Patentability/Literature Research

The probiotic-based solution to combat cholera as presented here comprises of two major innovative components: (1) the metabolite-dependent pathogen inhibition by a probiotic bacterium, and (2) *in situ* pathogen detection using an engineered living diagnostic with a novel hybrid receptor.

On Part (1), we performed a patent and literature search on the patentability of using a probiotic bacterium to combat an enteric pathogen. We found multiple patents and applications (Citation I.) disclosing the use of single species, or combinations of those, of natural microbes to treat or prevent *Clostridium difficile* infections. Citation II. discloses the use of a specific strain of *Lactobacillus Johnsonii* to treat or prevent intestinal infections, including those caused by *Vibrio* species. Citation III. discloses nutritional components that contain *Lactococcus* strains used for allergy amelioration. Citation IV. discloses the combination of probiotics and prebiotics and their synergy through potential functional interactions when co-localized. Finally, Citation V. is a research article that discovered the ability of *Ruminococcus obeum* to repress colonization factors of *V. cholerae* in mammalian intestines. There seems no directly comparable studies or proposed methods. Thus, the idea of using *Lactococcus lactis* to control *V. cholerae* infection through acidifying the gut environment has good novelty. In addition, our method of oral administration with validated dosing range and administration schedule in a mouse model are also patentable given the identified prior art.

Below is a list of the above-mentioned patents and literature we found relevant:

I. a. Patent 9,533,014 (US9533014 B2)

b. Patent application PCT/US2014/014744 (WO2014121301 A1)

These two entries, and a number of other patents or patent applications, disclose the use of natural microbes to prevent or reduce pathogenic bacteria in the GI tract, notably *Clostridium difficile*.
In (a), claims cover a synergistic combination of two different microbes that display cytocoxicity agains *Clostridium difficile*, whereas in (b), the proposed microbial composition compete with the pathogenic *Clostridium difficile* for nutrients and thus inhibit the pathogen's growth.

II. Patent application US 12/109,159 (US20080299098 A1)

This patent application discloses the broad-spectrum antibacterial and antifungal activity of *Lactobacillus Johnsonii* D115.

Relevant claims:
6. A composition, comprising:
(a) bacterial cells of the genus *Lactobacillus* species *johnsonii* strain D115 that produce an anti-microbial metabolite that is heat stable at temperature up to 121° C. for at least 15 minutes and is acid-tolerant in the range from neutral to pH 1 for at least 30 minutes; and
(b) a physiologically acceptable carrier for the bacterial cells and metabolite, suitable for oral administration.
7. The composition according to claim 6, wherein the metabolite has anti-microbial activity against human and animal pathogens.
8. The composition according to claim 7, wherein the human and animal pathogens are selected from the group consisting of *Brachyspira* spp., *Shigella* spp., *Vibrio* spp., ……
9. A method for the prophylaxis of the effects of an infection of microbes selected from the group consisting of *Brachyspira* spp., *Shigella* spp., *Vibrio* spp., …… comprising the step of administering an effective amount of the composition of or metabolite(s) of the strain of claim 6.

III. Patent application US 13/391,174 (US20120148629 A1)

This patent application discloses a complete nutritional composition comprising *Lactococcus* strains or probiotic that is provided for reducing the symptoms of allergies in different groups of patients such as allergies originating from food allergens in young children or infants and respiratory allergens in children, adults and household pets.

IV. Patent application US 14/952,894 (US20160271188 A1)

This patent application discloses the use of probiotic and prebiotic compositions, and methods of use thereof for treatment of gastrointestinal disorders

Relevant claims:

1. A method of reconstituting, modulating, or creating a beneficial bacterial flora in the gastrointestinal tract of a subject, the method comprising administering to the subject a composition comprising at least one isolated population of bacterial cells and a first isolated prebiotic, thereby reconstituting, modulating or creating a beneficial bacterial flora in the gastrointestinal tract of the subject.
15. The method of claim 1, wherein the bacterial cells and first isolated prebiotic are formulated to functionally interact when co-localized in the gastrointestinal tract of a human subject.

V. A. Hsiao *et al.*, Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. *Nature*. **515** (2014), doi:10.1038/nature13738.

- This research article described a commensal bacteria species, *Ruminococcus obeum*, which inhibits *Vibrio cholerae* colonization, through a quorum-sensing mediated repression of *V. cholerae* colonization factors.

By comparing with the identified patents and literature, we concluded that the idea of using *Lactococcus lactis* to inhibit the pathogen *Vibrio cholerae* development in the GI tract is novel. Furthermore, the method of using the natural metabolite (e.g. lactic acid) of a probiotic to inhibit a pathogen growth through co-localization and inter-species biochemical interaction, as well as the detailed dosing scheme also have good patentability given the identified art.

On Part (2), we have filed a patent application, "Synthetic hybrid receptor and genetic circuit in bacteria to detect enteric pathogenic microorganisms" (PCT/US2016/036703), on June 9th, 2016, with claims covering the design of the hybrid receptor for specific pathogen signal detection, the integrated genetic circuit that includes an inverter element to enable coupled sense-and-response, and the use of antimicrobial agents and/or enzymatic reporters as output functions of the genetic circuit. The PCT application further claims priority date of US application 62/172,971 filed on June 9th, 2015. We did a thorough search for prior art to date (June 4th, 2017), and conducted a patentability analysis based on the most relevant references. Notably, for prior art purpose, we can claim the benefit of our previous PCT application, filed on June 9^{th,} 2016, which further claims the priority date of June 9th, 2015. Therefore, any intervening prior art that came out between June 9th, 2016 and the filing date of our current invention may be antedated.

We found similar patent applications and research articles with our engineered diagnostic probiotic, which are cited below. Citation I. and II. both disclose engineered *E. coli* that detect *Pseudomonas aeruginosa* through its quorum-sensing molecules, and activate certain pathogen-killing functions in response. Notably, Citation I. has claimed a wider variety of possible host species for engineering, as well as potential pathogens to target. Citation III. discloses an engineered *E. coli* that detect the quorum-sensing molecule of *V. cholerae*, and claims on recombinant microbes that have genetic components for sensing, signal processing, and expression of a detectable marker. In addition, a few research articles describing engineered microbes (mostly *E. coli*) for bio-sensing in the gut are also listed in Citation V. Importantly, none of the identified art has described a hybrid receptor that enables pathogen-sensing by a distantly-related species, such as a food-associated lactic-acid bacterium.
With regard to the hybrid receptor, Citation IV. discloses a similar concept, which describes the method of building a chimeric protein which can respond to a specific ligand by changing its

interaction with another biomolecule. However, it is not obvious nor straightforward to apply such chimeric proteins for bio-sensing, such as the one we built in our system.

Below is a list of the references mentioned in the summary above:

I. Patent application US 13/033,233 (US 20120027786 A1)

S. Gupta, E. E. Bram, R. Weiss, Genetically programmable pathogen sense and destroy. *ACS Synth Biol*. **2**, 715–723 (2013).

This research article discloses the creation and *in vitro* testing of an engineered bacterium *Escherichia coli*, which detects the pathogen *Pseudomonas aeruginosa* through its quorum-sensing molecules and activates the production of an antimicrobial agent CoPy that kills the pathogen specifically.

Relevant claims in the patent application based on this invention:

1. A cell that recombinantly expresses (1) a detection circuit, (2) optionally a signal amplifying circuit, and (3) a secretion circuit that secretes a factor that specifically recognizes and destroys a specific pathogen.
11. The cell of claim 1, wherein the cell detects one or more molecules produced by one or more specific pathogens, optionally wherein one or more of the specific pathogens is a bacterial pathogen and/or wherein one or more of the molecules produced by one or more of the specific pathogens is a quorum sensing molecule.
24. The cell of claim 11, wherein the bacterial pathogen is a *Vibrio* bacterial pathogen, optionally a *Vibrio cholerae* bacterial pathogen.
26. The cell of claim 24, wherein the cell detects CAI-1 that is secreted by the *Vibrio cholerae* bacterial pathogen, and optionally wherein the cell comprises a signal amplifying circuit that regulates production of a factor that is secreted by the cell to specifically destroy the *Vibrio* bacterial pathogen.

II. Patent 9,603,877 (US 9603877 B2)

N. Saeidi *et al.*, Engineering microbes to sense and eradicate Pseudomonas aeruginosa, a human pathogen. *Mol Syst Biol*. **7**, 521 (2011).

Hwang, I. Y. *et al.* Engineered probiotic Escherichia coli can eliminate and prevent Pseudomonas aeruginosa gut infection in animal models. *Nat. Commun.* **8,** 15028 (2017).

This group discloses an engineered *Escherichia coli*, which detects the pathogen *Pseudomonas aeruginosa* through its quorum-sensing molecules and activates the production of a bacteriocin pyocin, together with an autolysis of the host to release the

pathogen-killing molecules. These two articles demonstrate the functionality of this system *in vitro* and *in vivo* respectively.

The patent on this invention has the following relevant claims:

1. An isolated nucleic acid molecule comprising:
(a) a first nucleotide sequence encoding a protein that detects the presence, amount or both of a pathogenic microorganism by forming a complex with a quorum sensing molecule produced by said pathogenic microorganism;
(b) one or more second nucleotide sequences, wherein the one or more second nucleotide sequences are under the control of a first inducible promoter that is induced by the complex of the protein encoded by the first nucleotide sequence and the quorum sensing molecule produced by the pathogenic microorganism and encoding (i) an antimicrobial peptide, wherein the antimicrobial peptide is effective against the pathogenic microorganism detected by the protein encoded by the first nucleotide sequence; and/or (ii) an antibiofilm enzyme, wherein the antibiofilm enzyme is effective against the pathogenic microorganism detected by the protein encoded by the first nucleotide sequence; and (c) optionally a third nucleotide sequence encoding a protein that controls the motility of the host organism, wherein the protein that controls the motility of the host organism directs the motility of the host organism towards said pathogenic microorganism.
13. The isolated nucleic acid molecule of claim 1, wherein the pathogenic microorganism is selected from the group consisting of *Pseudomonas aeruginosa*, *Clostridium difficile*, *Escherichia coli*, *Helicobacter pylori*, *Salmonella*, *Vibrio cholera* and *Yersinia*.
25. A method of sensing and killing pathogenic microorganisms, the method comprising contacting a recombinant microorganism comprising the isolated nucleic acid molecule of claim 1 with the pathogenic microorganism.
28. The method of claim 24 [*should be 25 here], wherein the pathogenic microorganism is selected from the group consisting *of Pseudomonas aeruginosa, Clostridium difficile, Escherichia coli, Helicobacter pylori, Salmonella, Vibrio cholera* and *Yersinia*.

III. Patent application PCT/SG2015/050428 (WO 2016072936 A1)

M. B. Holowko, H. Wang, P. Jayaraman, C. L. Poh, Biosensing Vibrio cholerae with Genetically Engineered Escherichia coli. *ACS Synth. Biol.* **5**, 1275–1283 (2016).

This research article discloses the creation of an engineered bacterium *Escherichia coli*, that detects *Vibrio cholerae* through expressing the native quorum-sensing receptor of *Vibrio cholerae*, and produces a fluorescent protein in response.

Relevant claims:

19. Use of a recombinant expression system comprising at least:
(i) a first nucleotide sequence encoding for at least one protein of a quorum sensing system capable of detecting the presence, amount or both of a microorganism of interest by forming a complex with a marker molecule indicating the presence of said microorganism; (ii) a second nucleotide sequence encoding for at least one detectable marker, (iii) a third nucleotide sequence encoding for a genetic inverter that inhibits expression of the second nucleotide sequence, wherein the genetic inverter is under control of an inducible promoter and wherein the inducible promoter is induced if the complex of the at least one protein encoded by the first nucleotide sequence and the marker molecule indicating the presence of said microorganism is below a threshold concentration and is not induced if the complex of the at least one protein encoded by the first nucleotide sequence and the marker molecule indicating the presence of said microorganism exceeds a threshold concentration, for detection of at least one type of microorganism.

IV. Patent application US 11/705,565 (US20070196816 A1)

This patent describes the method of building a chimeric protein which can respond to a specific ligand by changing its interaction with another biomolecule. It does not mention a chimeric/ hybrid receptor that changes conformation upon ligand binding and switches on or off downstream signaling pathways by phosphorelay.

V. Other relevant research articles:

J. W. Kotula *et al.*, Programmable bacteria detect and record an environmental signal in the mammalian gut. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 4838–43 (2014).

- Described an engineered *E. coli* that has an inducible promoter which turns on a memory element when activated by tetracycline, and produces an enzymatic reporter intracellularly. The response is maintained so that recovery of these engineered bacteria from fecal samples, following subsequent culture analysis, can indicate tetracycline exposure in the host gut.

K. N. Daeffler *et al.*, Engineering bacterial thiosulfate and tetrathionate sensors for detecting gut inflammation. *Mol. Syst. Biol.* **13**, 923 (2017).

D. T. Riglar *et al.*, Engineered bacteria can function in the mammalian gut long-term as live diagnostics of inflammation. *Nat. Biotechnol.* (2017), doi:10.1038/nbt.3879.

- Both groups designed inflammation-sensing functions in *E. coli*, and evaluated their performance in mice. Daeffler *et al.* used a fluorescent reporter, and Riglar *et al.*

coupled the sensing with the memory element and enzymatic output described in the Kotula *et al.* 2014 study.

F. Duan, J. C. March, Engineered bacterial communication prevents Vibrio cholerae virulence in an infant mouse model. *Proc Natl Acad Sci U S A*. **107**, 11260–11264 (2010).

- This study used engineered *E. coli* to produce the *Vibrio cholerae* quorum-sensing molecules and thus inhibit the pathogen's virulence genes.

With this patent/ literature search and analysis, we concluded that:

The concept of a hybrid receptor (e.g., CqsS/NisK) for detecting the quorum sensing molecules of a pathogen was not disclosed in the identified art. All of the above-mentioned bio-sensing designs are based on naturally occurring receptors, whereas our hybrid receptor is an artificial design, plus a functional point mutation in the original protein sequence, which is necessary to make the pathogen-sensing possible with a distantly-related microbial host.

The concept of detecting and reporting a pathogen using an engineered bacterium, wherein the engineered bacterium comprises a synthetic hybrid receptor that detects a biochemical signal from the pathogen, and a genetic circuit that controls the production of an enzymatic reporter appears to be novel over the identified art. Most of the previous bio-sensing designs are linked to pathogen-killing functions, or restricted to conventional laboratory-based reporters like fluorescent proteins. Here we adopted a diffusible enzymatic reporter which can be easily detected with a colorimetric assay, directly from fecal samples. Such a living diagnostic design appears to be patentable from our prior art search.