prophylactic protection that improve infection prognosis through lowering mortality and reducing severity of symptoms.

Further, the results showed that constitutive expression of Cbh in the no-sensor group did not improve survival of mice compared to the no-actuator control ($P = 0.247$). Conversely, the expression of Cbh from dysbiosis sensor in the no-amplifier group showed an improvement in survival over the no-sensor group ($P = 0.0305$). This is in spite of expression level of Cbh from constitutive promoter being higher than that from pNanA promoter *in vitro*. The outcomes of the various control groups suggest that the dysbiosis sensing module that drives on-demand *in vivo* expression of Cbh is necessary in achieving the intended function of modulating *C. difficile* infection. The nutrient level in lower gastrointestinal tract is expected to be poorer, and inefficient allocation of nutrient towards continuous expression of enzymes might have worked against the no-sensor probiotics. The result highlights the importance of the dysbiosis sensor in controlling the expression of Cbh from engineered probiotics to achieve high activity against CDI *in vivo*.

Taken together, these results demonstrate that engineered probiotics expressing dysbiosis sensor-controlled bile salt hydrolase Cbh are able to provide prophylactic resistance against *C. difficile* infection. Both the restoration of bile salt metabolism and the

dysbiosis-sensing module were demonstrated to be critical in providing protection against infection. Taken together, the probiotics demonstrated high efficacy as prophylaxis against infection of *C. difficile*.

5 **EXAMPLE 8**

Expression of the genetic circuit in other probiotic strains to achieve similar therapeutic functions

The genetic circuit in this invention can be easily expressed in other probiotic species, both of gram negative and gram positive. Many species of native probiotics can be
10 engineered as live biotherapeutics [O'Toole, P. W., Marchesi, J. R., & Hill, C. *Nat. Microbiol.* 2: 17057. doi: 10.1038/nmicrobiol.2017.57 (2017)]. Examples of such species include, but are not limited to, *Bacteroides sp.*, *Clostridium sp.*, *Faecalibacterium sp.*, *Lactococcus lactis*, and *Lactobacillus sp.* This invention addresses difficult technical issues of enzymatic expression and response to dysbiosis. The expression can be grafted onto other probiotic
15 species to achieve similar therapeutic functions. This can enable the engineered probiotics to colonise and target other locations of the gastrointestinal tract such as the duodenum, jejunum or ileum.

EXAMPLE 9

The antibiotic selection-free probiotic chassis can be applied to deliver other genetic 20 circuits *in situ*

An antibiotic selection-free probiotic chassis was engineered through the generation of auxotrophic phenotype in *E. coli* Nissle strain. This chassis is accompanied by a plasmid consisting of alanine racemase gene as selection marker. This chassis enables the delivery of engineered genetic circuit *in situ* and can be utilised for other purposes such as, but not
25 limited to, pathogen targeting, cancer targeting, and metabolites/biologic synthesis and delivery.

EXAMPLE 10

The sialic acid-based sensor functions as a proxy to dysbiosis and can be applied to other dysbiosis-associated diseases and infections

30 This invention responds to a dysbiosis event based on a sialic acid-responsive promoter. The sialic acid-responsive promoter can be engineered to respond to either upregulation or downregulation of sialic acid. Dysbiosis of the microbiome is also associated with a number of other diseases such as, but not limited to, inflammatory bowel disease,

pathogenic infections, type-2 diabetes mellitus, asthma, obesity, autism, and rheumatoid arthritis [Packey, C., D., & Sartor, R. B. *Curr. Opin. Infect. Dis.* 22(3): 292-301 (2009)]. The sialic acid-based sensor can be applied to engineered biotherapeutics that target such diseases.

5 **EXAMPLE 11**

The genetic circuit can be optimised and integrated into EcN genome to confer further stability

In this invention, the genetic circuit is expressed on plasmids. Alternatively, the genetic circuit can be integrated into the genome for further stability. This will avoid unnecessary but potential horizontal gene transfer to the microbiome. Multiple sites of integration have been identified in the EcN genome [Isabella, V. M., et al., *Nat. Biotech.* 36: 857-864 (2018)]. Integration of this genetic circuit can be performed at these sites without disrupting the genome stability of the probiotic strain, whilst resisting spontaneous loss or inactivation of the integrated genetic circuit.

15 **Summary**

Engineered probiotic chassis EcN

An embodiment of the invention provides *E. coli* Nissle with two alanine racemase genes deleted from the genome, which is able to maintain plasmids containing an alanine racemase gene as selection marker for an extended period without additional selection. This avoids unnecessary exposure of antibiotic resistance genes to the microbiome. EcN can then be co-administered with *C. difficile*-targeting antibiotics regimens to colonise the gastrointestinal tract and exert antimicrobial activity against *C. difficile*. The engineered probiotic can remain in the GI tract for an extended period, enabling prophylactic applications.

25 Sialic acid inducible system

In an embodiment of the invention, a sialic acid inducible system is provided which consists of a genetic circuit including pNanA promoter and optional NanR transcription factor, CadC transcriptional factor and its promoter pCadBA. NanR reverses the inducibility of pNanA and CadC-pCadBA amplifies the overall expression level. This system responds to changes in sialic acid depending on the parts used in the system. Elevated sialic acid levels are associated with dysbiosis of the gastrointestinal microbiome, so the system provides a timely response to dysbiosis events through the expression of therapeutic proteins limited to the occurrence of the dysbiosis event.

CadC amplification system

In an embodiment of the invention, the element CadC protein and pCadBA promoter are provided to amplify expression from the sialic acid-responsive promoter through an intermediate transcription activator expression. The primary function is to amplify expression of bile salt hydrolase to a therapeutically significant level. A secondary function is to enable the genetic circuit to be sensitive to pH; which provides an additional layer of control to bile salt hydrolase expression.

Bile salt hydrolase expression

Bile salt hydrolase is expressed to catalyse the deconjugation of taurocholate into cholate in order to reduce the endospore germination efficiency of *C. difficile* caused by the elevated bile salt during dysbiosis, an event that precedes CDIs. The enzyme inhibits germination of endospores and leads to an overall reduction of toxins secreted by *C. difficile*.

By expressing bile salt hydrolase preemptively in response to dysbiosis, the probiotic is able to function as an autonomous prophylaxis against CDIs. This strategy will be effective against the prevention of rCDIs as well. Hence, this probiotic address a gap in CDI management and can be targeted at patients who are at risk of CDIs and rCDIs.

An advantage of the invention is that it provides a non-bactericidal approach to controlling CDI and rCDI; thereby avoiding resistance towards this method.

References

Any listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that such document is part of the state of the art or is common general knowledge.

Begley, M., Gahan, C. G. M., & Hill, C. (2005) The interaction between bacteria and bile. *FEMS. Microbiol. Rev.* 25: 625-51.

Buffie, C. G., Bucci, V., Stein, R. R., McKenny, P. T., Ling, L., Gobourne, A., et al. (2015) Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nat. Lett.* 517: 205-8.

Carter, G. P., Chakravorty, A., Pham Nguyen, T. A., Mileto, S., Schreiber, F., Li, L., et al. (2015) Defining the roles of TcdA and TcdB in localised gastrointestinal disease, systemic organ damage, and the host response during *Clostridium difficile* infections. *mBio.* 6(3): e00551. doi: 10.1128/mBio.00551-15.

Chen, X., Katchar, K., Goldsmith, J. D., Nanthakumar, N., Chekins, A., Gerding, D. N., et al. (2008) A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology.* 135: 1984-1992.

- Chilton, C. H., Pickering, D. S., & Freeman, J. (2018) Microbiological factors affecting *Clostridium difficile* recurrence. *Clin. Microbiol. Infect.* Published ahead of print. doi: 10.1016/j.cmi.2017.11.017.
- 5 Coleman, J. P., & Hudson, L. L. (1995) Cloning and characterization of a conjugated bile acid hydrolase gene from *Clostridium perfringens*. *Appl. Environ. Microbiol.* 61(7): 2514-20.
- Gebhart, D., Lok, S., Clare, S., Tomas, M., Stares, M., Scholl, D., et al. (2015) A modified R-type bacteriocin specifically targeting *Clostridium difficile* prevents colonization of mice without affecting gut microbiota diversity. *mBio.* 6(2): doi: 10.1128/mBio.02368-14
- 10 Gupta, P., Yakubov, S., Tin, K., Zea, D., Garankina, O., Ghitan, M., et al (2016) Does alkaline colonic pH predispose to *Clostridium difficile* infection? *South. Med. J.* 109(2): 91-6.
- Huang, Y. L., Chassard, C., Hausmann, M., von Itzstein, M., & Hennet, T. (2015) Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice. *Nat. Commun.* 6: 8141. doi: 10.1038/ncomms9141.
- 15 Hwang, I. Y., Koh, E., Wong, A., March, J. C., Bentley, W. E., Lee, Y. S., & Chang, M. W. (2017) Engineered probiotic *Escherichia coli* can eliminate and prevent *Pseudomonas aeruginosa* gut infection in animal models. *Nat. Commun.* 8: 15028. doi: 10.1038/ncomms15028.
- Isabella, V. M., Ha, B. H., Castillo, M. J., Lubkowitz, D. J., Rowe, S. E., Anderson, C. L., et al. (2018) Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nat. Biotech.* 36: 857-864.
- 20 Kalivoda, K. A., Steenbergen, S. M., Vimr, E. R., & Plumbridge, J. (2003) Regulation of sialic acid catabolism by the DNA binding protein NanR in *Escherichia coli*. *J. Bacteriol.* 185(16): 4806-15.
- Keseler, I. M., Mackie, A., Peralta-Gil, M., Santos-Zavaleta, A., Gama-Castro, S., Bonavides-Martinez, C., et al. (2013) EcoCyc: fusing model organism databases with synthetic biology. *Nuc. Acids. Res.* 41: 605-12.
- 25 Maurer, J. M., Schellekens, R. C. A., van Rieke, H. M., Wanke, C., Iordanov, V., Stellaard, F., et al. (2015) Gastrointestinal pH and transit time profiling in healthy volunteers using the IntelliCap system confirms ileo-colonic release of ColoPulse tablets. *PLoS. One.* 10(7): e0129076 doi: 10.1371/journal.pone.0129076
- 30 May, T., Mackie, R. I., Fahey Jr, G. C., Cermin, J. C., & Garleb, K. A. (1994) Effect of fiber source on short-chain fatty acid production and on the growth and toxin production by *Clostridium difficile*. *Scand. J. Gastroenterol.* 29(10): 916-22.
- Nelson, R. (2007) Antibiotic treatment for *Clostridium difficile*-associated diarrhea in adults. *Cochrane. Database. Syst. Rev.* 18(3): CD004610.
- 35 Ng, K. M., Ferreyra, J. A., Higginbottom, S. K., Lynch, J., Kashyap, P. C., Gopinath, S., et al. (2013) Microbiota-liberated host sugars facilitate post antibiotic expansion of enteric pathogens. *Nat. Lett.* 502: 96-99.
- O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017) Next-generation probiotic: the spectrum from probiotics to live biotherapeutics. *Nat. Microbiol.* 2: 17057. doi: 10.1038/nmicrobiol.2017.57.

- Packey, C., D., & Sartor, R. B. (2009) Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. *Curr. Opin. Infect. Dis.* 22(3): 292-301.
- 5 Petrosillo, N. (2018) Tackling the recurrence of *Clostridium difficile* infections. *Med. Mal. Infect.* 48(1): 18-22.
- Ridlon, J. M., Kang, D., & Hylemon, P. B. (2006) Bile salt biotransformation by human intestinal bacteria. *J. Lipid. Res.* 47: 241-59
- Ridlon, J. M., & Hylemon, P. B. (2012) Identification and characterization of two bile acid coenzyme A transferases from *Clostridium scindens*, a bile acid 7 α -dehydroxylating intestinal bacterium. *J. Lipid. Res.* 53: 66-76.
- 10 Roberts, A. P., & Mullany, P. (2010) *Clostridium difficile*: no longer an enigmatic pathogen? *Clostridium difficile: methods and protocols*. Ed: P.Mullany, A. Roberts. Springer US, New York. 3-8
- Schultz, M. (2008) Clinical use of *E. coli* Nissle 1917 in inflammatory bowel disease. *Inflamm Bowel Dis.* 14(7): 1012-8.
- 15 Shelby, R. D., Tengberg, N., Conces, M., Olson, J. K., Navarro, J. B., Bailey, M. T., et al. (2019) Development of a standardized scoring system to assess a murine model of *Clostridium difficile* colitis. *Int. J. Surg.* doi: 10.1080/08941939.2019.1571129.
- Sonnenborn, U., & Schulze, J. (2009) The non-pathogenic *Escherichia coli* strain Nissle 1917 – features of a versatile probiotic. *Micob. Ecol. Health Dis.* 21: 122-58.
- 20 Sorg, J. A., & Soenenshein, A. L. (2008) Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *J. Bacteriol.* 190(7): 2505-12.
- Watson, N., Dunyak, D. S., Rosey, E. L., Slonczewski, J. L., & Olson, E. R. (1992) Identification of elements involved in transcriptional regulation of the *Escherichia coli* cad operon by external pH. *J. Bacteriol.* 174(2): 530-40.
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CLAIMS:

1. An expression cassette comprising;
 - i. a bile salt hydrolase gene, and
 - ii. a sialic acid-responsive promoter operably linked to the bile salt hydrolase gene.
2. The expression cassette according to claim 1, wherein the bile salt hydrolase gene is a Choloylglycine hydrolase (Cbh) protein-encoding polynucleotide sequence from *Clostridium perfringens*, preferably encoding the amino acid sequence set forth in SEQ ID NO: 13 or a functional variant thereof.
3. The expression cassette according to claim 2, wherein the Cbh protein-encoding polynucleotide sequence comprises a nucleic acid sequence that has at least 80% sequence identity due to redundancy of the genetic code to the nucleic acid sequence set forth in SEQ ID NO: 8 or SEQ ID NO: 9.
4. The expression cassette according to claim 2 or 3, wherein the sialic acid-responsive promoter is pNanA from *E.coli*, preferably comprising the nucleic acid sequence set forth in SEQ ID NO: 4 or a functional variant thereof.
5. The expression cassette according to claim 4, wherein a repressor of pNanA is positioned upstream of pNanA when there is activation of pNanA in the absence of sialic acid, wherein preferably the repressor is a NanR protein-encoding polynucleotide sequence, preferably encoding the amino acid sequence set forth in SEQ ID NO: 11 or a functional variant thereof.
6. The expression cassette according to claim 5, wherein the NanR protein-encoding polynucleotide sequence comprises a nucleic acid sequence that has at least 80% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 5.
7. The expression cassette according to claim 5 or 6, further comprising a constitutive promoter operably linked to NanR, wherein the constitutive promoter is selected from the group comprising pBad with AraC; J23108 with rbs2, rbs 3 or rbs 5; and J23113 with rbs 4.
8. The expression cassette according to claim 7, wherein the constitutive promoter operably linked to NanR is J23113-rbs4.
9. The expression cassette according to claim 8, wherein the cassette comprises J23113-rbs4-NanR, preferably comprising the nucleic acid sequence set forth in SEQ ID NO: 6 or a functional variant thereof.

10. The expression cassette according to any one of claims 5 to 9, further comprising an activator and promoter to increase the expression of Cbh, such as the transcription activator CadC protein-encoding sequence and promoter pCadBA, wherein CadC is positioned downstream and under the control of pNanA and pCadBA is positioned downstream of CadC and operably linked to the bile salt hydrolase Cbh protein-encoding sequence.
11. The expression cassette according to claim 10, wherein the activator and promoter nucleic acid sequence is set forth in SEQ ID NO: 7 or a functional variant thereof.
12. The expression cassette according to any one of claims 1 to 11, wherein the cassette is comprised in one or more plasmid vectors.
13. The expression cassette according to claim 12, wherein the plasmid vector is pEaat, preferably comprising the nucleic acid sequence set forth in SEQ ID NO: 1 or a functional variant thereof.
14. The expression cassette according to any one of claims 1 to 13, further comprising an antibiotic resistance gene flanked by FRT sites to enable its removal.
15. The expression cassette according to any one of claims 2 to 14, wherein the gene polynucleotide sequence for *cbh* is codon-optimised for expression in a probiotic cell, such as set forth in SEQ ID NO: 9.
16. The expression cassette of claim 15, wherein the gene polynucleotide sequence for *cbh* is codon-optimised for expression in a probiotic cell selected from the group comprising *E. coli* sp., *Bacteroides* sp., *Clostridium* sp., *Faecalibacterium* sp., *Lactococcus lactis*, and *Lactobacillus* sp.
17. Use of an expression cassette according to any one of claims 1 to 16 for the recombinant production of bile salt hydrolase proteins.
18. A composition comprising:
 - a) a probiotic bacteria; and
 - b) an expression cassette of any one of claims 1 to 16,wherein the probiotic bacteria comprises the expression cassette for production of bile salt hydrolase.

19. The composition of claim 18, wherein the probiotic bacteria is selected from the group comprising *E. coli* sp., *Bacteroides* sp., *Clostridium* sp., *Faecalibacterium* sp., *Lactococcus lactis*, and *Lactobacillus* sp.
20. The composition of claim 18 or 19, wherein the probiotic bacteria is auxotrophic.
21. The composition of claim 20, wherein the auxotrophic bacteria has had Alanine racemase genes deleted and cannot divide in the absence of D-Alanine.
22. A composition of any one of claims 18 to 21 for use in a method of treating *C. difficile* infections (CDIs) and/or recurring CDIs (rCDIs).
23. The composition of claim 22, wherein the CDIs and/or rCDIs are caused by dysbiosis.
24. Use of a composition of any one of claims 18 to 21 for the manufacture of a medicament for the treatment or prophylaxis of CDI and/or rCDI.
25. The use according to claim 24, wherein the CDI and/or rCDI is due to dysbiosis.