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Supplementary Materials for

Probiotic strains detect and suppress cholera in mice

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/10/445/eaao2586/DC1)

Table S1 (Microsoft Excel format). Primary data.

Materials and Methods

Bacterial strains

Vibrio cholerae strain C6706 (O1 El Tor biotype) was grown in Luria-Bertani (LB) broth at 37°C for the animal experiments. *V. cholerae* C6706 containing an inactivating transposon insertion in the cholera toxin gene *ctxA* (C6706ctxA) (*36*) was used for the *in vitro* experiments. To make the *cqsA* deletion strain C6706ctxA Δ*cqsA*, *V. cholerae* genomic DNA surrounding *cqsA* was amplified by crossover PCR and cloned into pWM91 (*37*) for subsequent SacB-mediated allelic exchange, as described previously (*36*).

Lactococcus lactis subsp. cremoris MG1363 was used as the parental strain for circuit construction and animal experiments. *L. lactis* was maintained at 30°C in Difco M17 medium supplemented with 0.5% glucose (GM17), unless otherwise stated. A GFP-integrated strain of *L. lactis* (MG1363G) was constructed using the method described by Pinto *et al.* (*38*). The *L. lactis* codon-optimized GFP gene was inserted into the *llmg_pseudo_10* locus in a single recombination step, conferring erythromycin resistance. The same integration strategy was used to interrupt the coding region of the principal lactate dehydrogenase gene of *L. lactis* MG1363 (*llmg_1120*). *Escherichia coli* NEB 10-beta (New England BioLabs) cells were used as intermediate hosts for plasmid construction. *E. coli* cells were propagated at 37°C in LB broth.

Appropriate antibiotics were added to growth media for selection or maintenance of plasmids at the following concentrations: carbenicillin 100 μg/ml, chloramphenicol 10 μg/ml, and erythromycin 10 μg/ml. To enumerate wildtype *L. lactis* from a mixed culture or the mouse gut homogenate, GM17-agar plates with 2 μg/ml rifampicin and 5 μg/ml polymyxin B (modified from Ballal *et al*. (*39*)) were used. For wildtype *V. cholerae*, LB-agar plates with 70 μg/ml streptomycin were used.

Plasmid construction

All plasmids were constructed in *E. coli* 10-beta, sequenced (Quintara Biosciences) when necessary, and used for *L. lactis* transformation. *L. lactis* competent cells were prepared and transformed according to Holo and Nes (*40*). The plasmid housing the cholera-sensing circuit was derived from pECGMC (*41*). The backbone has a ColE1 origin of replication for propagation in *E. coli*, an AMβ1 origin of replication for *L. lactis*, and a chloramphenicol-resistance gene *cat* that can be used for selection in both *E. coli* and *L. lactis*. The vector also contains the endogenous *L. lactis* two-component system *nisR* and *nisK* driven by the constitutive *nisR* promoter, with a reporter mCherry under the control of *nisA* promoter. This circuit turns on mCherry expression when *L. lactis* cells are exposed to nisin (30 ng/ml). To screen for a functional CqsS-NisK hybrid receptor, the nisK gene was replaced by one of the hybrid receptor variant genes, which were fused and amplified by overlapping PCR from templates in the genomic DNA of *V. cholerae* (*cqsS*) and *L. lactis* NZ9000 (*nisK*). The RBS of the hybrid receptor gene was designed to contain four randomized nucleotides, NNNNGG, by the RBS Calculator algorithm (*42*, *43*) and cover a predicted range of 3-10^3 translation initiation rates (fig. S9B). The hybrid receptor plasmid library was screened by placing individual colonies into GM17 media with chloramphenicol on a 96-well plate, and co-culturing the clones with *V. cholerae* wildtype or Δ*cqsA* mutant. *L. lactis* and *V. cholerae* co-cultures were assayed on a BD LSRFortessa flow cytometer with a high-throughput sampler. The clone that exhibited a difference in the fluorescent output between the two co-cultures was propagated for plasmid extraction. The *L. lactis* plasmid extraction followed Qiagen miniprep kit instructions, with an extra lysozyme (2 mg/ml in P1 buffer) digestion step at 37°C for 10

min before lysing the cells. The isolated plasmid with the functional hybrid receptor was cloned back into *E. coli* for sequence verification and future cloning purpose.

The final cholera-sensing circuit had a *tetR* gene incorporated and driven by the *nisA* promoter, whereas the mCherry gene was placed after the TetR-regulated *xyltet* promoter. To maximize the dynamic range of mCherry expression upon CAI-1 activation, *tetR* expression was screened over a randomized RBS library (NNGNNG) of 10-10^4 translation initiation rates . The individual colonies from the library cloning were cultured, induced by co-culturing with *V. cholerae*, and assayed by flow cytometry. Clones with the highest folds of activation were isolated, propagated and sequenced (pHCR2). To provide a colorimetric readout, the output mCherry gene was replaced by β-lactamase gene *bla*, codon optimized for *L. lactis* and fused to the *L. lactis* signal peptide usp45 to enable secretion to the extracellular environment (pHTR2).

Hybrid receptor design

BLASTp (*44*), UniProt (*45*) and TMpred (*46*) were used to identify domain boundaries for the transmembrane and signal transduction domains of CqsS and NisK. Ten different fusion sequences were designed (fig. S9A). Each hybrid receptor variant was screened over an RBS library as described in the previous section. To provide structural diversity in the junction between the fused domains, randomized nucleotides (up to 6 Ns), were introduced at the junction of the hybrid receptor variants for further screening. The E182G mutation found in the functional hybrid receptor was outside of the target region for this randomization and thus was not considered to be result of the mutagenesis effort.

Co-cultures

L. lactis-*V. cholerae* (L-V) co-culture was used to test the hybrid receptor and cholera-sensing circuit performance *in vitro*. To maintain viable *V. cholerae* cells in the co-culture, GM17 was additionally conditioned with 50 mM phosphate buffer at pH 7.0 (GM17-buffered). 1M stock of the phosphate buffer was made with KH_2PO_4 4.68 g + Na_2HPO_4 16.4 g in 100 ml DI water (recipe from Sigma Aldrich). Overnight culture of *V. cholerae* was diluted 1:1000, and *L. lactis* 1:100 in GM17-buffered, and allowed to grow to exponential phase (OD_{600} 0.2-0.4). Then a new tube of 2 ml GM17-buffered was inoculated with 100 µl *L. lactis* and 1 µl *V. cholerae*, and incubated at 37°C with shaking. Samples were taken at 2-hour intervals for flow cytometry analysis of fluorescent output. For high throughput screening, the co-culture was inoculated in 96-well plates, and the volumes were scaled accordingly.

The steady-state cell density of *V. cholerae* in an L-V co-culture in GM17 medium with no additional buffering was 2 logs lower than a *V. cholerae* monoculture or an L-V co-culture in GM17-buffered (fig. S2A). To examine the true efficacy and dynamics of *V. cholerae* killing by *L. lactis*, a GM17 medium with no buffer was made according to BD Difco M17 broth recipe, but contained no disodium-β-glycerophosphate. pH 7.0 phosphate buffer was added at 12.5 mM to eliminate the self-inhibitory effect of *V. cholerae* growing on glucose (*47*) (fig. S2B). This minimally buffered GM17 was inoculated with *L. lactis* and *V. cholerae* at a 100:1 ratio and sampled every hour for drop-plate CFU count of each species.

Hybrid receptor specificity

The specificity of signal activation of the hybrid receptor to other bacteria was evaluated under co-culture conditions in GM17 supplemented with 100 mM NaCl at 30 °C. The CAI-1 autoinducer

synthases (CqsA) are present in most *Vibrio* spp. and they can produce various CAI-1 moieties that have different acyl chain lengths and modifications (*22*). CqsS receptors in different *Vibrio* spp. display different affinities to distinct CAI-1s variants (*22*). The native CqsS receptor from *V. cholerae* is able to detect three chemical variants of CAI-1 molecules: (*S*)-3- hydroxytridecan-4-one (CAI-1), Enamino-CAI-1 (Ea-CAI-1) and (*Z*)-3-aminoundec-2-en-4-one (Ea-C8- CAI-1) (*22*). To test the specificity of HR4M to other CAI-1 producing and non-producing bacterial species, we evaluated circuit activation in response to a set of diverse environmental *Vibrio spp.* known to carry the CqsA/CqsS system, as well as in co-culture with distantly related bacteria that do not encode *cqsA* homologs (fig. S11). The panel included *V. alginolyticus* as a positive control, which has been shown to produce Ea-C8-CAI-1 in spent media (*22*).

Flow cytometry

A BD LSRFortessa cell analyzer (BD Biosciences) with a high-throughput sampler was used to measure the fluorescent output from *L. lactis* during hybrid receptor screening, and a BD FACSAria II (BD Biosciences) was used for the fluorescent output time-course measurement. Cell cultures were sampled and diluted 40-100 times in PBS, depending on cell density, for flow cytometry analysis. Each sample had at least 10,000 events collected, and was analyzed with FlowJo (FlowJo, LLC). For L-V co-culture, the GFP-integrated *L. lactis* strain carrying the cholera-sensing circuit was used, and the population was gated by GFP^+ to obtain mCherry expression levels in *L. lactis*.

Beta-lactamase assays

Nitrocefin (BioVision) was dissolved in DMSO to make a 1 mM stock. It was added to samples (bacterial culture supernatant or fecal samples in PBS) in a clear-bottom 96-well plate to a final concentration of 0.1 mM. For bacterial culture supernatant, the plate was placed in a spectrophotometer SpectraMaX M5 (Molecular Devices) to read absorbance at 486 nm for 1 hour. For fecal samples, the plate was incubated statically at room temperature overnight, and the liquid in each well was transferred to a new plate for absorbance measurement.

Animal husbandry

All mice in this study were treated in accordance with protocol IS00000852, approved by Harvard Medical School Institutional Animal Care and Use Committee and the Committee on Microbiological Safety. A litter of white CD-1 infant mice were purchased from Charles River Laboratories at postnatal day 1 with mother in a micro-isolator cage, and housed in BL2 animal facility for 72 hours of acclimation before each study.

V. cholerae infection experiments

The cholera survival protocol was modified from Duan and March (*5*). Four-day old suckling mice were separated from mother and kept in a 30°C incubator for 2 hours to digest the milk in stomach. Overnight bacterial cultures were washed and resuspended in GM17 to appropriate density (L concentrated by 5 fold, V diluted by 5 fold). 25 μl of the bacteria was administered to each mouse intragastrically, containing (~1-5) × 10^7 *V. cholerae* cells and (~0.5-1) × 10^9 *L. lactis* cells, at time 0 hours. Subsequent *L. lactis* doses were given every 10 hours three times. After each feeding, the mice were monitored for at least 15 min until they recovered from anesthesia, and then returned to the dam. The mice were monitored for 42 hours for survival and sacrificed at the end of study. The small and

large intestines of the mice were collected in 1 ml PBS and homogenized with an HT Mini high-throughput homogenizer (OPS Diagnostics). Serial dilutions of the homogenate were plated for *L. lactis* and *V. cholerae* cell counts. The survival studies were performed in a randomized cross-litter manner (*48*). Sample sizes were determined by power analysis to reach a statistical power of 0.8 or higher, using the pwr package (*49*) in R (*50*).

For the experiments that evaluated the direct administration of lactic acid by oral gavage on the colonization of *V. cholerae* in infant mice, *V. cholerae* cell counts in the intestines was determined at 26 h post-infection, and compared among three groups: fed with medium-only, *L. lactis*, and lactic acid at 167 mM (referenced maximum safe level in feed for livestock species (*51*)). For experiments that evaluated the survival of infant mice to *V. cholerae* infection under acid-neutralizing conditions, the oral gavage treatments were modified to modify the intestinal pH by supplementing the inoculation medium for *L. lactis* and the medium for the mock-fed group with 167 mM phosphate buffer at pH 7.4. Each of the treatments was administered at the 10-hour interval as described in Fig. 1C for the co-administration regimen.

Mouse fecal sample collection

Mice were inoculated with *V. cholerae* and *L. lactis* CSL as described above, returned to the dam for 10 hours, and fed with a second dose of *L. lactis* CSL. Then the pups were kept in a cotton-padded container at 30°C for up to 12 hours to collect their fecal samples. After collection, fecal samples were dropped in 90 μl of PBS in a 96-well plate for β-lactamase assay with 0.1 μM of nitrocefin.

L. lactis heat inactivation

Stationary-phase *L. lactis* cells were collected, washed, and resuspended to appropriate density for inoculation. Then the culture was aliquoted to 50 μl and heated at 70°C for 30 min. The heated bacteria were plated for viability testing before inoculation. The final inoculum contained less than 10 viable units of *L. lactis* administrated to the mouse as confirmed by drop-plate cell counts in GM17 agar.

Lactate quantification

Homogenized gut tissue was pelleted, supernatant filtered with a 0.2 μm filter, and then spun in a 10K Microcon centrifugal filter unit (EMD Millipore) to eliminate lactate dehydrogenase. Filtered samples were stored at -80°C until assayed. The lactate assay kit from Sigma Aldrich (MAK064) was used, and the quantification procedure followed the kit instructions. Briefly, samples were diluted to 3/100 of original concentrations in the assay buffer, so that the final concentrations fell into the range of standard curve. The diluted samples were mixed 1:1 with the enzyme master mix prepared from the kit and incubated at room temperature for 30 min. The absorbance at 570 nm was read on a SpectraMaX M5 (Molecular Devices). The concentrations of the samples were calculated from the standard curve. Each sample, including the standard curve, was run in duplicate.

Supplementary Figures

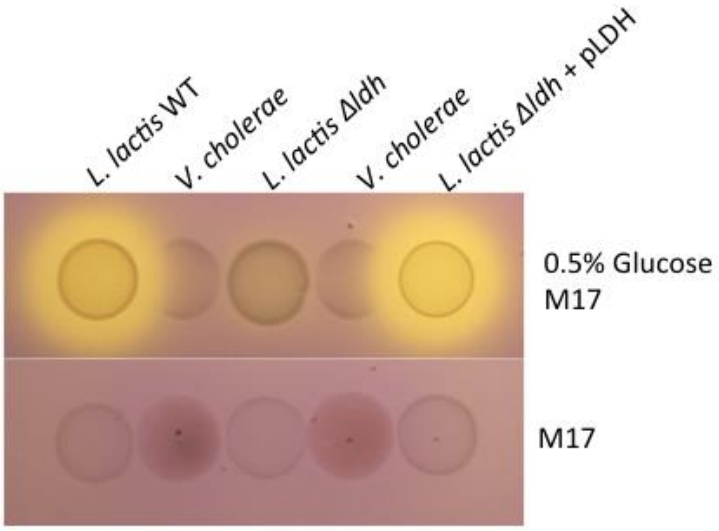

Fig. S1. Homolactic fermentation of glucose by *L. lactis* leads to growth inhibition of *V. cholerae*. Agar diffusion assay for adjacent *L. lactis* and *V. cholerae* strains in M17 media supplemented with glucose (top panel) and without glucose (bottom panel). Antagonistic effect is evidenced by the inhibition of growth in *V. cholerae* colonies adjacent to acidification-proficient *L. lactis* colonies. WT: wild type, Δ*ldh:* lactate dehydrogenase mutant, Δ*ldh (pLDH):* plasmid-complemented lactate dehydrogenase mutant. Minimally buffered M17 agar plates contain pH indicator bromocresol purple that turns yellow when pH drops below 5.2.

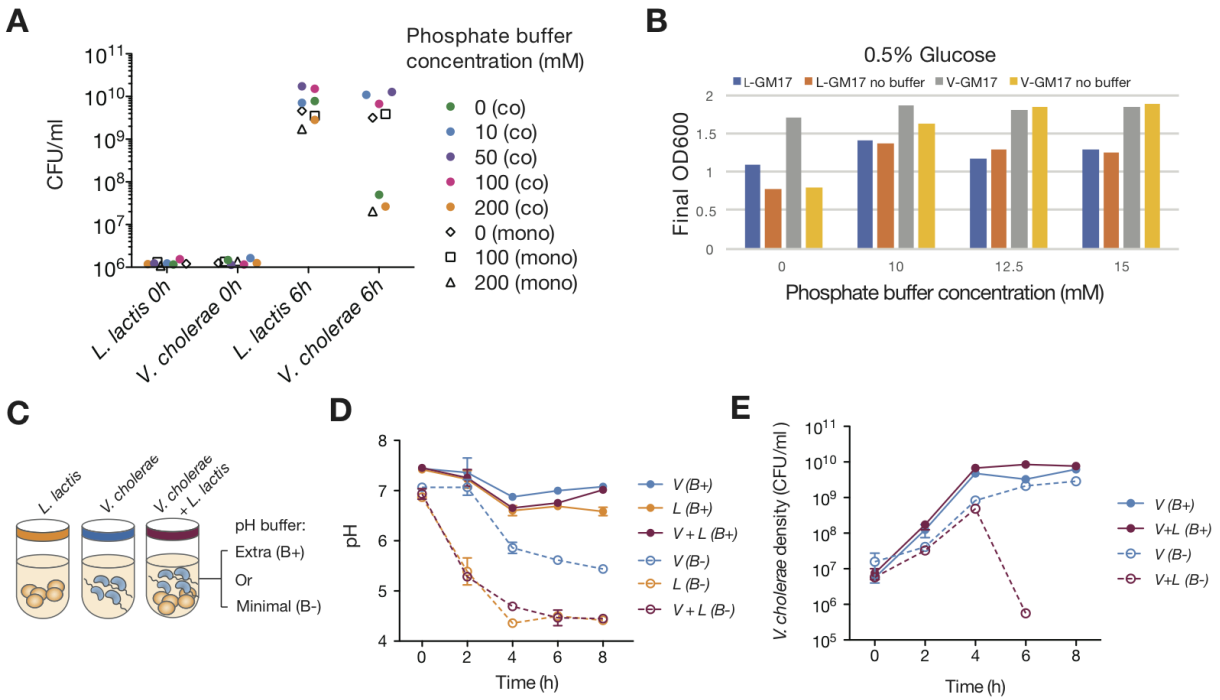

Fig. S2. *L. lactis*–*V. cholerae* coculture system.

(**A**) Optimization of extra-buffered GM17 for *L*-*V* co-culture. (**B**) Optimization of minimally-buffered GM17 for *L*-*V* co-culture. (**C**) *L*-*V* co-culture system. GM17 media with extra buffer (B+) contains 87.95 mM disodium-β-glycerophosphate and 50 mM phosphate (HPO_4^{2-}/$H_2PO_4^-$). Minimal buffer medium (B-) contains 12.5 mM phosphate buffer. (**D**) Acidification dynamics of *L. lactis* and *V. cholerae* under mono- and co-culture conditions. (**E**) *V. cholerae* density dynamics in mono- and co-cultures with *L. lactis*. Bars represent range of technical duplicates.

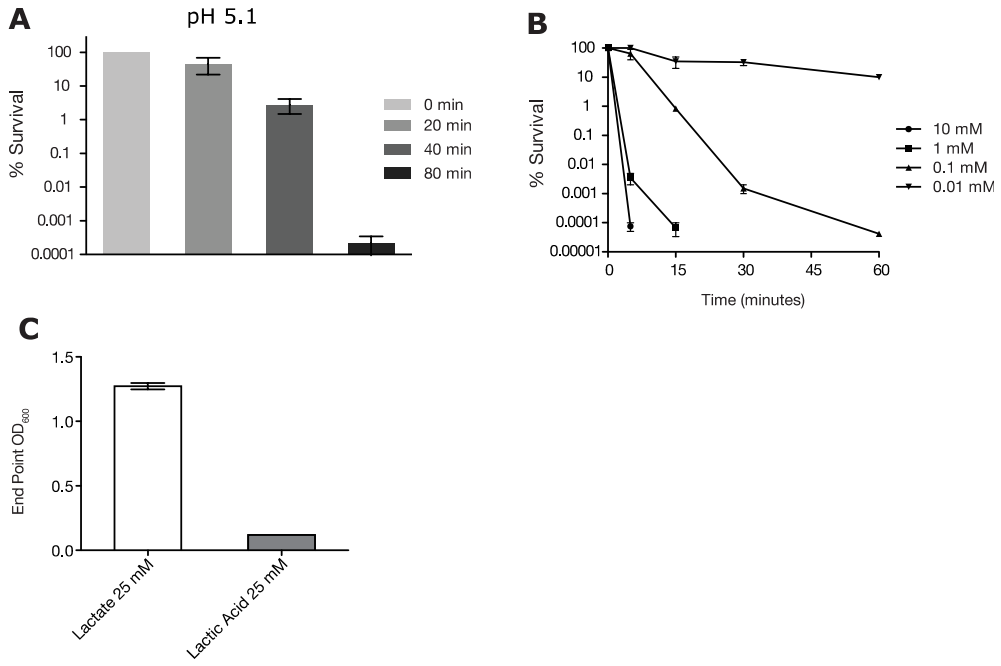

Fig. S3. Lactic acid displays a bactericidal effect on *V. cholerae.*

(**A**) Viability of *V. cholerae* cells shifted to growth media conditioned to pH 5.1 with lactic acid. The survival was calculated relative to cells shifted to control growth media at pH 7. Bars represent the standard deviation of three technical replicates. (**B**) Time-kill curve of *V. cholerae* cells exposed to lactic acid in a non-buffered saline solution (100 mM $MgCl_2$). The survival was calculated relative to cells that did not receive lactic acid treatment. Bars represent the range of technical duplicates. (**C**) End point optical density of *V. cholerae* cultures after 24 hours of incubation with lactate, the conjugate base of lactic acid, or with lactic acid. Bars represent the standard deviation of three technical replicates.

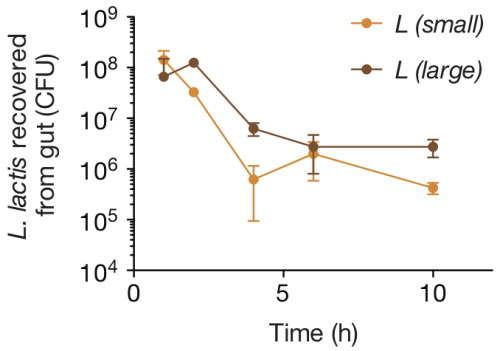

Fig. S4. *L. lactis* transit dynamics in mouse gut.

L. lactis population dynamics in the small and large intestine of infant mice after an initial inoculation of $3*10^8$ CFU. Bars represent the range of biological duplicates.

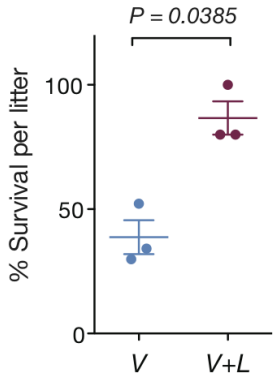

Fig. S5. Litter variation in infant mouse survival study.

Effect of litter variation on the significance of the protective effect of *L. lactis* against *V. cholerae* infection. Each data point represents a litter of infant mice. *P*(treatment)=0.0385, *P*(litter)=0.6325 with two-way ANOVA. Bars represent the standard error of the mean.

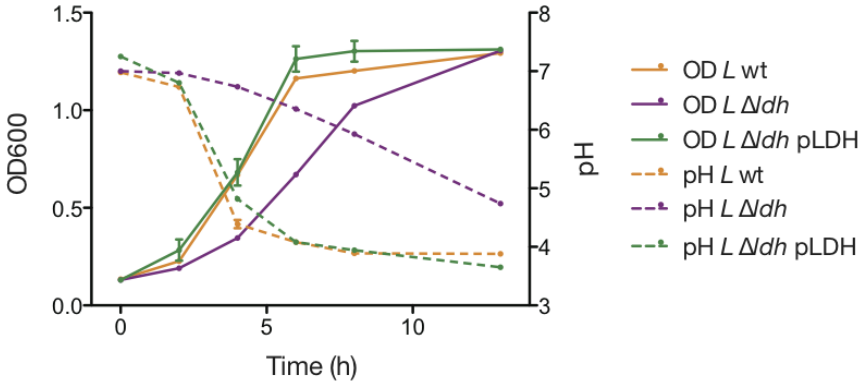

Fig. S6. Growth and acidification capability of *L. lactis* strains used in this study.

L. lactis wild type (wt), lactate dehydrogenase (Δ*ldh*) mutant and plasmid-complemented lactate dehydrogenase mutant Δ*ldh (pLDH)*. Bars represent the range of technical duplicates.

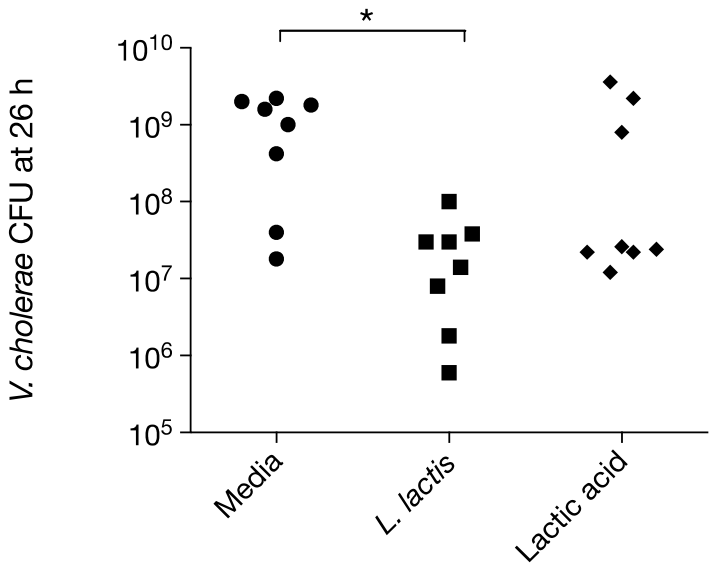

Fig. S7. *V. cholerae* colonization in infant mice treated with lactic acid.

Comparison was done with Kruskal-Wallis test with Dunn's post test. $n = 8$, $N = 4$ for each group. * $P = 0.0120$. No significant differences were observed between the direct lactic acid administration and the media control.

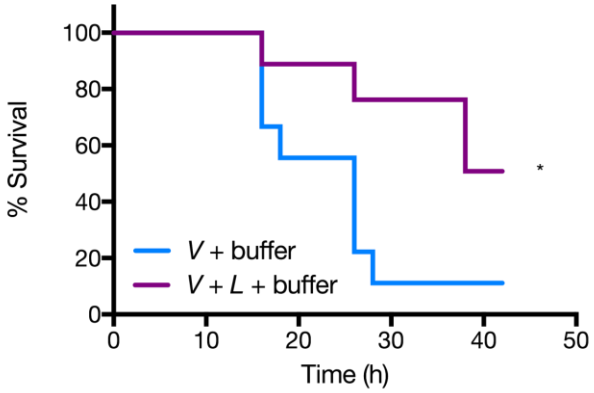

Fig. S8. Survival of *V. cholerae*–infected infant mice under acid-neutralizing feeding regimens.
The medium for *L. lactis* treatment and the medium for the mock-fed group were supplemented with 167 mM phosphate buffer at pH 7.4**.** The mock-fed group (n = 9, N = 2) had only 11.1% survival rate, whereas the *L. lactis*-treated group (n = 10, N = 2) were improved to 50.8%. * P = 0.0221 with log-rank test.

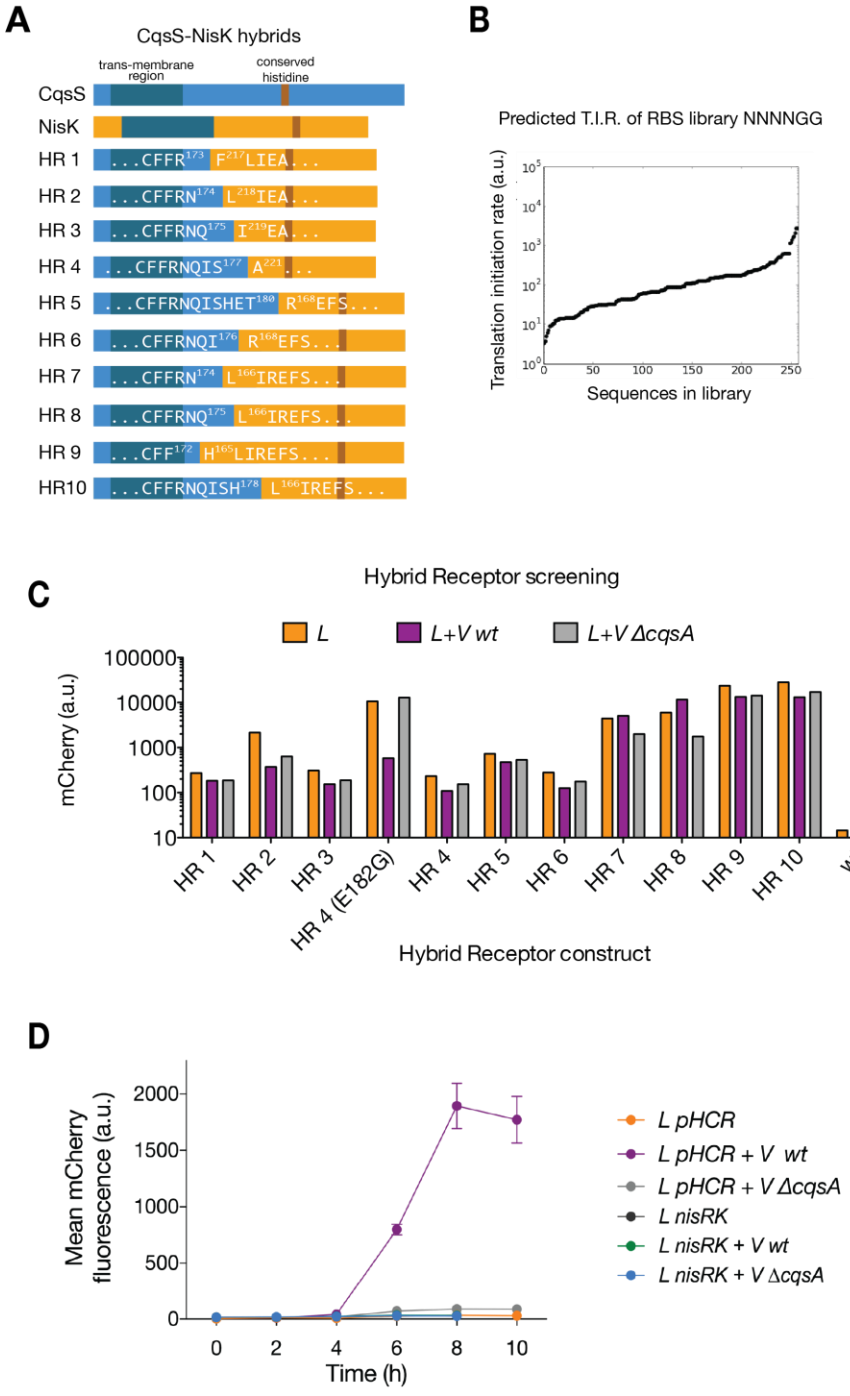

Fig. S9. HR design and screening.

(**A**) Fusion strategies of the 10 hybrid receptor variants. Amino acid numbers at fusion sites indicate positions in the original CqsS and NisK receptor sequences, respectively. (**B**) RBS library of hybrid receptors. (**C**) Hybrid receptor screening with the *L. lactis*-*V. cholerae* co-culture system. Hybrid Receptor 4 (E182G) is a variant that repressed the output gene by more than one order of magnitude. Hybrid Receptors 7 and 8 activate gene expression in response to CAI-1, but only by a small fractional increase due to high basal expression levels. (**D**) The native nisRK two-component system of *L. lactis* does not respond to CAI-1. Bars represent the standard deviation of three technical replicates.

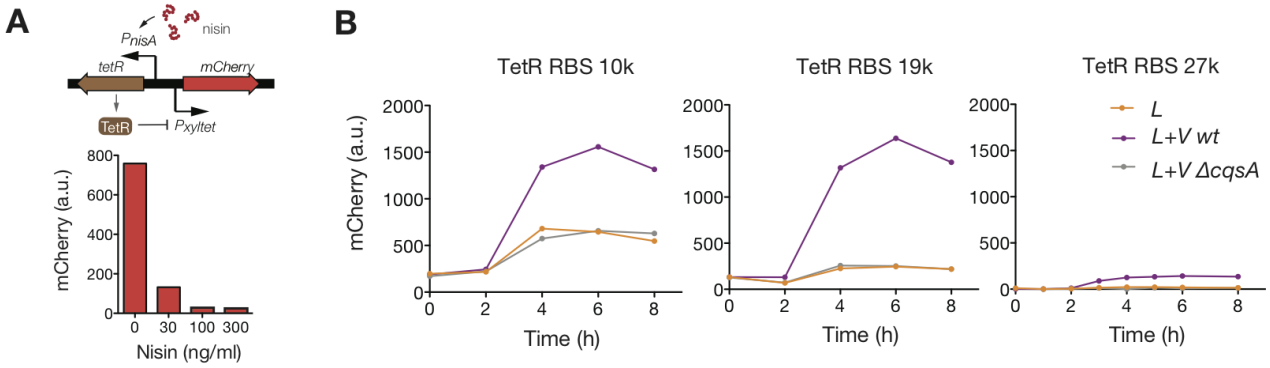

Fig. S10. *xyltet* promoter testing in *L. lactis* and circuit-tuning with different TetR translation rates.

(**A**) Expression of the *B. subtilis xyltet* promoter in *L. lactis* with a nisin-induced *tetR* expression circuit. (**B**) Fine-tuning of TetR expression in *L. lactis* under mono and co-cultures conditions. Shown in figure S10B are examples of low (10k), medium (19k) and high (27k) levels of TetR RBS strengths. An appropriate TetR translation rate allows full activation of the output under induced conditions, and keeps a low background in the off state. WT: wild type, *ΔcqsA*: non-CAI-1 producing *V. cholerae*.

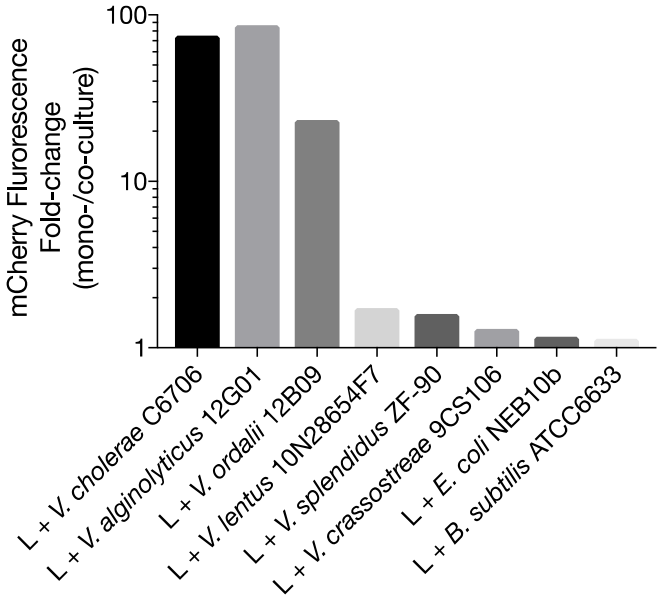

Fig. S11. Specificity of signal activation of the HR4M HR to other *Vibrio* spp. and non–*Vibrio* spp.

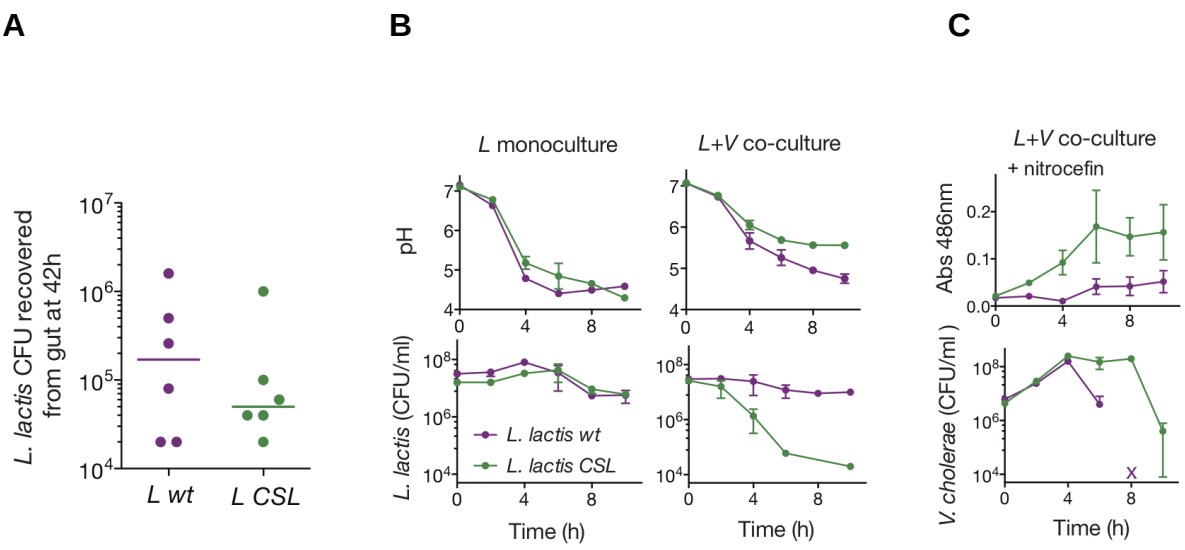

Fig. S12. Metabolic burden of *L. lactis* harboring engineered circuits.

(**A**) Viable cell count of wildtype and engineered *L. lactis* from mouse gut. Horizontal bar represents the median. (**B**) *L. lactis* wildtype and CSL strain in monoculture and co-culture with *V. cholerae* under anaerobic conditions. (**C**) Nitrocefin assay with the supernatant and *V. cholerae* cell viability in the *L*-*V* co-culture. Increased absorbance at 486 nm demonstrates activation of cholera-sensing circuit in CSL strain. Bars represent range of technical duplicates. "X" indicates undetectable level of bacteria.

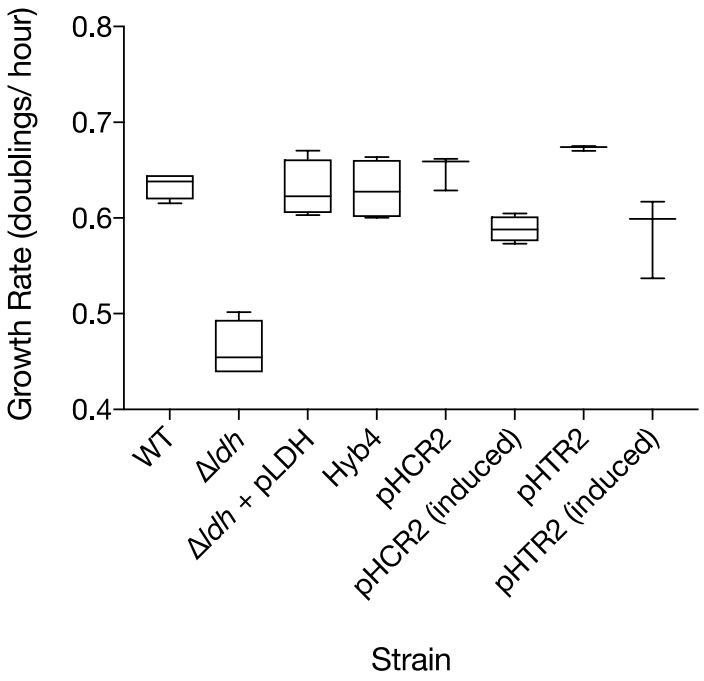

Fig. S13. Growth rate of *L. lactis* strains used in this study.

Induction of reporter genes with 50 μg/ml of aTC in engineered strains leads to a decrease in the growth rate. Cultures were grown in GM17 medium (BD) at 30 °C. WT: wildtype; *Δldh*: lactate dehydrogenase mutant; *Δldh* + pLDH: lactate dehydrogenase mutant complemented with a plasmid encoded *ldh* gene; Hyb4: plasmid-encoded HR4M; pHCR2: HR4M with inverter module and mCherry output; pHTR2: HR4M with inverter module and β-lactamase output. Average growth rates were determined for the logarithmic phase of triplicate cultures of three 10-fold serial dilutions of starting cells. Bars represent the standard deviation of four technical replicates.