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PROTOCOL

In vitro fermentation test bed for evaluation of engineered probiotics in polymicrobial communities

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Abbreviations used: ANOVA, one-way analysis of variance; Bf, *B. fragilis*; BHI, brain heart infusion broth; BHIS-NQ, supplemented BHI; Bo, *B. ovatus*; Bt, *B. thetaiotaomicron*; CAM, chloramphenicol; CCM, complex colonic medium; CFU, colony forming unit; Ct, cycle threshold; DBTL, design-test-build-learn; EcK12, *E. coli* MG1655; EcN, *E. coli* Nissle 1917; EcNWT, *E. coli* Nissle wild type; EcN(cGFP), *E. coli* Nissle GFPa1; Er, *E. rectale*; Fp, *F. prausnitzii*; La, *Lactobacillus acidophilus*; LB, Luria broth; MRS, *Lactobacillus* MRS broth; LTX, Lysozyme-Triton X-100 lysis solution; OD_{600}, optical density, 600 nm; PBG, phosphate buffered glycerol; PCA, principle component analysis; PYG, peptone-yeast extract-glucose broth; qPCR, quantitative PCR; RCM, reinforced clostridial medium; RPK, reads per kilobase; RT, room temperature; HSD, Tukey's honest significant difference; WGS, whole genome sequencing; YE, yeast extract

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ABSTRACT

In vitro fermentation systems offer significant opportunity for deconvoluting complex metabolic dynamics within polymicrobial communities, particularly those associated with the human gut microbiome. *In vitro* gut models have broad experimental capacity allowing rapid evaluation of multiple parameters, generating knowledge to inform design of subsequent *in vivo* studies. Here, our method describes an *in vitro* fermentation test bed to provide a physiologically-relevant assessment of engineered probiotics circuit design functions. Typically, engineered probiotics are evaluated under pristine, mono- or co-culture conditions and transitioned directly into animal or human studies, commonly resulting in a loss of desired function when introduced to complex gut communities. Our method encompasses a systematic workflow entailing fermentation, molecular and functional characterization, and statistical analyses to validate an engineered probiotic's persistence, plasmid stability and reporter response. To demonstrate the workflow, simplified polymicrobial communities of human gut microbial commensals were utilized to investigate the probiotic *Escherichia coli* Nissle 1917 engineered to produce a fluorescent reporter protein. Commensals were assembled with increasing complexity to produce a mock community based on nutrient utilization. The method assesses engineered probiotic persistence in a competitive growth environment, reporter production and function, effect of engineering on organism growth and influence on commensal composition. The *in vitro* test bed represents a new element within the Design-Build-Test-Learn paradigm, providing physiologically-relevant feedback for circuit re-design and experimental validation for transition of engineered probiotics to higher fidelity animal or human studies.

Keywords: *in vitro* fermentation, synthetic biology, simplified polymicrobial communities, engineered probiotics

BACKGROUND

The design-build-test-learn (DBTL) paradigm is commonly used in synthetic biology to guide circuit design, assembly, and evaluation to inform iterative re-design. Microbes have been engineered for various functions including: combatting pathogens, immune function modulation and altering host metabolism [1]; sensing and diagnosing disease [2]; gut inflammation detection [3,4]; analyte detection [5]; and as therapeutics [6]. However, engineered organisms often do not perform as desired during *in vivo* studies [7,3]. Typically, engineered bacteria are developed in monoculture, under optimal growth conditions that inaccurately reflect the highly complex *in vivo* environments (*e.g.*, animal or human gut microbiome studies) [8]. Testing of engineered organisms in a fully complex, competitive environment is rarely reported [1], which may explain an increased risk of failure for many engineered organisms due to lack of translation of laboratory testing to the intended environment. For instance, testing engineered *E. coli* strains under aerobic conditions may not translate to an anaerobic en-

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vironment; growth rate, gene expression and metabolic pathways differ between aerobic respiration and anaerobic fermentation [9,10]. While *in vivo* studies have absolute physiological relevance, interpretation of results is complicated by a large number of confounding variables. For example, hundreds of bacterial species concurrently compete for nutrients in which the microbial interactions and system dynamics are not well understood. These environments are vastly different from the monoculture testing conditions typically employed, posing a significant challenge to evaluate an engineered organism's ability to persist and function within the intended environment.

In vitro fermentation systems that simulate the human large intestine offer the ability to extend beyond simplistic mono- or co-culture testing regimens to a competitive environment with increased physiological relevance employing simplified or complex microbial communities. Simulated gut models have increased experimental capacity to allow rapid evaluation of multiple parameters by generating knowledge that can inform design and outcomes of *in vivo* studies. *In vitro* systems have been used to study temporal microbial compositional and bacterial-derived metabolite changes in response to system perturbations including introduction of dietary inputs, pathogens, probiotics and antibiotics [11]. These systems are generally inexpensive to operate, allow precise control and manipulation of environmental parameters (*e.g.*, temperature, pH, agitation, growth medium), and are useful for mechanistic studies [12].

Use of defined synthetic communities with *in vitro* fermentation studies offer less complexity than with fecal inoculum, yet mimic a complex environment to conduct screening studies not feasible using *in vivo* methods. This can serve to bridge the gap between testing of engineered organisms in monoculture and in a full, highly complex microbial community. *In vitro* investigations with defined communities include effects of antibiotics [13], pathogen colonization [14], genetic [15] and metabolic studies [16], employing both commercially available and laboratory isolates.

The test bed work flow presented here entails fermentation, molecular and functional characterization, and statistical analyses (**Fig. 1**). To demonstrate the test bed utility, an engineered probiotic *E. coli* Nissle 1917 (EcN) was tested in simplified communities of gut bacteria using a parallel bioreactor. Batch fermentations were conducted in nutrient rich medium designed to proliferate a host of gut commensals under anaerobic conditions at 37°C simulating the distal colon (pH = 6.8; residence time = 24 h) [17]. Mock polymicrobial communities offer a more complex, competitive environment than monoculture, and when used with *in vitro* fermentation can function as an intermediate step before testing in the desired, complex environment (**Fig. 2A**). In the workflow presented here, candidate mock community organisms were chosen based on the following: (1) nutrient metabolism; (2) commercial availability; and (3) inocula preparation feasibility. Choice of specific commensals utilized would depend on the engineered organism being tested. **Figure 2B** lists the commensals used with engineered EcN. The goal was to evaluate an engineered organism by introducing EcN to mock communities assembled to generate polymicrobial cultures with increasing complexity and competition, using 4-member (EcN + 3 commensals), 6-member (EcN + 5 commensals) and 8-member (EcN + 7 commensals) communities. For EcN, the gut commensals selected were mixtures of bacteria with synergistic metabolic function (*i.e.*, microbes that utilize more complex carbohydrates than EcN to generate substrates for EcN), metabolic competitors (*i.e.*, bacteria that utilize similar oligosaccharides and monosaccharides as EcN but may not compete for identical substrates) and direct competitors of the same species/genus but different strains (*i.e.*, nutrient utilization of identical substrates as EcN). Characterization of EcN was not started in complex communities already at steady state, to ensure comparable nutrient access to all members of the community. Engineered organism persistence and plasmid stability were determined using qPCR, while fluorescence determined reporter functionality. Molecular characterization and biostatistical analysis of replicate fermentation results are also presented. While not fully physiologically-relevant, utilization of this test bed can address the following research questions: (1) Does an engineered organism persist with a stable plasmid in a complex microbial community?; (2) Is the reporter production and function altered in presence of commensal bacteria?; and (3) Does the engineered organism cause any changes to the commensal composition?

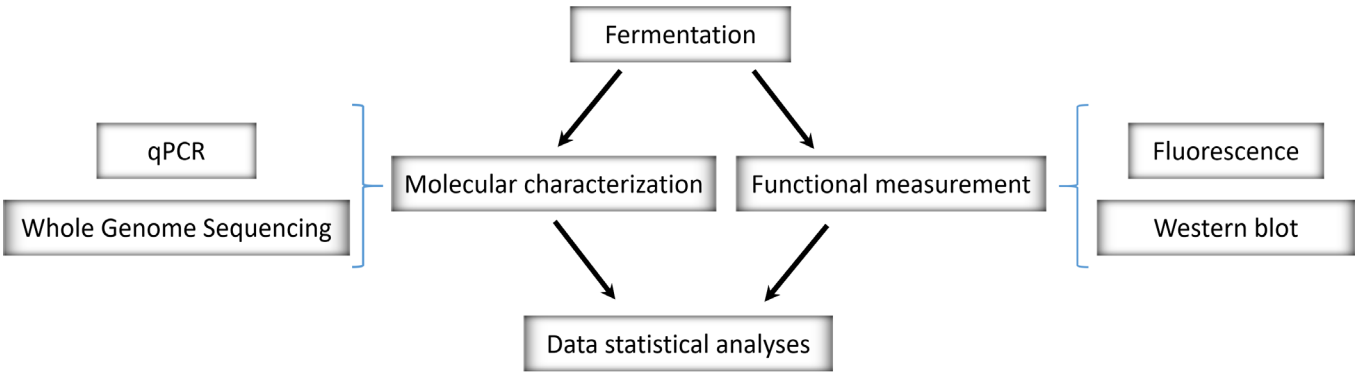


Figure 1. ***In vitro*** **fermentation test bed workflow.**

MATERIALS

Microorganisms

- ✓ *Escherichia coli* Nissle 1917 wild type (US Air Force Research Laboratory, Dayton, OH); designation EcNWT
- ✓ *Escherichia coli* Nissle 1917 engineered to produce GFPa1 (US Air Force Research Laboratory, Dayton, OH); designation EcN(cGFP)

NOTE: The following organisms were purchased from ATCC (Manassas, VA)

- ✓ *Bacteroides thetaiotaomicron* 29148
- ✓ *Bacteroides fragilis* 25285
- ✓ *Bacteroides ovatus* 8483
- ✓ *Lactobacillus acidophilus* 53544
- ✓ *Escherichia* coli K12 700926
- ✓ *Eubacterium rectale* 33565
- ✓ *Faecalibacterium prausnitzii* 27768

Reagents

NOTE: Culture media and chemicals were purchased from Millipore-Sigma (St. Louis, MO) unless noted.

- ✓ Luria Broth (LB)
- ✓ Lactobacilli MRS Broth (MRS)
- ✓ Difco Reinforced Clostridia Medium (RCM), (BD Biosciences, Sparks MD)
- ✓ Proteose peptone
- ✓ Yeast Extract (YE); (Fisher Scientific, Waltham, MA)
- ✓ Glucose
- ✓ K_2HPO_4
- ✓ KH_2PO_4
- ✓ Cysteine-HCl
- ✓ Hemin (98%)
- ✓ NaOH (JT Baker, Philipsburg, NJ)
- ✓ Vitamin K
- ✓ Ethanol
- ✓ Brain Heart Infusion (BHI) broth (BD Biosciences, Sparks MD)
- ✓ 1,4 naphthoquinone
- ✓ Na_2HPO_4
- ✓ NaH_2PO_4
- ✓ Glycerol
- ✓ Resazurin
- ✓ NaCl (American Bioanalytical, Natick, MA)
- ✓ Tween 80 (Fisher Scientific, Waltham, MA)
- ✓ Bacto Peptone (Fisher Scientific, Waltham, MA)
- ✓ Tryptone (Fisher Scientific, Waltham, MA)
- ✓ $NaHCO_3$
- ✓ $MgSO_4$-$7H_2O$ (American Bioanalytical, Natick, MA)
- ✓ $FeSO_4$-$7H_2O$
- ✓ $CaCl_2$-$2H_2O$
- ✓ Potato starch
- ✓ Pectin
- ✓ Guar gum
- ✓ Xylan (oat spelt)
- ✓ Arabinogalactan
- ✓ Inulin (dahlia)
- ✓ Casein (bovine milk)
- ✓ Bile salts #3
- ✓ Mucin (porcine type III)
- ✓ Lysozyme
- ✓ Tris
- ✓ EDTA
- ✓ Triton X-100
- ✓ HCl
- ✓ KCl (Thermo Fisher Scientific, Waltham, MA)
- ✓ Chloramphenicol (CAM)
- ✓ PopCulture lysis solution (EMD Millipore, Danvers, MA)
- ✓ PowerFecal DNA Isolation Kit (QIAGEN, Inc., Germantown, MD)
- ✓ 2X Forget-Me-Not qPCR Master Mix (Biotium Inc., Hayward, CA)
- ✓ Distilled, deionized water
- ✓ Ultrapure™ DNase/RNase Free Water (Invitrogen, Carlsbad, CA)
- ✓ Qubit™ dsDNA HS (High Sensitivity) Assay Kit (Invitrogen, Carlsbad, CA)
- ✓ Nextera DNA Flex Library Preparation Kit (Illumina, San Diego, CA)
- ✓ Agencourt AMPure XP Beads (Beckman Coulter, Brea, CA)
- ✓ Miseq Reagent Kit v2 (300 cycles) (Illumina, San Diego, CA)

Recipes

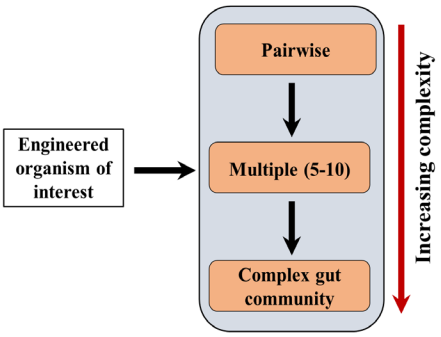
- ✓ Reduced phosphate buffered glycerol (PBG), per liter: 140 ml 0.2 M NaH_2PO_4, 360 ml 0.2 M Na_2HPO_4, 150 g glycerol, 8 g cysteine-HCl, 0.1% resazurin.
- ✓ Peptone-Yeast Extract-Glucose broth (PYG): Basal medium, per liter: 20 g proteose peptone, 5 g YE, 5 g sodium chloride. Supplement basal medium with (per liter): 20 ml 25% ($^{w}/_{v}$) glucose, 20 ml 25% ($^{w}/_{v}$) K_2HPO_4, 10 ml 5% ($^{w}/_{v}$) cysteine-HCl, 1 ml 0.5% ($^{w}/_{v}$) hemin in 1N NaOH, 500 µl 5 ug/ml vitamin K in ethanol [18].
- ✓ Supplemented Brain Heart Infusion broth (BHIS-NQ) [19], per liter: 37 g BHI. Supplement BHI with (per liter): 5 g YE, 10 mg hemin, 1 mg 1,4 naphthoquinone, and 0.8 g cysteine-HCl.
- ✓ MRS, RCM reduced with 0.8 g/L cysteine-HCl.
- ✓ LTX lysis solution: 20 mg/ml lysozyme, 20 mM Tris-Cl pH 8, 2 mM EDTA, 1.2% Triton X-100.
- ✓ Complex Colonic Medium (CCM): **Table S1**.
- ✓ CAM stock: 25 mg/ml in 100% ethanol.
- ✓ qPCR reactions: 10 µl Biotium Master Mix, 2 µl primers at 5 pmol/µl, 2 µl DNA template, 4 µl DNAse free water.
- ✓ 80% ethanol: 8 ml absolute ethanol into 2 ml distilled water.

Equipment

- ✓ Coy Vinyl "A" & "C" dual anaerobic chamber (Coy Labs, Green Lake, MI) with HEPA filtration system, incubator, airlock, and oxygen monitors
- ✓ 100 ml glass serum bottles with 20 mm grey rubber septa and 20 mm aluminum caps with center tear out (Wheaton, Millville, NJ)
- ✓ 16 × 100, 16 × 125 mm Hungate tubes (Thermo Fisher Scientific, Waltham, MA); 13 mm rubber septa and open top screw caps (Bellco Glass, Vineland, NJ)
- ✓ HEL BioXplorer 100 (HEL Group, Borehamwood, UK) with acid/base pump modules and mass flow controllers
- ✓ Redox potential probes (Applikon Biotechnologies, Foster City, CA)
- ✓ pH probes (Applikon Biotechnologies, Foster City, CA)
- ✓ Nanodrop One^{c} (ThermoFisher Scientific, Inc., Waltham, MA)
- ✓ iCycler iQ Optical module (BioRad Laboratories, Hercules,

CA)
- ✓ HJY Fluorolog 3 fluorimeter (Horiba Scientific, Piscatawy, NJ)
- ✓ WPA CO8000 Cell Density Meter (Biochrom Ltd, Cambridge, UK)
- ✓ Qubit assay tubes (Life-technologies, Carlsbad, CA)
- ✓ PCR tubes (Molecular Bioproducts, San Diego, CA)
- ✓ Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA)
- ✓ Agencourt Magnetic stand (Beckman Coulter, Brea, CA)
- ✓ 4200 Tapestation System (Agilent, Santa Clara, CA)
- ✓ Applied Biosystems Veriti 96-Well thermal cycler (Thermo Fisher Scientific, Waltham, MA)
- ✓ MiSeq sequencer (Illumina, San Diego, CA)

A



B

	Mock community organisms for evaluating engineered *E. coli* Nissle		
	Organism	**Substrate utilization**	**Community dynamics**
Probiotic	*Escherichia coli* Nissle GFPa1	Mono- and oligosaccharides	Engineered organism
	Escherichia coli Nissle wild type	Mono- and oligosaccharides	Control organism
Commensals	*Bacteroides thetaiotaomicron*	Simple starch, complex/simple carbs	Synergistic/competitor
	Lactobacillus acidophilus	FOS, GOS, MOS	Competitor
	Escherichia coli K12	Mono- and oligosaccharides	Direct competitor
	Bacteroides fragilis	Dietary glucans, glucose	Synergistic/competitor
	Eubacterium rectale	Simple starch, monosaccharides, XOS	Synergistic/competitor
	Bacteroides ovatus	Dietary glucans	Synergistic
	Faecalibacterium prausnitzii	Simple carbs, mono-, oligosaccharides	Competitor

Figure 2. Mock community design and build. A. *In vitro* fermentation test bed approach to testing in a polymicrobial community with increasing complexity and competition. **B.** Mock community design. Organisms chosen based on substrate and metabolic competition with engineered organism of interest.

PROCEDURE

Microorganism growth and storage

The organisms used in this study include seven commensal bacteria from ATCC, engineered EcN(cGFP) and EcNWT as a control. The EcN(cGFP) construct that constitutively expresses the fluorescent protein GFPa1 was made by transforming *E. coli* Nissle 1917 wild type (EcNWT) with the pAME200 plasmid (See supplemental information, **Fig. S1** for construct details).

1. Microorganisms from ATCC were reconstituted in recommended growth medium.
2. EcN(cGFP) and EcNWT were grown in LB + 25 ug/ml CAM and LB, respectively.
3. Each organism was grown to an optical density at 600 nm (OD_{600}) = 0.5–1, representing a mid-log concentration of ~1×10^8 Colony Forming Units (CFU/ml).

   NOTE: OD_{600} = 0.5–1 has previously been determined by viable plate count to be ~1×10^8 CFU/ml. If using other organisms, OD_{600} should be validated by plate count.

4. Reduced 50% (w/v) glycerol was added to a final concentration of 15% glycerol.
5. 100 µl aliquots were stored at −80˚C as single use frozen stocks.

   CAUTION: All bacterial cultures should be treated as potentially pathogenic to the laboratory worker and colleagues. Use of appropriate aseptic techniques, and the wearing of appropriate personal protective equipment are strongly recommended.

   CRITICAL STEP: For facultative anaerobes (EcN(cGFP), EcNWT, *E. coli* K12, *L. acidophilus*) prepare under anaerobic conditions.

Mock community preparation

6. Equilibrate sterilized glassware, 250 ml centrifuge bottle with stir bar, serological pipets and pipet tips in chamber under 85% N_2:10% H_2:5% CO_2 mixed gas for at least 24 h prior to use. Equilibrate culture plates for 48 h.
7. Sterilize reduced liquid growth medium to ensure anaerobicity.

8. Transfer media immediately to anaerobic chamber.

NOTE: Tightly close cap to prevent boil over in the chamber airlock during gas exchange.

9. Grow each organism individually in an anaerobic chamber at 37˚C: *B. thetaiotaomicron*, *B. fragilis* and *B. ovatus* in PYG; *E. coli* MG1655 and EcNWT in LB; EcN(cGFP) in LB + 25 ug/ml CAM; *E. rectale* in BHIS-NQ; *F. prausnitzii* in RCM and *L. acidophilus* in MRS.

NOTE: ATCC recommended media not optimal for growth to achieve desired OD_{600} in step 10; alternative media needed for *B. thetaiotaomicron*, *B. fragilis*, *B. ovatus* and *E. rectale*.

10. Inoculate 5 ml starter culture of each organism from single use glycerol stocks stored at −80˚C. Grow to OD_{600} = 0.5–1 (~10^8 CFU/ml) in chamber at 37˚C (**Fig. 3**). Also incubate growth media (no cells) as a sterility control and OD_{600} blank. OD_{600} determined using the portable WPA CO8000 Cell Density Meter in the chamber.
11. Inoculate 200 ml medium (in 250 ml Pyrex bottle) with 5 ml starter culture. Grow to OD_{600} = 0.5–1 in chamber at 37˚C.
12. Transfer culture to equilibrated 250 ml centrifuge bottle in chamber and seal tightly. Remove sealed bottle from chamber, centrifuge 15 min, 12000 *g* at room temperature (RT) and return to chamber.
13. Remove culture supernatant by pipetting. Resuspend pellet by stirring in 200 ml reduced PBG. Determine OD_{600}; quantitate by viable plate count.

CRITICAL STEP: Growth media employed for commensal stock cultures must be removed through centrifugation and cell resuspension in reduced PBG. This is essential to mitigate any contribution from monoculture media when combining to generate fermentation inocula.

NOTE: Pipetting is used instead of decanting to prevent potential loss of cells.

14. Aliquot 5 and 10 ml cells into sterile Hungate tubes, seal using cap with septa and store at −80˚C.

NOTE: Cap Hungate tubes tightly to ensure cultures maintain anaerobicity when removed from chamber.

TIP/HINT: Aliquot volume will be dependent on inoculation volumes required for particular fermentation vessels (*e.g.*, 5 and 10 ml aliquots generated to support 150 ml fermentation vessels).

TIP/HINT: Capping tubes while maintaining sterility can be challenging; assemble septa and caps prior to sterilization and equilibration to minimize handling. Use sterile tweezers to pick up septa with caps to cap tubes in the chamber.

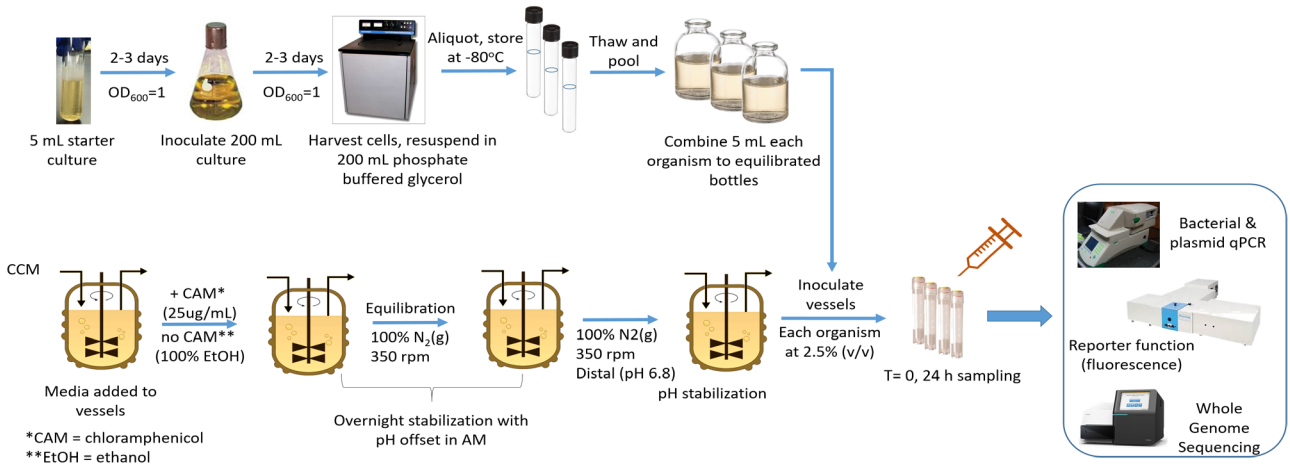


Figure 3. Test bed experimental set-up.

Fermentation inoculum preparation

The overall fermentation process is shown in **Figure 3**. Mixtures of bacteria were assembled in a stepwise fashion with increasing complexity. EcN(cGFP) was tested in monoculture (as a baseline), plus 3 commensals (4-member culture), plus 5 commensals (6-member culture) and plus 7 commensals (8-member culture). EcNWT was run as 4-, 6-, and 8-member cultures for direct comparison. Although not within the example provided herein, EcNWT monoculture can be included as a comparative sample.

15. Thaw frozen organism starter cultures (step 14) in anaerobic chamber. For each organism, pool sufficient volume for uniform concentration in each serum bottle when aliquoting for inocula assembly (step 16).

16. Add 5 ml each organism to equilibrated, sterile serum bottles and bring to 40 ml with reduced PBG, pH 7.2. The final inoculum volume for each organism was 12.5% ($^{v}/_{v}$). Bacterial composition for each bioreactor vessel can be found in **Table 1**.

17. Crimp serum bottles containing bacterial mixtures to maintain anaerobicity; remove from the chamber for vessel inoculation.

TIP/HINT: Cell-free PBG control prepared as an inoculation control.

CRITICAL STEP: Anaerobic conditions must be maintained during growth and assembly of bacterial mixtures to represent a physiologically relevant environment found in the gut. Presence of oxygen may inhibit the growth of commensal anaerobes, as well as change the growth and metabolism of facultative anaerobes (*e.g.*, *E. coli* Nissle 1917, *L. acidophilus*).

TIP/HINT: Do not combine monocultures prior to storage at −80°C. Store commensals individually and combine on day of study. Separate storage allows flexibility in commensal assembly.

In vitro fermentation

18. Add nutrient-rich CCM medium to HEL BioXplorer 100 parallel bioreactor vessels (100 ml/vessel) equipped with oxidation-reduction potential and pH probes.

NOTE: Numerous other growth media used for *in vitro* gut fermentation could be utilized as an alternative to CCM; resulting organism growth may be different.

19. Autoclave 35 min at 120 psig.

20. Sterilize and prime acid/base pump lines using clean in place procedure as follows: 10 ml 70% ethanol; 10 ml 2 N NaOH, 10 ml sterile deionized water, 10 ml 1 N NaOH for base and 0.2 N HCl for acid lines. Collect flow through in a beaker and discard appropriately.

Table 1. Fermentation vessel setup showing assembly of bacterial mixtures*.

Bioreactor vessel	Community	EcN(cGFP) (ml)	EcNWT (ml)	Bt (ml)	La (ml)	EcK12 (ml)	Br (ml)	Er (ml)	Bo (ml)	Fp (ml)	PBG (ml)	Final volume (ml)
V1	EcN(cGFP) monoculture	5									35	40
V2	EcNWT 4-member		5	5	5	5					20	40
V3	EcNWT 6-member		5	5	5	5	5	5			10	40
V4	EcNWT 8-member		5	5	5	5	5	5	5	5	0	40
V5	EcN(cGFP) 4-member	5		5	5	5					20	40
V6	EcN(cGFP) 6-member	5		5	5	5	5	5			10	40
V7	EcN(cGFP) 8-member	5		5	5	5	5	5	5	5	0	40
V8	CCM medium										40	40
Total		20	15	35	35	35	20	20	10	10	135	

*A vessel with CCM only was the fermentation control. Volume of each organism in the mixture is 12.5% (v/v).

21. Attach vessels to bioreactor platform by connecting redox and pH probes, inserting temperature probe, connecting to gas line that feeds 100% O_2-free N_2 (g) at 20 psig and rate of 5 ml/min and installing condenser.

CRITICAL STEP: Store vessels awaiting connection to bioreactor on stir plate at 250 rpm. When attaching vessels to bioreactor platform, apply gentle manually-activated stirring to avoid settling of insoluble medium components.

TIP/HINT: Connection of vessels from back to front of bioreactor platform facilitates working with small vessel within the tight space available.

22. Add 25 ug/ml CAM to vessels that to be inoculated with EcN(cGFP); ethanol only was added to vessels assigned to EcNWT.

NOTE: The plasmid encoding the GFPa1 contains a chloramphenicol resistance gene; addition of CAM prevents plasmid loss.

23. Equilibrate vessels containing medium overnight at 37˚C with constant agitation (350 rpm) and under constant headspace flush without pH adjustment.

24. Adjust calibration drift that occurs as pH probes cool during overnight equilibration by manual verification of pH by removing 5 ml aliquots from each vessel using a sterile 5 ml syringe.

25. Activate acid/base pumps to adjust and maintain pH in vessels to emulate the distal colon (pH 6.8 ± 0.1) by addition of 1 N NaOH and 0.2 N HCl and allow to reach steady state for 30 min.

26. Using a sterile 10 ml syringe with a 23-gauge sterile needle, inoculate vessels from crimped serum bottles (step 17) with 20 ml of each bacterial mixture (final 2.5% ($^{v}/_{v}$) inoculum; 5×10^6 CFU/ml for each bacteria/vessel), final vessel volumes ~120 ml.

NOTE: Collect inoculum aliquots for quality control purposes: (1) plating (1 ml); (2) baseline GFP (4 ml); (3) baseline composition (4 ml); and (4) baseline plasmid stability (4 ml).

CAUTION: Spray diluted bleach solution on the vessel and serum bottles septa to avoid contamination during inoculation and add full volume to vessel before aliquoting inoculation controls.

27. Run fermentation for 24 h. Remove 4 ml aliquots in duplicate at 0 and 24 h using sterile 5 ml syringes for post-fermentation analyses: (1) GFP fluorescent function; (2) organism growth persistence and plasmid stability by qPCR; and (3) Whole Genome Sequencing (WGS) to determine commensal abundance. Store samples for fluorescence at −20˚C, others at −80˚C.

NOTE: Additional temporal aliquots are recommended if growth rate and flux comparisons are desired. Fermentation occurs rapidly due to nutrient-rich medium; typically reaching mid-log growth at ~6–7 h and stationary phase at ~10–12 h. Unless performing a fed-batch fermentation, sampling beyond 24 h is unnecessary as nutrient-limiting conditions are typically reached after 12 h.

Fluorescence measurements

28. Transfer 1.8 ml from GFP fermentation aliquots at each residence time from each vessel to 2.0 ml centrifuge tubes.

29. Centrifuge 5 min, 16000 *g* at RT.

30. Remove supernatant, discard and wash pellet by resuspending in equal volume of 0.9% NaCl by vortexing.

NOTE: If SCFA or metabolomics analysis desired, save and store supernatant at 4°C prior to subsequent processing.

31. Centrifuge 5 min, 16000 *g* at RT; discard supernatant and repeat wash.

32. Resuspend cells in 800 µl LTX lysis solution (filter sterilized using a 0.2 um syringe filter) to lyse cell pellets 30 min at 37°C.

TIP/HINT: Lysis in a water bath is ideal for even heating across tubes.

NOTE: LTX solution is stable for 1 month stored at 4°C.

33. Add 200 µl 0.5× PopCulture lysis solution (final concentration = 10%); incubate 20 min at RT.

NOTE: POP detergent can be used independently to lyse cells; however, lysozyme has been shown to enhance lysis efficiency of the Gram-positive bacteria (*e.g.*, *Lactobacilli*). LTX solution suggested by QIAGEN [20] to ensure that difficult to lyse Gram-positive microorganisms are efficiently extracted.

34. Centrifuge 5 min, 16000 *g*; transfer clarified lysate to sterile 2 ml centrifuge tube.

35. Measure fluorescence by adding 700 µl of each sample to a semi-micro quartz cuvette and inserting into a cuvette holder for right angle analysis on HJY Fluorolog 3 fluorimeter.

TIP/HINT: Centrifuge samples immediately prior to measurement to pellet any cellular debris inadvertently transferred with lysate.

36. Fluorescence parameters for GFP: excitation = 480 nm; slits = 1.5 nm; increment = 1 nm; integration time = 0.5 s; emission scan = 485–550 nm; anticipated λ_{max} = 510–512 nm.

NOTE: The fluorescent parameters will vary based on the fluorescent reporter. Negative control (lysis buffer) should be run in addition to media only vessel control to effectively determine background associated with CCM.

CAUTION: Do not agitate sample prior to transfer into cuvette to avoid adding particulates that will scatter light and confound interpretation of results.

CAUTION: Wash cuvette between samples thoroughly with DI-water to avoid cross contamination.

CRITICAL STEP: Always run a positive fluorescent control to determine if compensation from fluorescent variation due to instrumental parameters that may vary between runs is required. If possible, run samples from all 8 vessels during the same instrument on/off cycle. If not, adjustments may be needed based on fluorescent control values to accurately compare measurements between analysis days/cycles.

DNA extraction

37. DNA from fermentation aliquots for persistence and plasmid stability was isolated using PowerFecal DNA Isolation Kit with LTX solution modification.

38. Thaw 4 ml fermentation aliquots (step 27) at RT.

39. Pellet bacteria by centrifugation for 2 min, 10000 *g* at RT.

40. Remove all but 1 ml supernatant.

41. Vortex; transfer sample to PowerFecal Kit bead tube.

NOTE: Vortexing resuspends the bacterial pellet for transfer to bead tube.

42. Centrifuge 30 s, 10000 *g*; remove supernatant.

43. Add 200 µl LTX solution, vortex; incubate 30 min, 37°C.

NOTE: Extraction protocol modified by addition of LTX lysis to ensure that difficult to lyse Gram-positive microorganisms (*e.g.*, *Lactobacilli*) are efficiently extracted.

44. Add 550 µl kit Bead Solution; vortex.

45. Follow remainder of PowerFecal DNA Kit protocol.

46. Quantitate DNA using a Nanodrop.

NOTE: Other instruments such as the Qubit can also be used for quantitation.

47. Store DNA at −20°C.

qPCR of organism and plasmid persistence

48. Preparation of qPCR standard.

48.1. Grow 5 ml EcN(cGFP) to OD_{600} = 0.5–1.

48.2. Determine CFU/ml using viable plate count as in step 3.

48.3. Extract DNA using PowerFecal Kit with LTX modification.

48.4. Quantitate by Nanodrop.

48.5. 10-fold serially dilute DNA with DNAse-free water. Seven concentrations used for standard curve.

49. qPCR reactions.

49.1. Organism persistence: Mut7f, Mut8r primers (**Table S2**), iCycler program 95˚C, 2 min, then 45 cycles of 95˚C for 10 s, 60˚C for 10 s, 72˚C for 25 s.

49.2. Plasmid stability: GFPa1-f and GFPa1-r primers (**Table S2**), iCycler program 95˚C, 10 min, then 45 cycles of 95˚C for 15 s, 51˚C for 1 min, 72˚C for 25 s.

49.3. From the extracted DNA samples in step 47, determine absolute abundance (CFU/ml culture) of EcNWT and EcN(cGFP) using a standard curve generated from the target organism of known CFU/ml concentration.

49.3.1. Standard curve is generated by qPCR reactions of a series of 10-fold dilutions (step 48.5) with known CFUs.

49.3.2. Plot log CFU against PCR cycle threshold (Ct) values to generate a linear curve.

NOTE: Using log CFU is necessary for generating a linear curve.

49.3.3. Sample Ct is then interpolated on the standard curve to find the sample log CFU. (**Fig. S2**).

49.3.4. Convert log CFU to log CFU/ml culture.

49.3.5. GFPa1 plasmid concentrations (ng/ml culture) are calculated in a similar manner using the same DNA extracted (step 47).

Whole genome sequencing

50. Quantitate DNA (step 47) using Qubit dsDNA HS Assay Kit.

51. Dilute samples to 100 ng total DNA in 10 mM Tris HCl (pH 8.0) to a final sample volume of 30 µl.

CRITICAL STEP: Accurate concentration is crucial for bacterial DNA genomic library preparation.

52. Generate libraries using PCR with Nextera DNA Flex Library Preparation Kit.

53. Sequence libraries using Illumina Miseq Reagent Kit.

Biostatistical analyses

Analyses were done to identify statistically significant differences. Culture comparisons (from $n = 4$ fermentations) are identified in **Table 2**.

54. Within either Excel or statistical analysis software, create separate spreadsheets or tabs for fluorescence measurements (peak intensity at λ_{max}), plasmid qPCR (ng/ml), and organism qPCR (ng/ml). Compile the appropriate data from replicate experiments ($n = 4$) into each spreadsheet. Arrange data such that replicates of the same microbial community are in the same column, and such that data in each row is from the same replicate. Add columns denoting replicate identifier, sampling time, and presence of EcN(cGFP) or EcNWT control for each row.

NOTE: Requirements for arranging data for statistical analysis depend on the software program used. The above arrangement is usable by multiple spreadsheet-based analysis programs, including SigmaStat and JMP SAS used here, for analysis of variance (ANOVA).

Fluorescence measurement analysis

55. Determine that the data are normally distributed (Shapiro-Wilk test for normality or visual inspection of a histogram) and that variances between groups are equal (Brown-Forsythe test).

56. If data are normally distributed and variances are equal, perform an ANOVA test. Use the P value ≤ 0.05 to determine significant difference between groups.

57. If a significant difference was detected, perform Tukey's Honest Significant Difference (HSD) test to evaluate pairwise differences between groups (threshold: $P \leq 0.05$) and isolate which group(s) differ from the others.

CAUTION: Results of parametric tests (*e.g.*, ANOVA) may not be accurate if data are not normally distributed and/or variances are not equal. If those criteria cannot be met, use a non-parametric equivalent test for accurate results. For the above procedure, use Kruskall-Wallis test in place of ANOVA.

Organism and plasmid qPCR analysis

58. Calculate the base-10 logarithm of each data point to create a normally-distributed data set.

59. Perform a Two-Way Repeated Measures ANOVA, setting sample time and member composition as fixed factors. Assess P value for each fixed factor as well as the interaction between the two factors (threshold for significant difference: $P \leq 0.05$).

60. If a significant difference was detected, perform Tukey's HSD as described earlier.

NOTE: A significant interaction P value indicates that differences detected between groups are due to the combination of sample time and member composition rather than either factor alone. The interaction and fixed factor P values should be used to inform interpretation of pairwise differences detected by Tukey's HSD.

NOTE: Paired data or repeated measures techniques, like the one employed here, mitigate inherent variability in biological data sets by linking data from the same experimental replicate together; however, they require that data from each combination of sample time and member composition is accounted for in each replicate. For data sets not meeting these criteria, a standard two-way ANOVA may be used, but this approach is less likely to yield statistically significant results.

Table 2. Culture comparisons used in statistical analyses (n = 4 fermentations).

Measure	Comparison	Bioreactor vessel: Community	Analysis
Community complexity on EcN(cGFP) fluorescence function, organism persistence, plasmid stability	EcN(cGFP) polymicrobial cultures *vs*. monoculture	V5: EcN(cGFP) 4-member V6: EcN(cGFP) 6-member V7: EcN(cGFP) 8-member V1: EcN(cGFP) monoculture	**Fluorescence**: one-way analysis of variance (ANOVA) **Organism persistence**: two-way repeated measures ANOVA; hypothesis tests (ANOVA followed by pairwise comparisons) to cultures *vs*. monoculture determine which, if any, communities of differing size show statistically significant differences in measures **Plasmid stability**: two-way repeated measures ANOVA
Effect of engineering on EcN persistence	EcN(cGFP) *vs*. equivalent EcNWT cultures	V5: EcN(cGFP) 4-member *vs*. V2: EcNWT 4-member V6: EcN(cGFP) 6-member *vs*. V3: EcNWT 6-member V7: EcN(cGFP) 8-member *vs*. V4: EcNWT 8-member	Pairwise *t*-tests to compare engineered *vs*. wild type cultures in communities of equal size
Effect of engineering on commensals	EcN(cGFP) *vs*. EcNWT polymicrobial cultures	V5 + V6 + V7: all EcN(cGFP) *vs*. V2 + V3 + V4: all EcNWT	Pairwise *t*-tests to compare engineered *vs*. wild type cultures when merging communities of all sizes

Bioinformatics analyses

The selection of bioinformatics software for WGS data analysis was as follows:

61. KneadData to perform quality control and determine fastq file of filtered sequences [21].

NOTE: Raw fastq file is taken as input and trimmed using 70% length of input reads and a quality-controlled fastq file is output.

62. HUMAnN 2.0 to determine gene families file, pathway abundance file and pathway coverage file [22].

NOTE: Quality controlled fastq file is input and organisms present are mapped.

63. MetaPhlAn2 to determine taxonomic profile [23].

NOTE: Performs species-level profiling and estimation of abundance.

64. ChocoPhlAn [HUMAnN2 proprietary] database to functionally annotate species pangenomes [24].

NOTE: Performs nucleotide-level search through collection of species pangenomes using genomes available from NCBI GenBank and RefSeq.

65. UniRef universal protein reference database to define gene families [25].

NOTE: Provides definitions of gene families for clustering organisms identified.

66. Principle Components Analysis (PCA) compared overall community between EcNWT and EcN(cGFP) using RStudio and a P value = 0.05 significance level.

ANTICIPATED RESULTS

Here we demonstrate an *in vitro* fermentation test bed approach to evaluate an engineered microorganism in a polymicrobial community. Engineered organism function and persistence, plasmid stability, and effects on commensal community were determined (results represent n = 4 fermentations). The function of *E. coli* Nissle constitutively producing the fluorescent protein GFPa1 was affected by commensal community complexity, with fluorescence dramatically increasing in the 8-member culture (and to a lesser extent the 6-member) relative to monoculture (**Fig. 4A**). These results were confirmed by Western blot detection of increased GFPa1 (**Fig. S3**). It is unlikely that the 8-member increase is due to autofluorescence from addition of organisms Bo and Fp, as signal from the corresponding EcNWT 8-member showed no additional fluorescence. It is possible that cross feeding of compounds produced by these additional organisms resulted in more efficient GFPa1 expression. Further investigation using multi-omics analysis could reveal the reason for the result. qPCR measured organism persistence and GFPa1 plasmid concentration as a function of community complexity. Engineered organism concentration (log CFU/ml) was not statistically different between vessels at 0 h. Increasing complexity exhibited no effect on EcN(cGFP) persistence; growth after 24 h showed no statistically significant difference when comparing mono-, 4-, 6-, and 8-member cultures (**Fig. 4B**). However, GFPa1 plasmid concentration continued to decrease compared to monoculture as more commensals were added (**Fig. 4C**). Engineering did not adversely influence EcN growth; direct complexity comparisons (EcN(cGFP) *vs*. corresponding EcNWT fermentation) revealed no statistical differences (**Fig. 4D**). The effect of EcN(cGFP) on commensal relative abundance was investigated by WGS after 24 h fermentation. With the exception of *E. rectale* in the 6-member culture, commensal relative abundances were not statistically different when comparing EcN(cGFP) and EcNWT cultures (**Fig. 5**). The differences in commensal abundance were likely due to differential growth of each organism. The PCA comparison of the entire community by grouping all the EcNWT and comparing to all EcN(cGFP) 24 h cultures showed no obvious clustering, suggesting that engineering EcN does not influence the community differently than wild type (**Fig. S4**). The ellipses on the PCA biplots are normal probability ellipsoids covering 95% of the probability distribution for each experimental group. The assumption is that each experimental group follows a bivariate normal distribution with regard to the first two principal components of the dataset. Overlapping ellipses indicate populations follow similar, often indistinguishable, probability distributions [26].

CONCLUSIONS

We have used a simplified mock community in an *in vitro* fermentation test bed to determine the persistence and function of an engineered probiotic. The workflow provides a framework for evaluating any microorganism in a polymicrobial community; it is agnostic regarding organism of interest, microbial community or analyses. This system was purposely not fully physiologically relevant, but designed to screen functionality of the proof-of-concept engineered organism EcN(cGFP). Numerous aspects of the test bed can be optimized to improve relevance, based on desired outcomes ranging from quick screening under batch conditions over 24 h, similar to described here, or more physiologically-relevant approach using a simulated human large intestine model that can be conducted over a 5-week period with a two-week dosing regimen under continuous culture conditions. Further optimization includes adjusting starting relative abundances of the simplified community to more accurately reflect *in vivo* conditions, or adding the engineered organism to an established commensal community. Genetic incorporation of the engineered organism functionality into the chromosome would eliminate antibiotic addition necessary for maintaining plasmid stability. Other functions including metabolic activity, antibiotic resistance, multi-'omics analysis or impact on microbiota can be determined and, if combined with a host model incorporating human cell lines, host response as a function of microbial function can be ascertained and the testing outcomes expanded. Community composition can be tailored to increase complexity or metabolic characteristics, or a fully complex community such as fecal inoculum can be used to enhance physiological relevance. The *in vitro* fermentation test method provides a more complex, competitive environment than monoculture and represents a new element of testing within the DBTL approach, generating feedback to inform the transition of engineered probiotics to higher fidelity evaluations in

animal models or human studies.

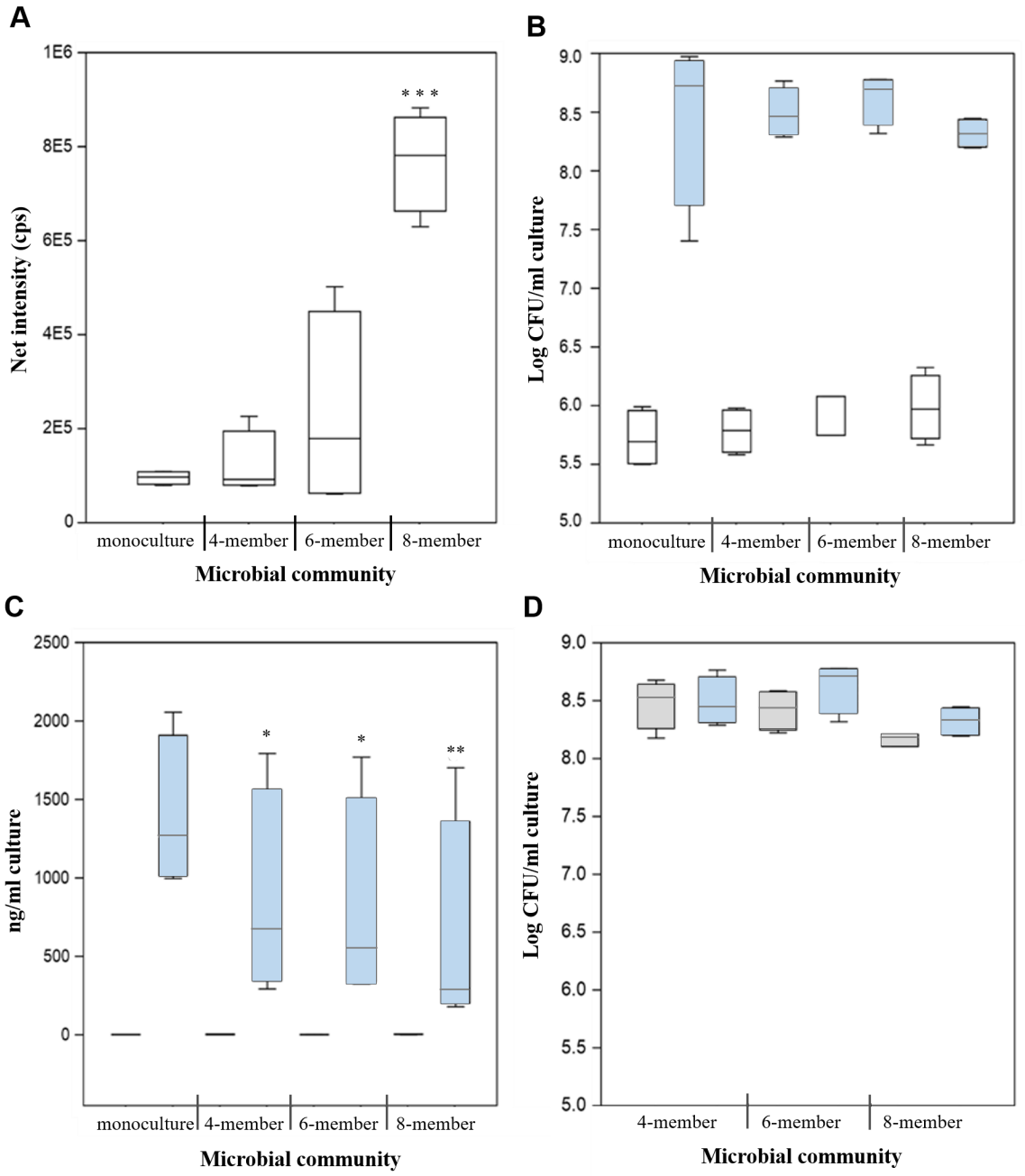


Figure 4. Engineered organism EcN(cGFP) function and persistence in complex communities (*n* = 4 fermentations). A. Net GFPa1 fluorescence at 24 h; ****P* value < 0.001 compared to monoculture. qPCR analysis of engineered *E. coli* Nissle in microbial communities, compared to monoculture. **B.** EcN(cGFP) organism persistence at 0, 24 h. **C.** EcN(cGFP) plasmid quantitation at 0, 24 h; ***P* value < 0.05, **P* value < 0.01. For (B) and (C), white bars are 0 h, blue bars are 24 h samples. **D.** EcNWT (gray bars) *vs*. EcN(cGFP) (blue bars) organism persistence at 24 h.

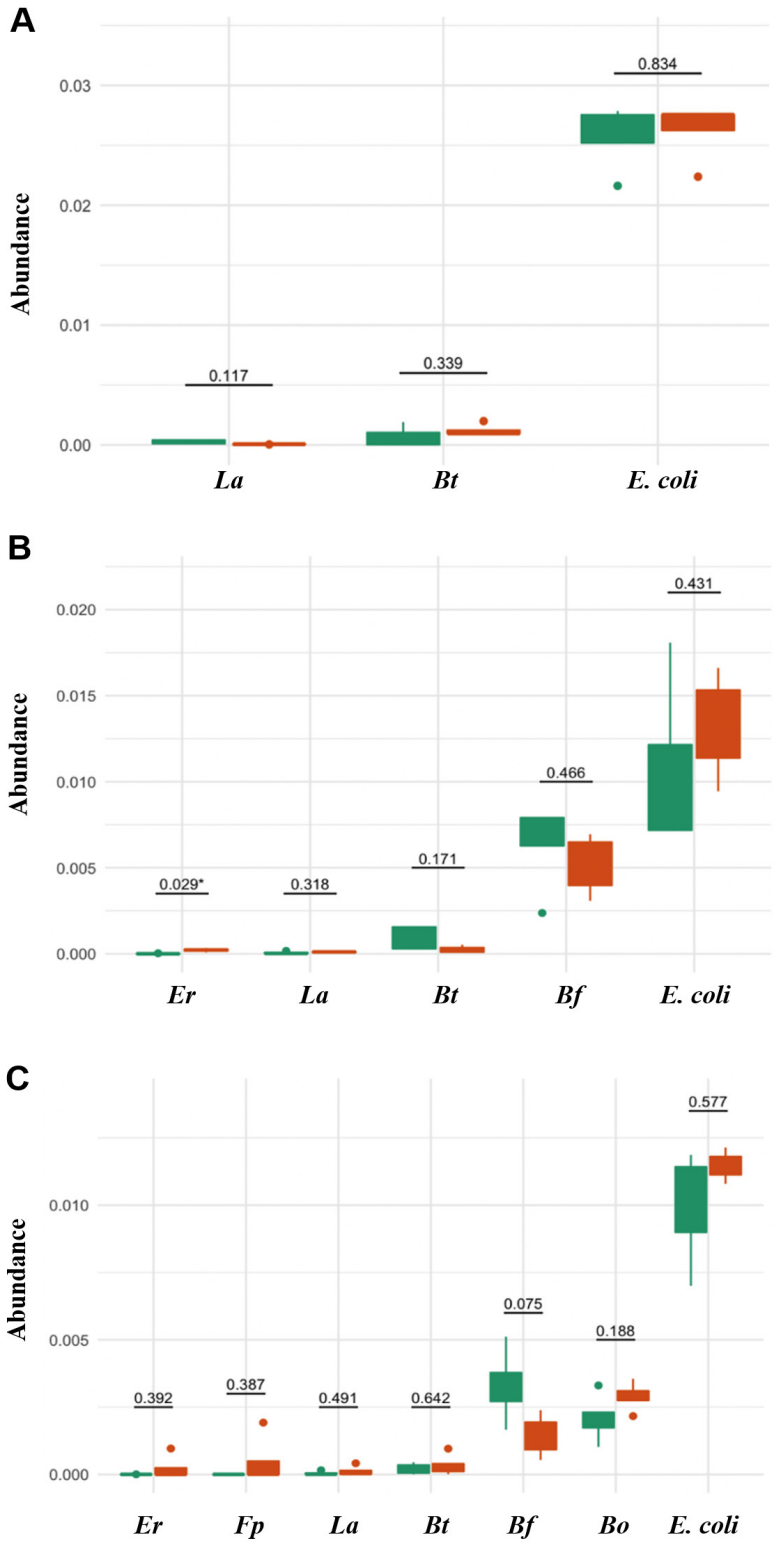


Figure 5. WGS to determine commensal species abundance comparing fermentations containing engineered EcN(cGFP) or wild type Nissle (EcNWT) at 24 h. A. 4-member, **B.** 6-member, **C.** 8-member fermentations. EcNWT: red bars; EcN(cGFP): green bars. It should be noted this analysis could not distinguish between the *E. coli* Nissile and *E. coli* K12 strains. Abundance is the aggregate sum of sample abundances (RPK) for all samples from the fermentation replicates of each experimental group (*i.e.*, 4-member, 6-member, 8-member). Underlined values above boxplots indicate the *P* values for two-sample *t*-tests comparing EcNWT and EcN(cGFP), with a *P* value = 0.05 significance level.

TROUBLESHOOTING

Possible problems can be experienced when using the protocol and their solutions are listed in **Table 3**.

Table 3. Troubleshooting table.

Step #	Problems	Causes	Suggestions
9	Growth of microorganisms. Culture growth using medium recommended by ATCC does not achieve desired OD	Media recommended by ATCC is the medium the deposited culture was grown in and may not be optimal	Test other media identified in literature for improved growth
28	Fermentation vessel failure. During the fermentation run, vessel malfunction in temperature, pH control or stirring creating an incomplete replicate for comparative analysis	Temperature threshold is exceeded, pH probe failure, or stirring module failure during installation are all system defaults that cause a shutdown	If this occurs, consider running a replicate but with only the engineered and WT mixture that is required, statistical analysis can be used to compensate for any differences between replicates
36	Florescent response on fluorimeter. Signal:noise of fluorescent signal too high	Measurement parameters not optimized to minimize signal:noise when comparing reporter signal to WT or media only controls	Prior to fermentation, perform baseline parameter optimization with monocultures of engineered and WT bacteria using ideal growth parameters. If needed, run an emission scan at optimal excitation wavelength under varying slit openings until fluorescent value is about 300 x 10^3 intensity (CPS)
36	Fluorescent positive control variance between replicates. Instrumental variation can artificially alter fluorescent intensity between analysis of fermentation replicates	Age, temperature and use of xenon flash lamp employed to excite fluorescent molecules alters signal artificially	Run monoculture positive control, to baseline fluorescent measurements or sample with ample intensity from previous replicate fermentation, and normalize values if the positive control varies by more than 10% between analysis days

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Supplementary information

Figure S1. Plasmid construction and strain engineering of *E. coli* Nissle 1917 for constitutive production of the fluorescent protein GFPa1.

Figure S2. qPCR workflow to calculate log CFU/ml culture organism persistence from sample Ct values.

Figure S3. Immunodetection of GFPa1 protein expression.

Figure S4. PCA of overall community dynamics comparing EcN(c-GFP) to EcNWT.

Table S1. Complex colonic medium recipe.

Table S2. Primers sequences used for qPCR.

Supplementary information of this article can be found online at https://jbmethods.org/jbm/article/view/347.