High-Throughput Isolation and Culture of Human Gut Bacteria with Droplet Microfluidics

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

2019

ABSTRACT

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Abstract

The hundreds of microbes that inhabit the human gut play a large role in human health and disease. The ability to isolate and culture bacteria from within the human gut allows us to interrogate their role in causing disease and therefore may lead to the discovery of new therapeutics. However, characterization of these microbes represents a complex and substantial problem based on the diversity and magnitude of unique microbes present in the human gut: there are hundreds of unique microbes present within an individually and potentially billions of individual bacterial cells within one gram of their stool. While microbiologists have been working on this problem for decades, current approaches have been unable to experimentally study individual bacterial taxa at the depth necessary to obtain comprehensive insight into their function within hosts. Here, we combine advances in droplet microfluidics and high-throughput DNA sequencing to develop a platform (MicDrop) for isolating and assaying microbiota members in picoliter droplets. MicDrop can be used to create millions of distinct bacterial colonies in a single experiment while using off-the-shelf parts that are compact enough to fit in an anaerobic chamber. In proof-of-concept experiments, we used the platform to characterize antibiotic sensitivity in a set of gut microbes. We also used MicDrop to test the hypothesis that growth kinetics of individual gut bacterial taxa are associated with long-term community dynamics in an artificial gut. These demonstrations suggest that the MicDrop platform

could support future diagnostic efforts to personalize microbiota-directed therapies, as well as to provide comprehensive new insights into the ecology of human gut microbiota.

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To Patricia Pukkila

"Instructions for living a life. Pay attention. Be astonished. Tell about it."
– Mary Oliver

1. Introduction

We are inhabited, surrounded, and interact with thousands of different microbes every day. The importance and contribution of these microbes to human health has been widely recognized, and research into these effects is growing. More and more, the importance of therapeutics targeting microbes, as well as using microbes as therapies themselves is being investigated.

The advent of next generation sequencing (NGS) technologies has made the discovery and cataloging of large numbers of microbes easier and cheaper than ever. As more data become available, there is a greater call for functional assays of the microbiome. While defining the function and phenotypes of individual microbes is important, this remains elusive due to the diversity of microbes that live in and around us. Many of these microbes remain uncultured, and we lack comprehensive culture collections to study them. Moreover, sequencing-based assays remain popular because of the low-cost of sequencing.

In this dissertation, I present a novel method leveraging the throughput of NGS and droplet microfluidic techniques in order to isolate and phenotype the growth of microbes from the gut microbiome. I was then able to apply the knowledge ganed from

this novel dataset about the growth of bacterial growth from the human gut to an artificial gut system, where I could then identify an individual bacterium's contribution to the community composition. This approach has potential applications in the development and promotion of microbial therapeutics, and our findings lay the groundwork for optimizing in vitro modeling systems of the gut microbiome, as well as applying these models to other complex microbial systems with functions relevant to their host or environment.

1.1 Human gut microbiome

The human microbiome (the collection of microbes that live in and on us) has long been recognized for its role in human health and disease. Since the revolution of affordable sequencing, we now have access to unprecedented catalogs of microbes from diverse sources: gut microbiomes from people around the world (with different cultures with diets), as well as samples from different body sites. These studies reveal a diverse set of microbes that we coexist with. Moreover, these studies have begun to reveal how microbes contribute to overall human health. Microbes are integral to the development of our immune system: studies involving gnotobiotic (germ-free) mice show animals with severely compromised immune systems (Goodman et al., 2011). Changes in microbiome

composition have been associated with diet change and obesity (David et al., 2014; Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2009; Turnbaugh et al., 2006). Microbes have been shown to be causative in stomach ulcers, and found to have associations with IBD and colon cancer (Garrett, 2015). Moreover, a healthy microbiome is shown to have protective effects in hospital associated infections such as *Clostridium difficile* (Lawley et al., 2012). More recently studies have shown a role for the human gut microbiome in drug metabolism (Maier et al., 2018b; Zimmermann et al., 2019). These studies show the important role that gut microbes may have in the diagnosis, treatment, and maintenance of human health.

A major tool for identifying these associations has been NGS (Overview of bacterial community profiling, Figure 1). NGS allows for the multiplexing of many samples, which allows the profiling of hundreds of bacterial communities at one time. This throughput combined with the ease of sample collection from patients and subjects (most often stool samples) makes analyzing the microbiome affordable and accessible to many researchers. Community profiling by NGS has led to many disease and health associations and is now a valuable part of microbiome research.

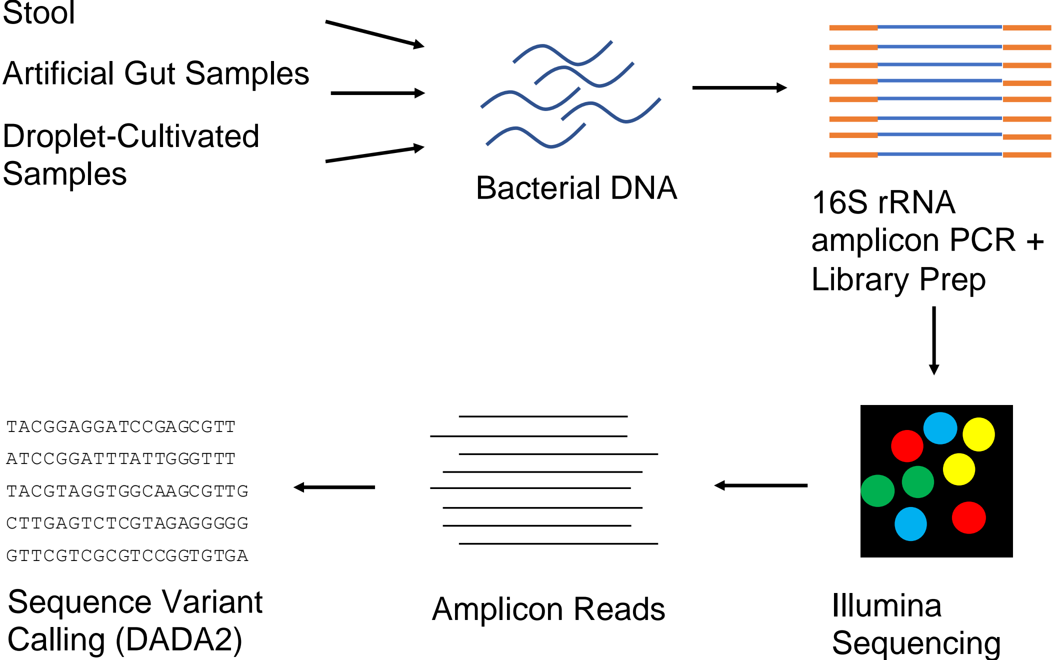

Figure 1. Overview of 16S rRNA Amplicon Sequencing. Stool and artificial gut samples are the primary samples used. DNA is extracted from the samples then the 16S rRNA gene is amplified and the products are uniquely barcoded for pooled sequencing. Sequence variants from the amplicon reads are then used in all downstream analyses.

While identifying associations with health outcomes is important in the microbiome field, recently there has been more of a push for identifying mechanisms the microbiome use to exert an effect on human health in order to develop therapeutics and treatments targeting the microbiome. The most notable of these therapeutics include probiotics (inserting a living organism into the community, as a specific replacement treatment, or in order to promote or inhibit growth of other members of the community)

and prebiotics (inserting a dietary supplement such as fiber in order to promote the growth of beneficial microbes). Other therapies include antibiotics, with potential detriments to the microbial community, as well as other xenobiotics that change the composition of the microbial community.

Efforts to target the microbiome therapeutically will require identifying the individual contributions of microbes as well as to the greater community. Identifying individual contribution of microbes often requires direct isolation of a microbe, for the purposes of genetic manipulation or other experiments.

1.2 *Cultivating gut microbes with microfluidics and an artificial gut system*

Cultivation has long been a staple of microbiology. However, many of the gut microbes have been thought to be unculturable due to the "great plate count anomaly (Staley and Konopka, 1985)." As our understanding of microbiology has grown however, many microbes initially thought to be fastitidious or to be evadiding current culturing methods just need more focused effort in order to culture them. Most of the gut microbes are obligately anaerobic, where even the slightest bit of oxygen is toxic; by utilizing anaerobic culture more gut bacteria are able to be cultured. Moreover, there has been a

greater emphasis on optimizing nutritional requirements has been made in order to culture different microbes.

Novel culturing methods have allowed scientists to access some of the human microbiome projects “most wanted taxa”. These taxa are of high value in the lab as isolates for multiple reasons. They are important for developing reference genomes for future metagenomics efforts. Culture collections are also useful to identify phenotypes associated with individual strains and in order to identify nutrient of signalling interactions between microbes. Furthermore, culture collections would allow for tolerance testing for many xenobiotics, many of which are taken orally and interact with the gut microbiome in still unknown ways.

Two different ways to address the problem of cultivating difficult-to-grow gut microbes are droplet microfluidics and artificial gut systems. These two techniques represent alternate strategies towards cultivating gut microbes. One reason that it may be hard to grow some microbes on plates is due to competition: When a fast-growing microbe and a slow-growing microbe are present on the same plate, the fast-growing microbe out competes the slow-growing microbe for both space and nutritional resources; therefore only the fast-growing microbe survives to form a colony. Droplet microfluidics, which involves the precise manipulations of very small volumes of liquid (micro- to pico-liter

volumes), is a subset of microfluidics that involves mixing immiscible liquid (often an aqueous phase through an oil-phase) to create separate, compartmentalized droplets. Isolating individual bacteria within these droplets is a way to separate disparate bacteria with disparate growth rates, thus reducing competition and allowing both fast-growing and slow-growing microbes to individually thrive. Droplet microfluidics have increasingly been recognized as a high-throughput way to isolate and assay bacteria in a microbiology context (Kaminski et al., 2016).

A second reason that it may be challenging to cultivate some microbes is hypothesized to be the complex nutritional dependencies and interaction networks that are present within heterogeneous environments such as the human gut. Artificial gut models have long been used to replicate the human gut environment in an effort to sustain complex microbial communities (McDonald et al., 2013). For example, there are continuous flow systems that can sustain complex communities for long periods of time, in part because the environmental parameters can be tightly controlled, such as temperature, pH, and media type. These in vitro systems have been used to mimic complex natural systems that are otherwise intractable to frequent sampling and manipulation, and have enabled the effects of nutritional supplements on human infant microbiomes to be probed (Gamage et al., 2017). They also experiments in scenarios where

precise sampling is required; for example, continuous-flow artificial gut systems have enabled exploration of how quickly gut bacteria metabolically respond to diet (growth medium) change (Aguirre et al., 2016). Both droplet microfluidics and artificial gut models have thus proven valuable to discovering the individual contributions of microbes to the whole community.

1.3 Overview of results

Our first contribution to the gut microbiome field includes developing a droplet microfluidic assay (MicDrop) for measuring the growth of gut bacteria directly from a stool sample. This MicDrop system (addressed in Chapter 2) allows the growth rate of compartmentalized bacteria to be monitored without a preculture step; this therefore reduce culturing bias and facilitates the growth of slow-growing bacteria. This droplet assay can be used to measure the growth of hundreds of bacteria in response to different conditions, such as different media types or environmental parameters. Here, we validate MicDrop by testing the susceptibility of gut microbes to different antibiotics.

In chapter 3, we then apply the data obtained from this novel culturing approach to an in vitro model of the human gut. These artificial gut systems are increasingly important in looking at whole microbial communities without the influence of host

factors. These systems provide a host-free way of sampling and testing different variables that could be prohibitive to test in animals or humans. We show that the individual growth of microbes contribute to the final community composition in these artificial gut systems. Overall, we therefore provide a platform that has optimized the use and integration of these two in vitro systems, laying the groundwork for future work in complex microbial systems.

2. Microfluidic cultivation of bacteria

2.1 Introduction to microfluidic methods

Bacterial culture was among the first techniques used to study human gut microbiota (Kendall, 1909). Bacterial isolation efforts beginning in the early 1900s identified key enteric genera such as *Bacteroides, Bifidobacterium*, and *Bacillus* (Rajilic-Stojanovic and de Vos, 2014). Microbes isolated since then have served as crucial reagents for experiments. Gut bacterial isolates allow testing causal roles for specific microbes in animal models of metabolic and auto-immune disorders (Le Barz et al., 2018; Mathewson et al., 2016; Zegarra-Ruiz et al., 2018). Bacterial isolates can also be genetically modified and tested *in vitro* to identify enzymatic machinery in processes like the fermentation of dietary fiber (Larsbrink et al., 2014), and cocktails of cultured bacteria are being explored as therapeutics for *C. difficile* infections and cancer (Buffie et al., 2014; Routy et al., 2018; Whiteside et al., 2015).

Yet a key challenge for current microbiota culturing efforts has been keeping pace with increasing knowledge and interest in gut microbial diversity. Culture-independent methods based on high-throughput 16S rRNA sequencing have revealed the average individual harbors hundreds of distinct enteric bacterial strains (De Filippo et al., 2010;

Eckburg et al., 2005; Human Microbiome Project, 2012; Yatsunenko et al., 2012). Moreover, unrelated individuals likely share no more than ~30% of bacterial strains (Faith et al., 2013). Prevailing culture techniques do not scale to the diversity of microbes spanning human populations. Because most taxa are rare, exhaustive capture of bacterial species from even a single stool sample requires laborious spotting of thousands of bacterial colonies (Goodman et al., 2011; Lagier et al., 2015). To reduce the human effort needed for such experiments, state-of-the-art culture assays leverage plate and liquid handling robots; but, even these automated efforts tend to be limited to tens of strains (Desai et al., 2016; Maier et al., 2018b). This limitation stems in part from the physical constraints of typical plate-based culture methods, which grow bacteria in wells ranging from centimeters to millimeters in diameter. Even relying on 96- and 384-well plates, conventional large-scale culture efforts require loading and handling hundreds of plates under anaerobic conditions (Maier et al., 2018b).

An alternative approach is to culture bacteria in small volumes (nano- to pico-liters) by separating microbes into microscale wells. Devices composed of thousands of such wells have been used to culture both lab strains of bacteria and fungi (Martin et al., 2003), as well as isolate previously uncultured bacteria from the gut and soil (Ma et al., 2014; Nichols et al., 2010). Even higher-throughput experiments are possible by

compartmentalizing microbes in droplets of media that are tens to hundreds of microns in diameter and separated by immiscible oils and engineered surfactants (Guo et al., 2012; Kaminski et al., 2016). Because droplets are not limited by the need to microfabricate physical wells or channels, millions of distinct culture volumes can be created on the order of minutes. Droplet techniques have so far been used to isolate uncultured microbes from seawater and soil communities (Liu et al., 2009; Ma et al., 2014; Zengler et al., 2002), assess microbial cross-feeding (Park et al., 2011), and examine antibiotic sensitivity and commensal-pathogen interactions of human gut and oral microbiota (Terekhov et al., 2018; Terekhov et al., 2017). Still, existing droplet microfluidic approaches for assaying bacteria have required combining complex emulsion techniques (water-oil-water) with flow cytometers or custom on-chip droplet sorting devices. These protocol requirements limit the accessibility of droplet technologies for bacterial assays and in their present form require equipment that does not fit into typical chambers for creating anaerobic conditions, which are needed to culture human gut bacteria (Browne et al., 2016).

Microfluidics has been around for decades, emphasized for the need to miniaturize assays, leading to increased throughput and decreased cost of reagents. There are many uses for microfluidics, the most notable and relevant for the life sciences are next generation sequencing (NGS) techniques which allow small volumes of liquid to flow

through capillaries, allowing for expensive reagents to be used sparingly, making many reads affordable. Moreover, microfluidics has improved sample processing times, increased the control of fluids, and made rapid improvements in clinical and research areas (Sackmann et al., 2014).

Here, we developed a platform to isolate and culture bacteria from human gut microbiota in droplets (MicDrop) using accessible techniques and equipment. A key challenge our method addresses is how to measure the growth of isolates within distinct microfluidic droplets, accomplished without sorting, culture, or picking individual droplets: all of these methods reduce the throughput that using droplet microfluidics enables. To accomplish this, we rely on 16S rRNA as intrinsic DNA barcodes that are shared between droplets carrying the same bacterial taxa, which we refer to here as a sequence variant or SV (Callahan et al., 2016). By loading our droplets such that there is only one SV in our droplets, we can assume that each SV is growing independently in our growth medium, separated into distinct clonal populations within droplets. This approach in turn allows us to measure SV growth in droplets without the need for double-emulsion techniques or droplet sorting. Instead, we combine single-emulsion (water-in-oil) microfluidic droplet protocols with molecular techniques (qPCR and 16S rRNA

sequencing). These simplified protocols allow us to employ off-the-shelf microfluidic pumps and chips, which are compact enough to fit within typical anaerobic chambers.

To verify MicDrop we grew an artificial consortium of gut microbiota, representative of the most common phylum represented in the gut, and grew them in droplets with and without the presence of antibiotics. We did the same thing growing bacteria in conventional well plates. In this way we were able to show that growth in the presence of antibiotics was equivalent in MicDrop and conventional ways of culturing microbes. This verification allowed us to identify the dynamic range of growth that can be detected in droplets. These proof-of-concept experiments showcase the potential for microfluidic droplet techniques to characterize growth and function of individual bacterial strains from complex gut microbial communities in high-throughput.

2.1.1 Overview of microfluidic methods for microbiology

2.1.1.1 Microfluidics and metagenomics

Metagenomics is a sequencing approach that sequences all the genes present in a microbiome sample. In contrast with 16S sequencing, the DNA is extracted from a microbiome sample and is then sheared, and the fragments are then sequenced to recover microbial genes in the sample (Gill et al., 2006). This approach requires computational

methods to assemble and assign taxonomy and function to each of the sequenced genes, yet the approach can yield a more comprehensive understanding of microbial function in an environmental sample. Microfluidic techniques have been combined with metagenomics as high-throughput screen used to identify novel microbes. A microfluidics method has been used to enrich for genes of interest, in order to target microbes that have been only previously identified with metagenomics. In this way novel microbes from the Human Microbiome's project "Most Wanted Taxa" have been cultured (Ma et al., 2014). Microfluidics have also been used to dilute environmental samples of microbes to amplify whole genomes from single cells. In this way, microfluidics has enabled shotgun and single-cell metagenomics analyses to be used on microbial samples with low abundances or that are unable to be cultured, and therefore may contain novel taxa, as well as aiding in the computational problem of assigning genes to specific taxa, as taxa are first isolated with microfluidics (Yu et al., 2017).

2.1.1.2 Microfluidic cultivation in chambers

The miniaturization aspect of microfluidics is especially alluring where in vivo systems are cumbersome and in vitro systems that address the host component to these communities are important. Microfluidic gut-on-a-chip are a recent development, using

organoid cultures and microfabrication of polymer substrates in order to mimic the physical and physiological environment of gut (Bein et al., 2018). The gut-on-a-chip has been used to model the complex interactions between commensal microbes and host processes such as inflammation and identify singular contributions of microbes to inflammatory bowel disease (IBD) (Kim et al., 2016). More broadly, microfluidic chips have been engineered into microscopic habitats for microbes, allowing for advanced studies in motility, sensing, and growth of bacteria (Rusconi et al., 2014).

2.1.2 Droplet microfluidics

Droplet microfluidics involves the mixing of immiscible fluids (often aqueous solutions such as media containing bacteria) into an oil phase. These droplets, or emulsions, can be made many different ways, including by mixing, extrusion and laminar flow. The key component of droplet integrity are the oil and the surfactants.

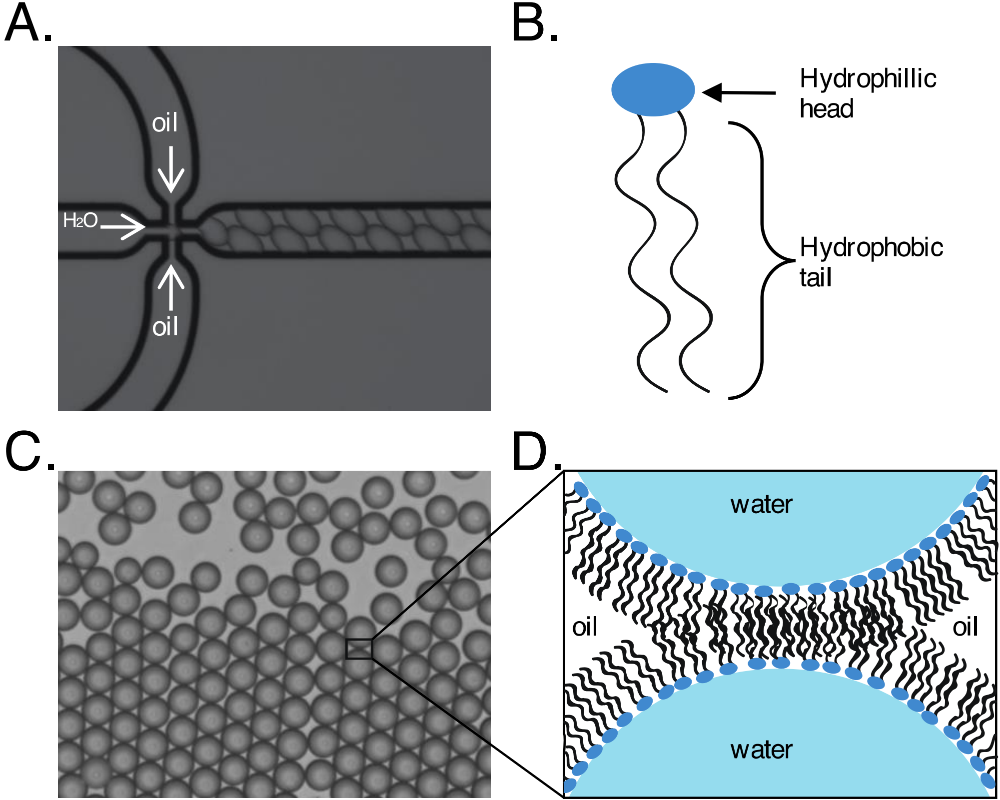

Figure 2. Droplet Schematic. A) An aqueous solution such as water or media flows through two streams of oil. B) The oil contains surfactants, which are amphiphilic molecules, meaning they have a have both a hydrophilic and hydrophobic parts. C) Liquid droplets cluster close together on a surface, yet maintain distinct boundaries. D) The surfactants interact at the interface between the water and the oil, creating a lipid bilayer between the droplets, which maintains their distinct shape, and prevents mixing between droplets.

Droplets are made by flowing aqueous solution through oil and surfactant solution. The liquids can be intermixed using a variety of ways, including physical mixing,

aire pressure, and physical pumps. Here we use syringe pumps that flow through oil and water through a chip that meet at a T-junction (Figure 2A.) By attenuating the flow rates, different sized droplets can be generated, ranging from tens to hundreds of microns in diameter. The size of the droplet is important for the surfactant chemistry and the integrity of the droplets, as well as the assay of interest. Digital droplet PCR for example generates nanoliter-sized droplets, while other millifluidic methods looking at larger microcroorganisms generate droplets less than a millimeter in diameter and capable of nanoliter scale volumes (Boitard et al., 2015).

Microfluidic droplets require surfactants instead of physical barriers to maintain integrity. Surfactants are amphiphilic compounds that help to lower the interfacial tension between two liquids. Soluble in oil, they contain both hydrophobic and hydrophilic groups, therefore they contain an oil- and water-soluble part. They therefore form at the interfaces of water and oil (Figure 2B,C).

To generate our droplets, we use a 6-junction microfluidic chip (Dolomite Microfluidics). The aqueous solution is made up of the medium chosen for the assay and the cell suspension. For the oil phase, we use a fluorinated oil and surfactant mixtures: 1% Picosurf (Sphere Fluidics) in Novec 7500 oil (3M). One day prior to performing the

droplet assay, all reagents including the oil surfactant mixture and culture medium were equilibrated to the anaerobic atmosphere in an anaerobic chamber.

2.2 Methods for assaying growth

2.2.1 MicDrop: a platform for culturing human gut microbiota in droplets

To isolate and culture individual gut bacteria from human gut microbiota, we merged concepts from prior microfluidic droplet protocols with high-throughput DNA sequencing (Figure 3).

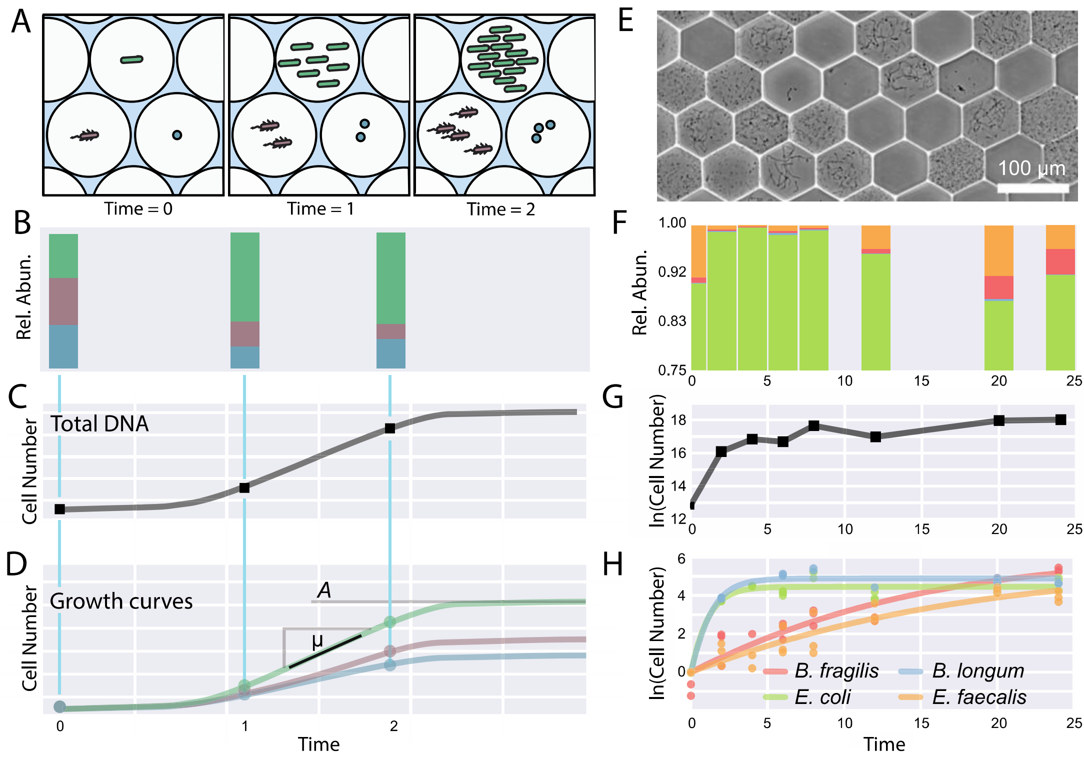

Figure 3. Concept and validation of droplet growth assay using mock communities. (A) Schematic of bacterial loading and growth in droplets over time. At each time point, droplets are destructively sampled and (B) the 16S rRNA gene is sequenced to establish the relative abundance of each sequence variant. (C) qPCR is used on the same samples to determine absolute abundance of the 16S rRNA gene. Relative abundance is combined with absolute abundance to form growth curves (D). An experimental example of MicDrop is depicted in (E-H), featuring an artificial community comprised of *B. fragilis*, *B. longum*, *E. coli*, and *E. faecalis* gut isolates. This community was loaded into the MicDrop platform (E), and then assayed by 16S rRNA composition (F) and qPCR (G) longitudinally using destructive replicate sampling. (H) Fitted growth curves were then inferred.

Our protocol first randomly encapsulates individual bacterial cells from gut microbiota into picoliter-sized droplets (Figure 3A). Gut microbiota samples are diluted

before encapsulation using the Poisson distribution, choosing a loading concentration that maximizes the number of droplets loaded with cells (~10-26%) while minimizing the number of droplets loaded with more than one microbe (~95-86% of loaded droplets contain single cells) (Figure 4) (Najah et al., 2012).

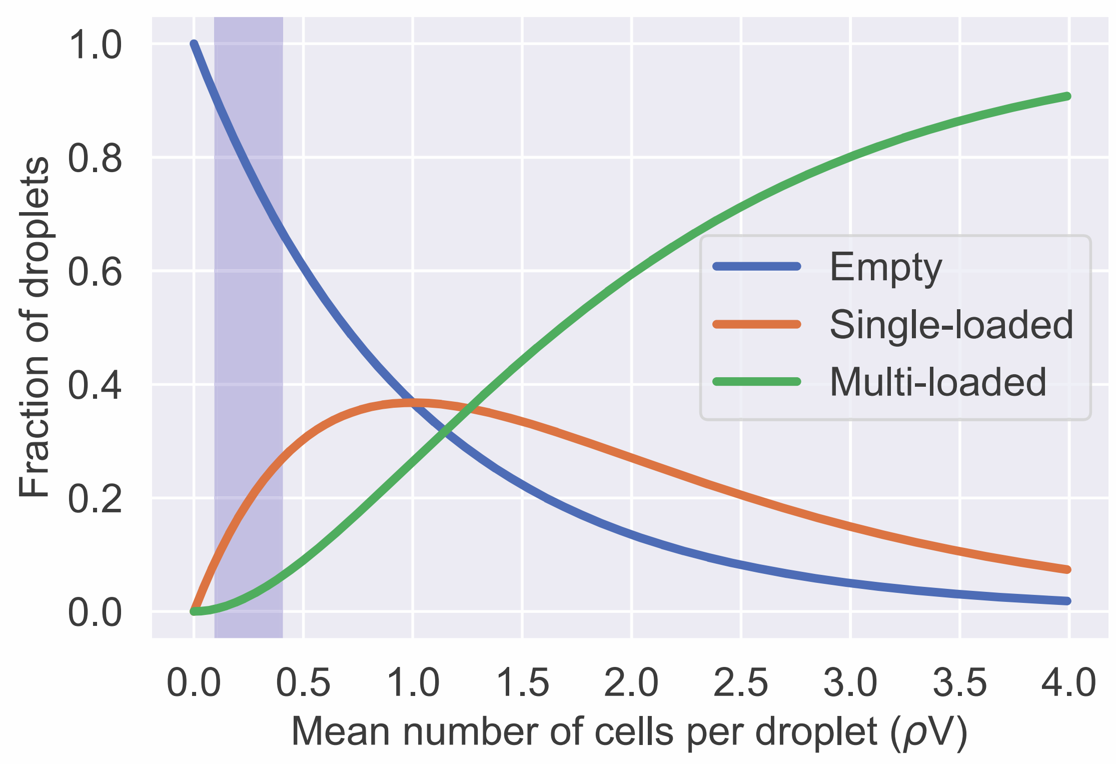

Figure 4. Poisson loading distributions. Tradeoffs exist between the number of droplets loaded with bacteria and how many of those loaded droplets are clonal. MicDrop protocols here balance these tradeoffs by loading droplets at means ranging from 0.1-0.3 (highlighted region).

Since many gut bacteria are obligate anaerobes, encapsulation takes place in an anaerobic chamber and droplets are subsequently incubated under anaerobic conditions (Figure 5). To track SV growth, we can avoid having to identify and sort bacteria by assuming that droplets are either empty or loaded with clonal isolates whose progeny share the same 16S ribosomal rRNA (rRNA) sequence, meaning genomic material accumulating across all droplets reflects the growth of SVs grown in isolation (Figure 1A-H). We therefore track isolate growth in droplets at a given time point using bulk bacterial DNA extraction without droplet sorting (Figure 6), followed by DNA sequencing and total quantification (qPCR) of 16S rRNA. The product of relative SV levels from 16S rRNA sequencing and total 16S rRNA levels yields an estimate of the absolute levels of each SV across all droplets at the time of sampling. Longitudinal data can be collected by generating replicate droplet pools and destructively sampling these pools over time.

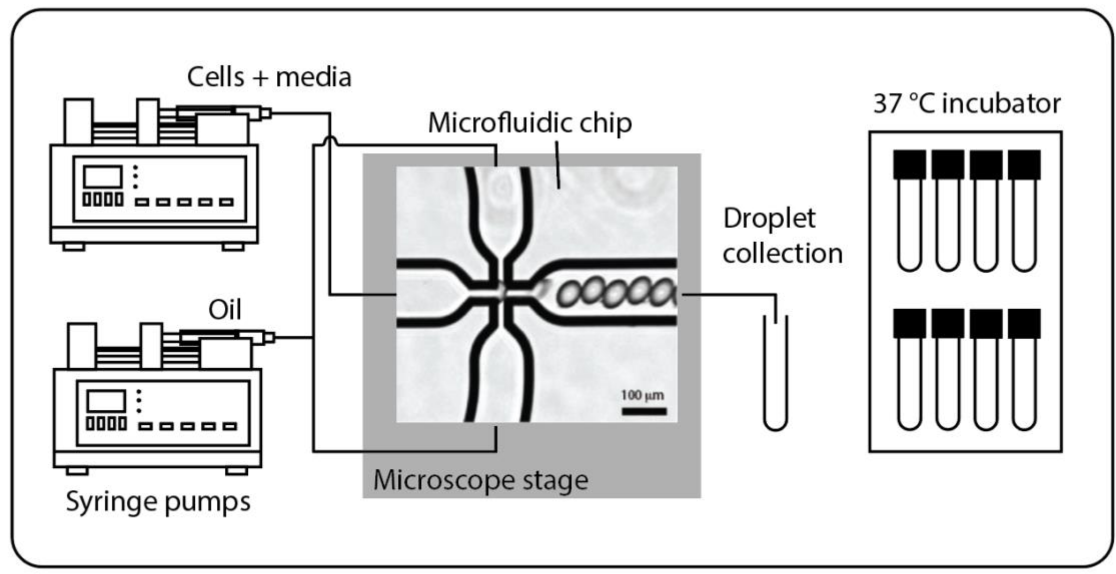

Figure 5. Schematic of droplet production in an anaerobic chamber. Cells are encapsulated in droplets, which are formed by flowing the aqueous bacterial suspension through an immiscible oil via a T-junction on a microfluidic chip (center). Flow is controlled by two syringe pumps (left). Droplet production may be monitored by a microscope equipped with an LCD display (center). After droplets are generated, they are incubated anaerobically (right) until destructive sampling.

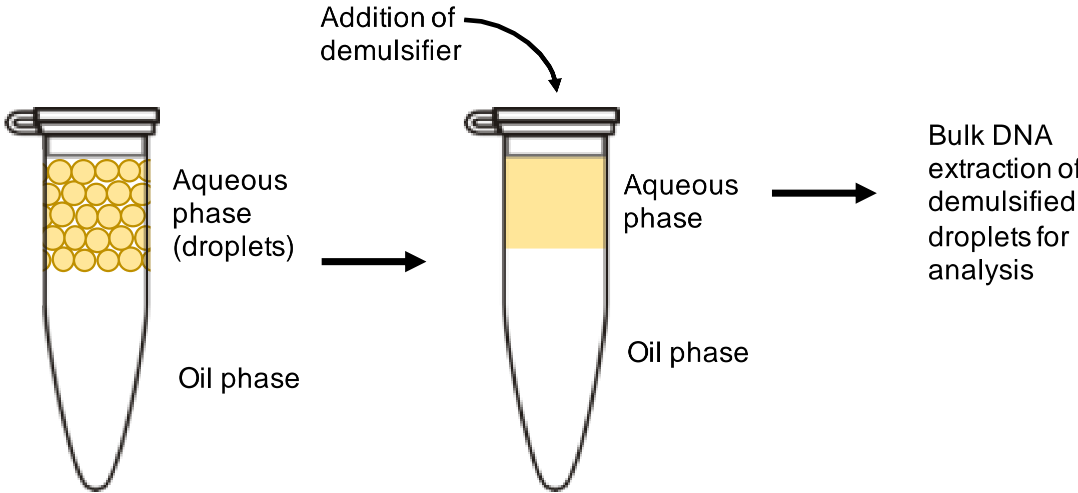

Figure 6. DNA extraction from droplets. Droplets are collected and incubated as separate compartments as described in text. When extracting DNA, a demulsifier is added that disrupts the surfactants separating the droplets. When these emulsions are broken, the aqueous phase containing the contents of the droplets is separate from the oil phase. DNA is extracted from the bacteria present in the aqueous phase.

2.2.2. Growth and incubation in droplets

To confirm that molecular methods could be used to measure bacterial growth over time in microfluidic droplets, we first investigated aerobic monocultures of fluorescent *Escherichia coli*. The *E. coli* replicated in droplets in a qualitative manner that resembled growth on conventional Petri dishes (Figure 7). Quantitative growth measurements using qPCR of the 16S rRNA gene sampled every two hours from liquid cultures were also similar to *E. coli* grown on plates (ϱ=0.95, p=8.7e-9; Spearman correlation; Figure 8).

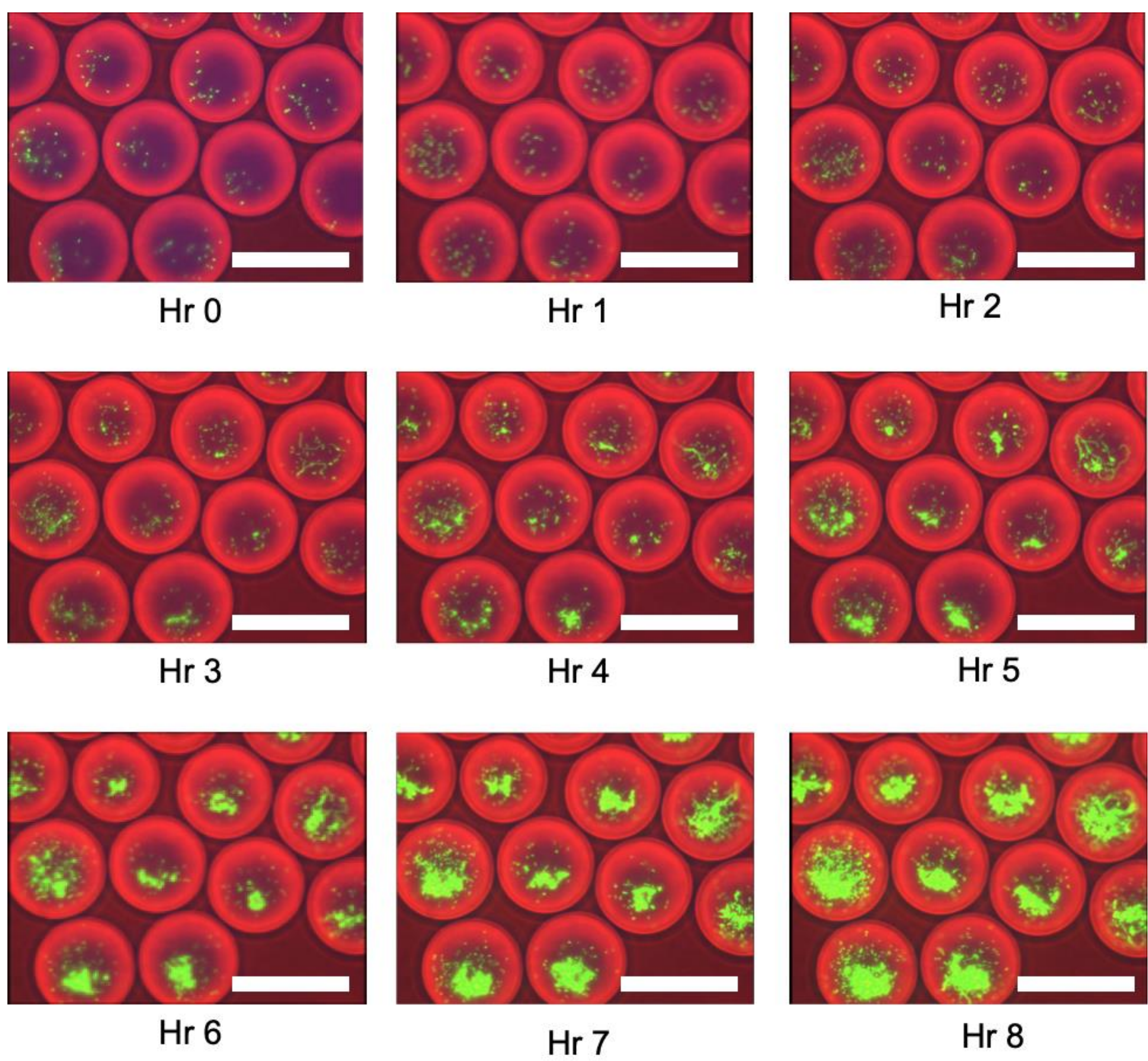

Figure 7. Fluorescently labeled *E. coli* growing in droplets growing from 0-8 hours. In these MicDrop experiments we facilitated imaging by loading *E. coli* at high concentrations (*i.e.* most droplets were therefore loaded with more than one *E. coli* cell). Scale bars are 100 μm.

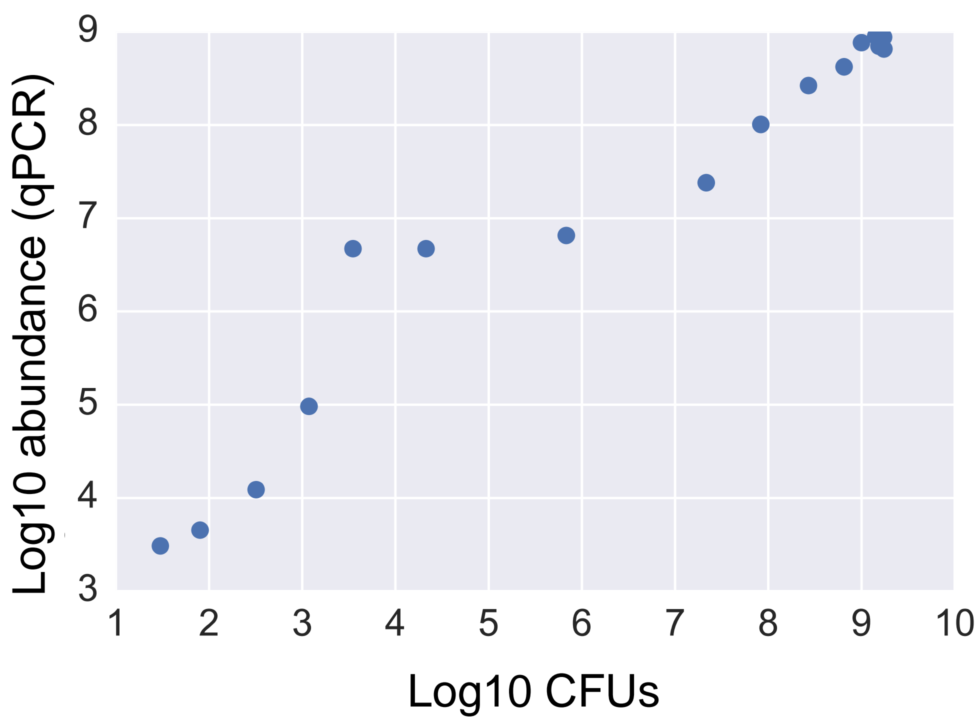

Figure 8. Comparison between *E. coli* grown in plates (measured in CFUs, x-axis) and in microfluidic droplets (measured by qPCR, y-axis; Spearman ρ=0.95, p=8.7e-9). Cultures of E. coli were grown overnight, then diluted to varying concentrations. These cultures were then simultaneously plated for CFU counting, and DNA was extracted from them for determining cell number via qPCR. These numbers were then compared, and determined that qPCR is comparable method to plate counting as a way to enumerate growing cells.

We next tested MicDrop using artificial microbial consortia. Microscopy showed droplets could be used to segregate clonal isolate populations with distinct morphology

and motility out of mixed microbial communities (Figure 9). An artificial community was assembled using five facultative gut anaerobes: *Streptococcus agalactiae*, *Stapholycoccus haemolyticus*, *Enterococcus faecalis*, *Enterbacter clocae*, and *Eschericia coli*. Individual isolates were: 1) grown aerobically overnight (~18 hours); 2) culture densities were determined by OD600; 3) cultures were diluted and mixed at equivalent concentrations; and, 4) mixtures were loaded into droplets. For ease of microscopic imaging, we loaded at a Poisson dilution such that > 50% of droplets contained clonal populations. To image, 5μl of droplets were placed on glass slide, and when exposed to air the oil phase began to dissolve, and droplets became flattened hexagons on the slide.

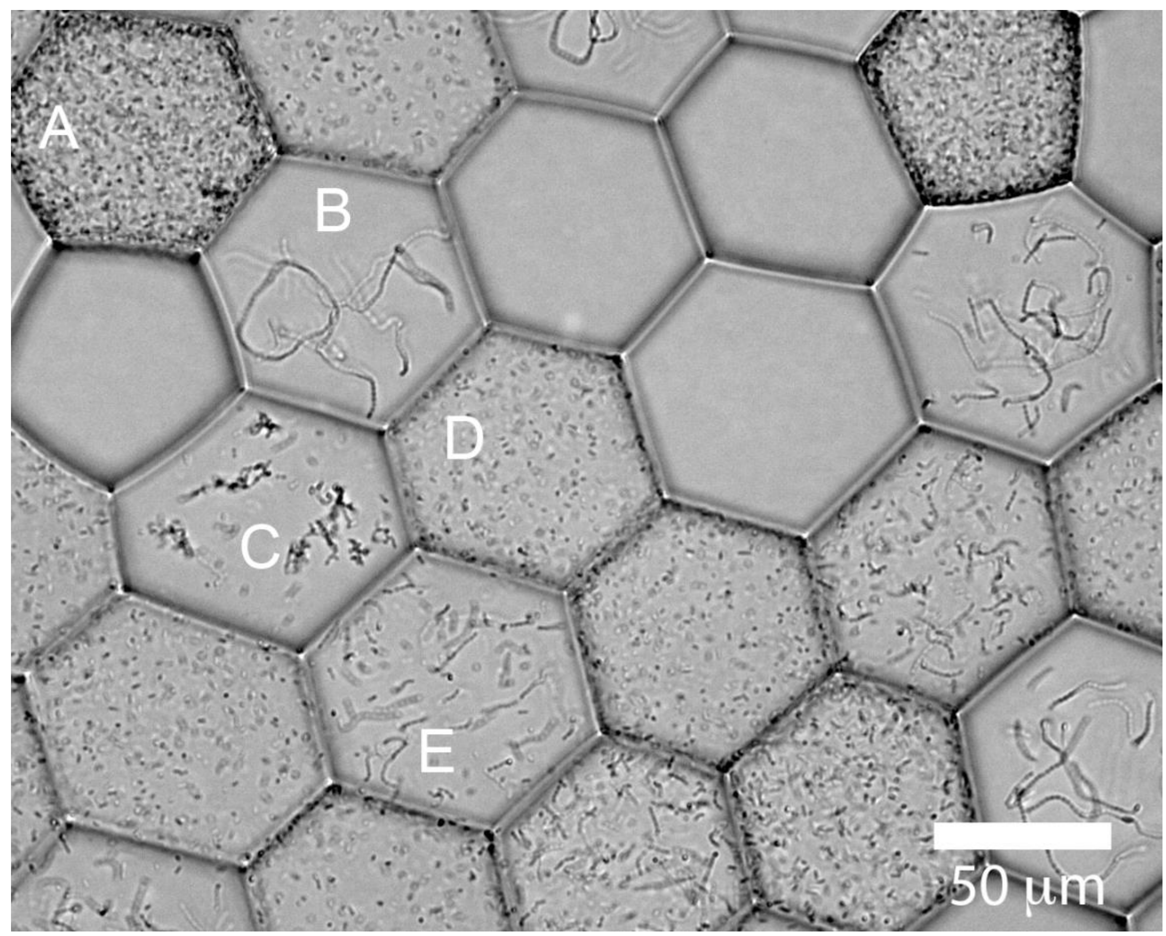

Figure 9. Distinct populations of bacteria observed in droplets after 6 hours incubation at 37℃. A-E mark clonal populations of bacteria that are distinguished by colony morphology.

We next tested whether the DNA-based technique of MicDrop could be used to track bacterial levels in droplets. To do this we chose a consortium of five bacteria isolated from the human gut (*Bacteroides spp. 1, Bacteroides spp*. 2, *Bacteroides thetaiotamicron, Bacteroides uniformis, Enterobacter clocae*) that grew well in conventional well-plate

methods. We grew these bacteria in mGAM and six different antibiotics concentrations (amoxicillin (100 μg/mL), amoxicillin + clavulanate (100 μg/mL), ampicillin (100 μg/mL), gentamicin (10 μg/mL), kanamycin (50 μg/mL), and ciprofloxacin (5 μg/mL)). Growth was measured via qPCR and sequencing (droplets) and OD600 (96-well plates) after 24 hours. To determine equivalent growth standards from the DNA-based measurements of growth in MicDrop, we analyzed different growth threshold cut-offs and generated an receiver operator characteristics (ROC) curve (Figure 10C). For this, we held constant a growth value in the wells (equivalent to OD600 of 1), and then for values ranging from the minimum DNA amount to the maximum DNA amount (in natural log space), and calculated the true positive rate (TPR) and false positive rate (FPR) at each growth threshold. For each growth threshold, plotting the TPR against the FPR, creates the ROC curve, representing the relationship between sensitivity and specificity at a given growth threshold. We picked the point that maximizes TPR (sensitivity) and minimizes the FPR (1-specificity) (denoted by star in Figure 10C). This point corresponds to a growth cut-off of doubling at least 2.14 times (Δ ln(SV DNA abundance) $\geq$ 1.48). This cut off was then used to draw the heatmap in Figure 10A, while Figure 10B shows the values used as a reference. With these values we found that the resulting bacterial DNA levels in droplets corresponded to isolates' optical densities in reference well-plates (accuracy=75%).

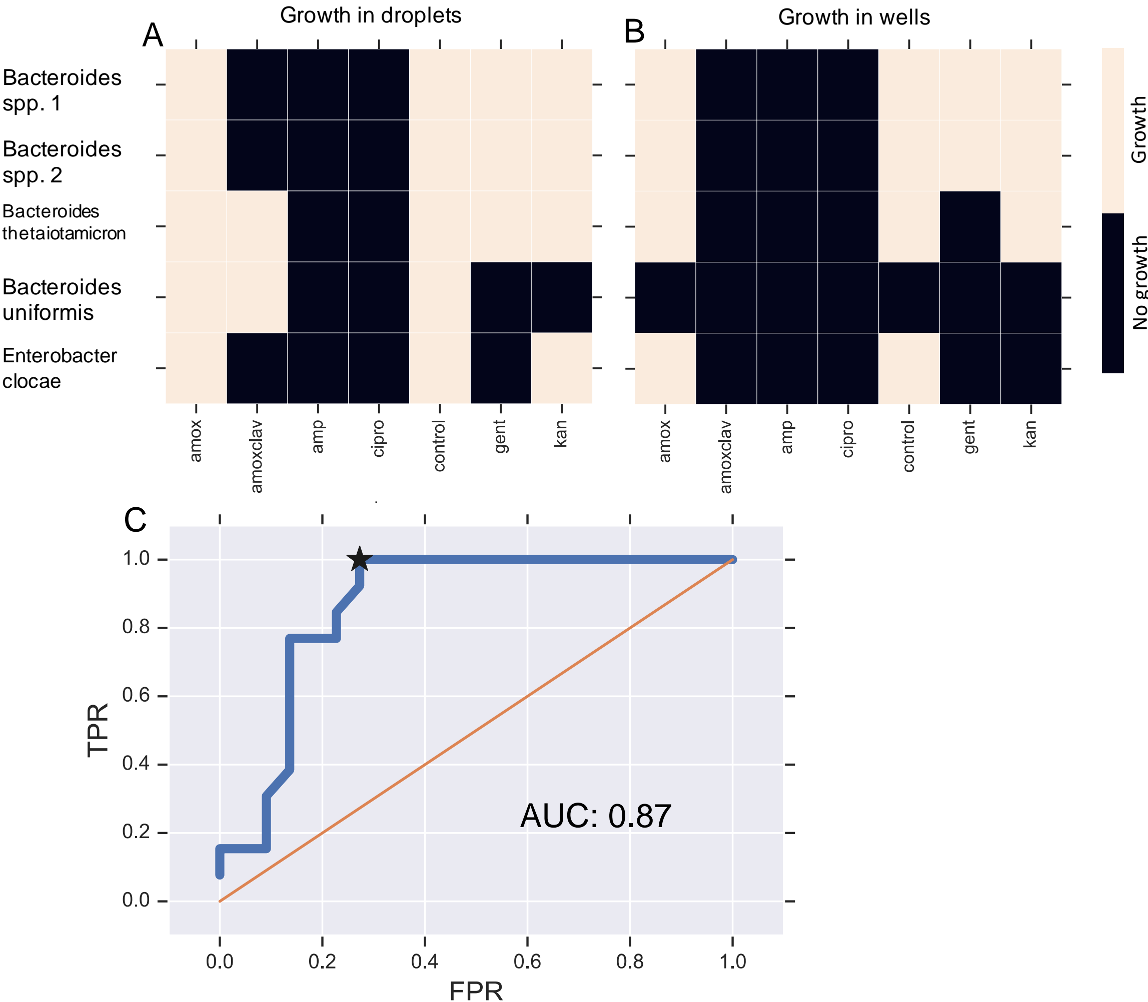

Figure 10. Results of growth with antibiotics in droplets (A) and 96-well plates (B), using five gut isolates (*Bacteroides spp. 1, Bacteroides spp. 2, Bacteroides thetaiotamicron*, *Bacteroides uniformis*, *Enterobacter clocae*) grown in mGAM and six different antibiotic combinations (amoxicillin (100 µg/mL) [amox], amoxicillin + clavulanate (100 µg/mL) [amoxclav], ampicillin (100 µg/mL) [amp], gentamicin (10 µg/mL) [gent], kanamycin (50 µg/mL) [kan], and ciprofloxacin (5 µg/mL) [cipro]). Growth was measured via qPCR and sequencing (droplets) and OD600 (96-well plates) after 24 hours. (C) ROC curve of MicDrop assay results at different growth threshold

cut-offs using (B) as a reference. A growth cut-off of doubling at least 2.14 times (Δ ln(SV DNA abundance) $\geq$ 1.48) maximized the true positive rate while minimizing the false positive rate (Youden's J; denoted by star on curve) and was used to draw the heatmap in (A).

2.2.3 Isolation of gut bacteria in droplets

Finally, we investigated the utility of MicDrop when applied to the human gut microbiota. Human fecal microbiota isolated and cultured in droplets exhibited 2.6 times more diversity than when grown in mixed conditions (Figure 11A), which is consistent with the hypothesis that droplet isolation enables slow-growing microbes to be sheltered from competition with fast-growing bacteria (Ma et al., 2014). Shannon diversity index is commonly used to measure diversity in an ecological community, taking into account both the number of species present (abundance) as well as the proportions in which they appear (evenness). Shannon diversity is known to strongly correlate with sequencing read depth (Goodrich et al., 2014), so we show that these diversity indices are not correlated

with number of sequencing reads for each sample (Figure 11B).

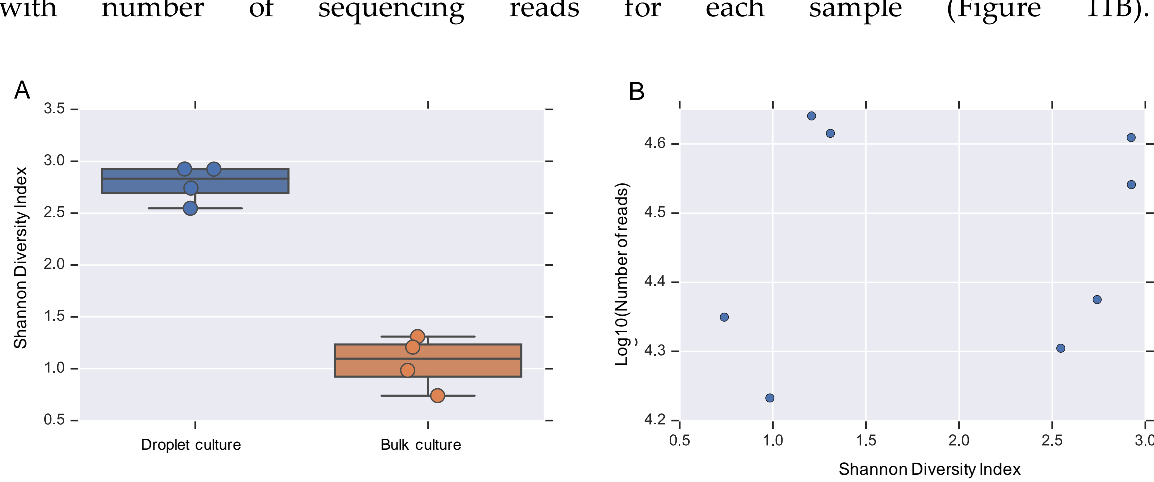

Figure 11. Diversity of microbial communities when isolated in droplets. A) Diversity (Shannon index) of microbial communities isolated and cultured in droplets culture compared with communities cultured without separation in standard bulk culture. B) Diversity (Shannon index) of these communities is not correlated with sequencing depth (y-axis, log10(number of reads)) ($p > 0.5$, Spearman correlation).

Droplets can be further useful for visualizing individual colony morphologies. Before sequencing became the primary method of identification of novel bacteria, novel bacteria were first isolated, then subjected to as many as twenty biochemical tests used to categorize and identify the microbe. However, in some cases, bacterial morphology is still an optimal way of differentiating different microbes, though this is less common of a practice now (Moore and Holdeman, 1974b). Even when this was common practice, the main concern was still throughput: the technical time needed to screen hundreds of colonies was overwhelming. While not a practical use of droplet microfluidics, because

in this case isolation after imaging is not possible, we were able to capture diverse bacteria from a fecal sample and image individual colony morphologies in droplets (Figure 12). Furthermore, we were able to identify colony morphologies from MicDrop and compare them with microscopy and illustrated images from some of the first attempts to document fecal microbiota (Holdeman, 1974; Moore and Holdeman, 1974a) (Table 1).

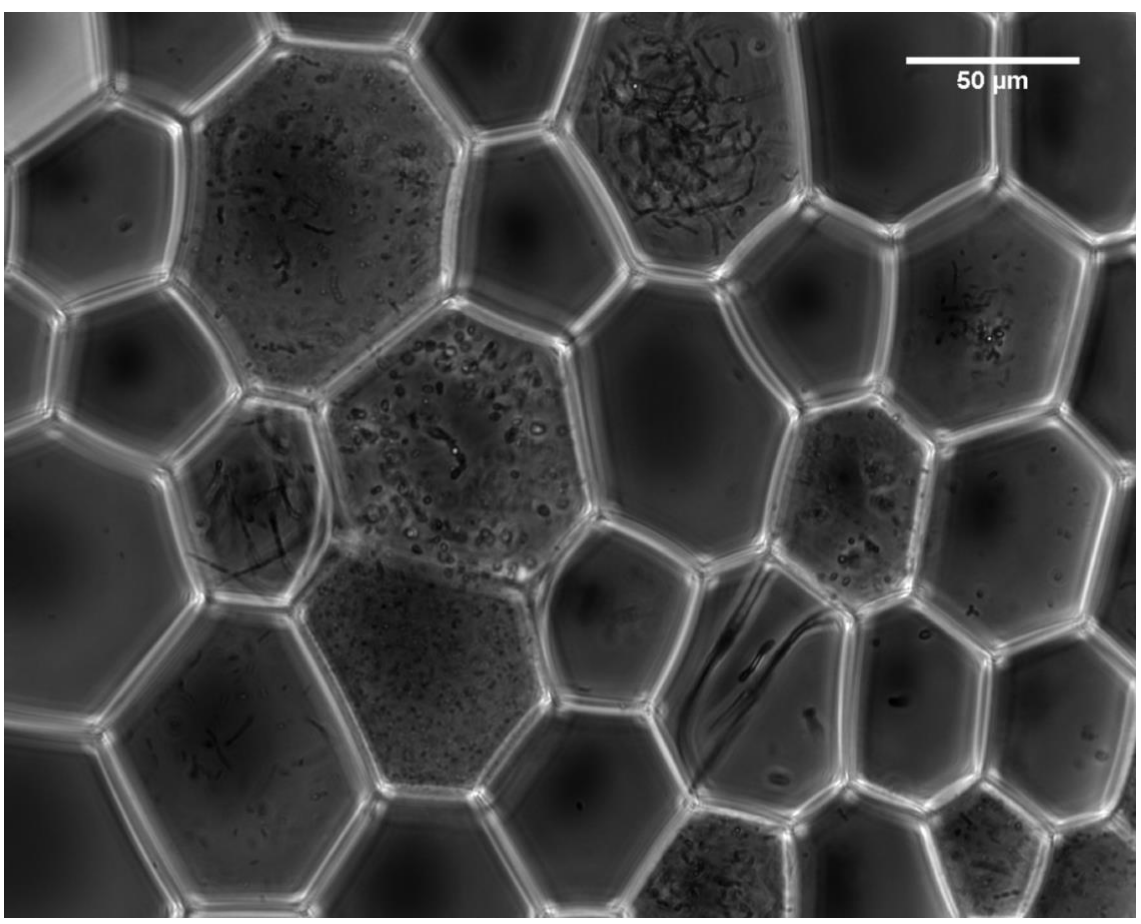

Figure 12. Gut bacteria isolated in droplets and incubated for 24 hours. We see many different colony morphologies.

Table 1. Visual identification of fecal bacteria. We compared colony morphology identified in literature (via microscopy or illustration) to colony morphology identified using MicDrop.

Species	Colony morphology identified in literature	Colony morphology from MicDrop	Reference
Coprococcus catus			(Holdeman, 1974)
Ruminococcus torques			(Holdeman, 1974)
Ruminococcus-AU			(Moore and Holdeman, 1974a)
Bacteroides-J			(Moore and Holdeman, 1974a)
Bacteroides-L			(Moore and Holdeman, 1974a)

2.2.4. Generation of growth curves

Droplets were made on a microfluidic chip (6-junction droplet chip, Dolomite Microfluidics). Bacterial media varied by assay; for the oil phase, we used a fluorinated oil and surfactant mixture 1% Picosurf (Sphere Fluidics) in Novec 7500 (3M). One day prior to performing the droplet assay, all reagents including carrier oil, culture media, and carbon solutions were equilibrated to the anaerobic atmosphere in an anaerobic chamber (Coy). The fecal inoculum optical density at 600 nm was recorded and diluted according to the Poisson distribution: $P(n, \bar{n}) = \frac{\bar{n}^n e^{-\bar{n}}}{n!}$, where n is the droplet occupancy (i.e. 0,1,.. cells/droplet) and $\bar{n}$ is the average number of cells per droplet given by: $\bar{n} = \rho V$, where V is droplet volume and ρ is cell density. Assays were performed at a $\bar{n}$ of 0.1-0.3 to minimize the number of droplets loaded with more than one cell (Figure 4). Thus, for a fixed droplet volume and $\bar{n}$, the target cell concentration can be obtained from: $\rho = K\frac{\bar{n}}{V}$, where K is a constant that converts CFUs/mL to OD_{600} determined from replicate CFU assays. Syringe pumps were used to control the flow rates of the oil and cell suspension (NE-1000 Single Syringe Pump, New Era Pump Systems). Following the culture period, droplets were loaded into chambered slides (C10283, Invitrogen) or directly onto glass slides and observed with Phase and/or Darkfield microscopy (Nikon) to examine growth and the

appropriate loading. All steps of cell encapsulation and culture were performed in an anaerobic chamber.

To generate growth curves in droplets, at hourly time point we then destructively sampled droplets by taking them out of the incubator in the anaerobic chamber and putting them at -20 °C (Figure 3A). This stalls all growth at this time point. After all the time points have been collected, we first remove excess oil from droplet generation, then we break the emulsions using PFO, then the aqueous layer is pipetted off, and then we extracted DNA from the bacteria found in the aqueous layer. From the DNA we sequence the V4 16S rRNA gene) to get relative abundance of each SV and perform qPCR (Figure 3C). For the sequencing data, we drop SVs with reads in less than five of the time points. For the rest of the sequencing data, we add a pseudocount of one to account for zeroes in sequencing count data. We then determine relative abundance of each SV by dividing each sample by the total number of reads. These values, the relative abundance of the SV and the absolute number of 16S rRNA genes are multiplied together to determine the absolute number of each SV. With a time series of absolute values of each SV, we are able to fit growth curves in order to determine growth rate.

2.2.4.1 Droplet DNA extraction, PCR amplification, and DNA sequencing

To extract DNA from droplets, excess oil was removed by pipetting and water-in-oil emulsions were broken by adding an equal amount of 1H,1H,2H,2H-Perfluoro-1-octanol (PFO, VWR) and briefly vortexed. Then, the samples were briefly centrifuged (<200 g) to separate the aqueous and oil phases by density. The aqueous solution was transferred to a new tube, and DNA was extracted using a kit (Qiagen #12224). DNA was extracted from artificial gut and stool samples using a 96-well PowerSoil kit (Qiagen #12888). For all samples, the V4 region of the 16S rRNA gene was barcoded and amplified from extracted DNA using with custom barcoded primers, using published protocols (Caporaso et al., 2011; Maurice et al., 2013). 16S rRNA amplicon sequencing was performed on an Illumina MiniSeq with paired-end 150 bp reads. We chose to only analyze samples with more than 5,000 reads to remove outlying samples that may have been subject to library preparation or sequencing artifacts. The 16S rRNA nucleotide sequences generated in this study will be made available at the European Nucleotide Archive under study accession number TBD. Total bacterial abundances from droplet cultures were estimated by qPCR for bacterial 16S rRNA using the same primers used in the DNA sequencing protocol. Amplification during the qPCR process was measured

with a Real-Time PCR system (CFX96 Real-Time System, BioRad) using *E. coli* DNA at a known cell concentration as a reference.

2.2.4.2 Identifying Sequence Variants and Taxonomy assignment

DADA2 was used to identify SVs (Callahan et al., 2016). Custom scripts were used to prepare data for denoising with DADA2 as previously described(Silverman et al., 2018). Reads were then demultiplexed using scripts in Qiime v1.9 (Caporaso et al., 2010). SVs were inferred by DADA2 using error profiles learned from a random subset of 40 samples from each sequencing run. Bimeras were removed using the function removeBimeraDenovo with tableMethod set to "consensus". Taxonomy was assigned to sequence variants using a Naïve Bayes classifier (Wang et al., 2007) trained using version 123 of the SILVA database (Quast et al., 2013). For growth dynamics of the human gut microbiota and microbiota dynamics in the artificial gut, only forward sequencing reads were analyzed. Downstream analysis on sequence variant tables was performed using R (ver. 3.4.2) and Python (ver. 2.7.6). PERMANOVA was run in R using adonis in the vegan package (ver. 2.5-2).

2.2.5. Fitting curves to sequence data

To estimate SV growth curves using MicDrop, we collected a total of 70 separate microfluidic droplet aliquots for destructive longitudinal sampling. Droplets were generated according to the MicDrop protocol described above. We used a modified Gifu Anaerobic Medium (mGAM) in our droplets (Gifu Anaerobic Medium, HiMedia, with the addition of 5 mg/L Vitamin K and 10 mg/L Hemin). Each aliquot of 200 μl of droplets was incubated at 37 °C in an anaerobic chamber. Aliquots were destructively sampled in triplicate, hourly, for hours 0-24 after droplet making and in duplicate once a day for hours 24-127 after droplet making.

Growth curves were fit using a combination of 16S rRNA qPCR and DNA sequencing data. To minimize the potential for poorly fit growth curves, SVs were required to have been detected by DNA sequencing in >5 samples to be included in curve fitting. To avoid numerical instabilities associated with taking the log or dividing by zero, a pseudocount of one was added to the sequence variant count table prior to normalization to relative abundances. Relative abundances of each SV were then determined by dividing the number of counts associated with each SV in each sample by the total read counts in the sample. Concentrations of each taxa were then estimated by multiplying the relative abundances of SVs by the 16S rRNA concentrations determined

by qPCR. Technical replicates constituted distinct data points in these calculations. We used the SciPy Python package (v0.19.1) to fit a modified Gompertz equation (Zwietering et al., 1990) to which we added an additional term to account for differences in starting abundance to the resulting dataset: $y = Aexp\left\{-exp\left[\frac{\mu \cdot e}{A}(\lambda - t) + 1\right]\right\} + A_0$, where μ is growth rate, A is carrying capacity, λ is lag time, or the time it takes for a bacteria to reach logarithmic growth, and A_0 accounts for the relative abundance of different SVs in the inoculum. We fit curves using the module scipy.optimize.least_squares with the robust loss function "soft_l1". Parameter bounds were also used to minimize the optimization search space. We set lower bounds of A=0, λ=-50, μ=0, A_0=0; and, upper bounds of A=15, λ=12, μ=2.6, A_0=15. We selected bounds by considering both biological feasibility and parameter sensitivity analyses (Figure S12). Our upper bound for growth rate (μ=2.6) represented a doubling time of 15 minutes, which we based on the fastest growth rates observed in an anaerobic bacterium (Sottile and Zabransky, 1977). The upper bounds on A and A_0 were set to the maximum amount of DNA measured across replicate MicDrop samples from the human fecal inoculum. The upper bound on λ, which represents the lag time until exponential growth (Monod, 1949) was set at 12. Lower bounds of 0 for A, A_0, and μ reflect our choice not to model negative growth. A lower bound for λ was selected

by sensitivity analysis (Figure 13), which revealed that a bound of zero led to fitted λ values regularly collapsing to our boundary limits. We also found that fitted curves were sensitive to starting parameters. To ensure a broad search of parameter space, we initialized each curve fit multiple times (n=100) with starting parameters randomly distributed between the bounds of each parameter. Fitted growth rates often collapsed to the maximum μ tolerated; we therefore only retained fits where growth rates were at least slightly below our upper bound for μ ($\mu < 2.5$) (Figure 14). Of the remaining fitted curves, we analyzed the one with the lowest loss function. In our analyses of SV growth in human fecal samples, we defined total SV levels as y(127 hours) - y(0 hours).

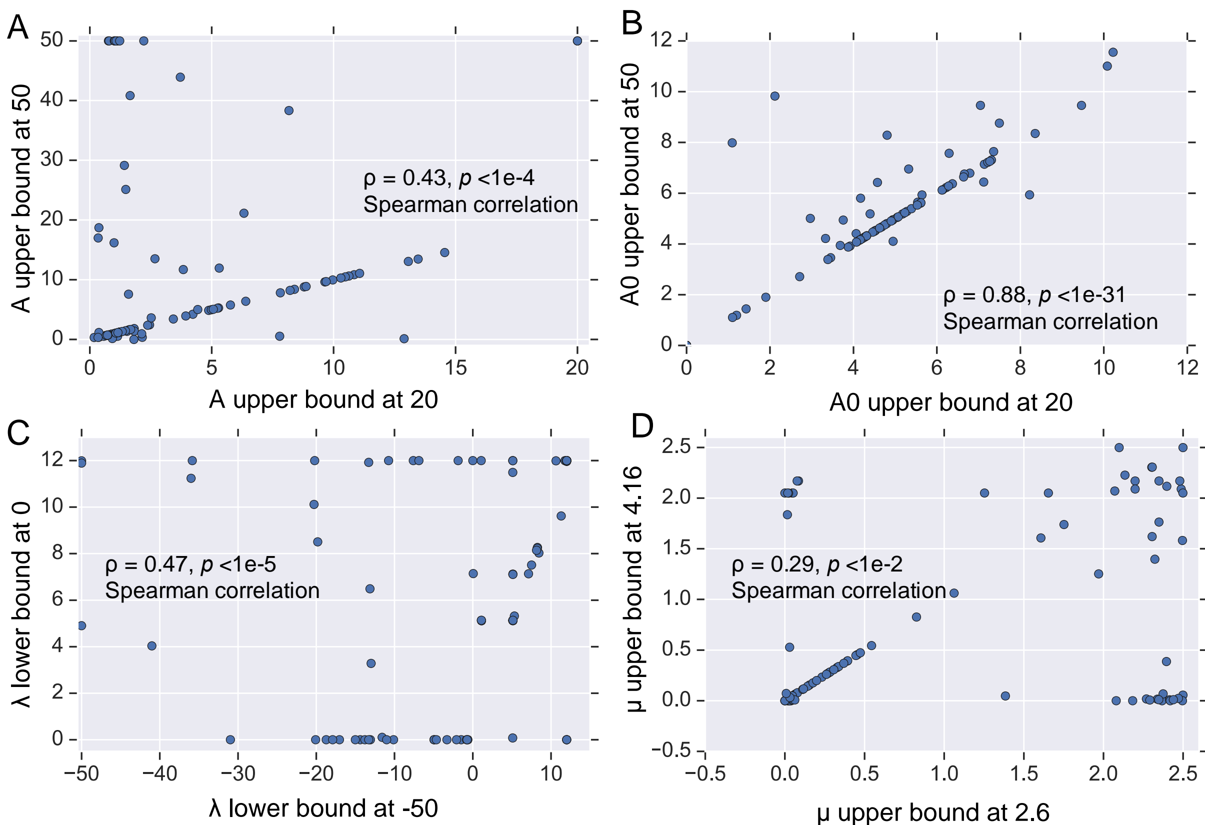

Figure 13. Sensitivity of curve fitting to parameter bounds. X-axis depicts each fit parameter as defined by bounds described in *Methods*. Y-axis reflects fit parameters with alternative parameter bounds. Each point represents a fit parameters for distinct SV.

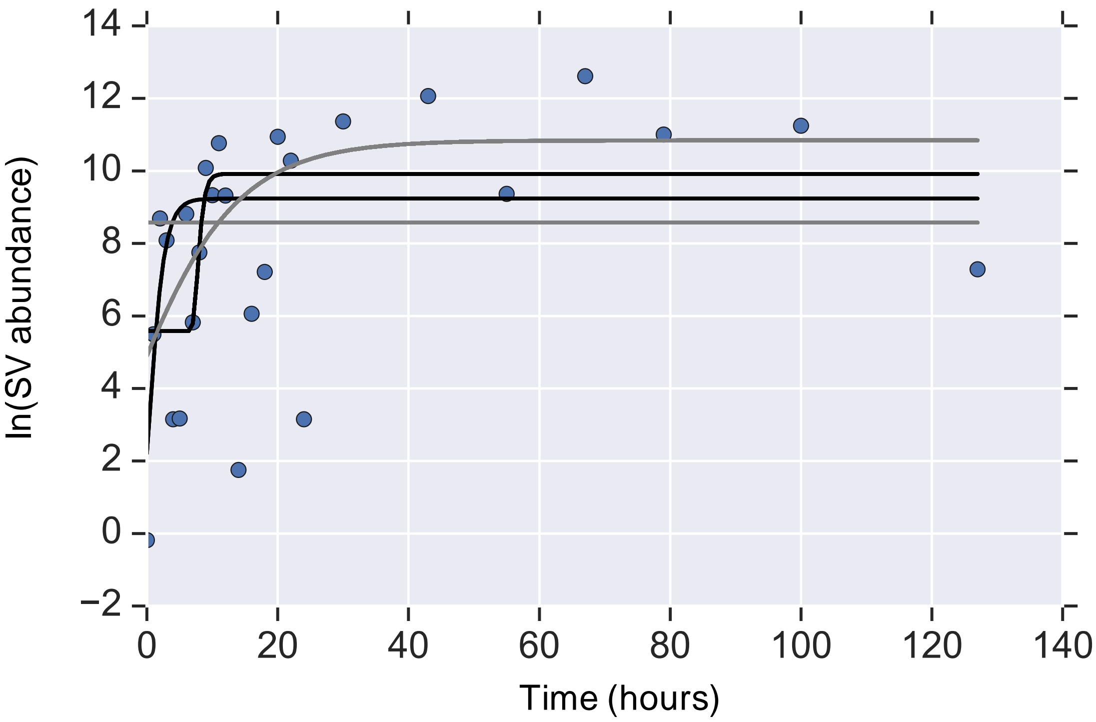

Figure 14. Example of optimal curve fitting. Gompertz curves were fit to longitudinal SV data using starting parameters that were randomly chosen from bounded uniform distributions. Fitted curves typically converged on a small number of distinct local minima (solid lines). We discarded fitted curves whose growth rates (μ) collapsed to our upper bound on μ (black lines).

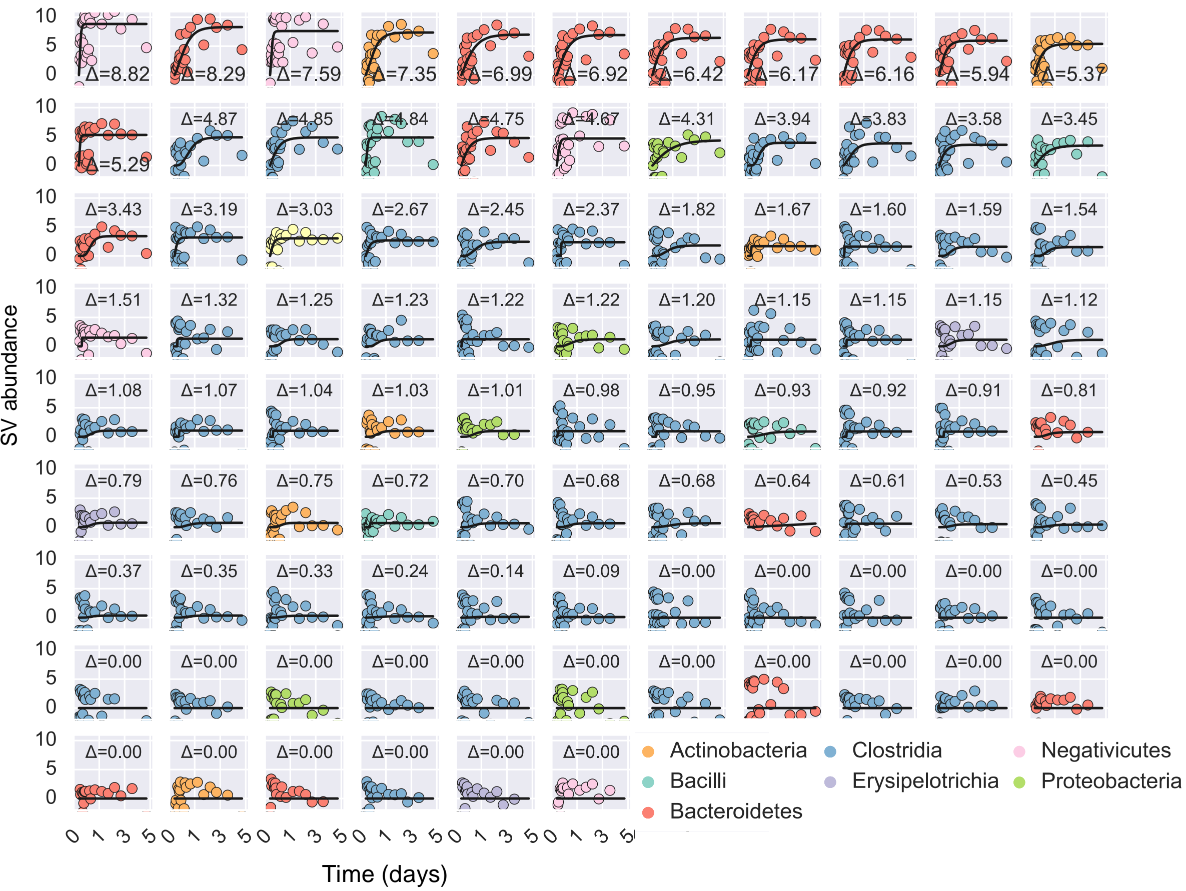

Figure 15. All detected SVs in MicDrop experiment using a human stool sample. Modified Gompertz growth curves are fit to time-series. SVs are colored by taxonomy and sorted according to total growth (curve asymptote height; indicated by Δ), which is denoted on each sub-plot. To ease viewing, curves are shifted vertically so y-intercepts are at the origin.

2.3 Discussion

We report here a microfluidic platform for isolating, culturing, and assaying component members of human gut microbiota (MicDrop) using accessible microfluidic and molecular techniques. With MicDrop we were able to measure the growth kinetics of dozens of microbial SVs from the human gut microbiome, an unprecedented number without comprehensive isolation and culture collections. The flexibility of the platform suggests its underlying concepts could be applied to assaying microbial responses to other compounds including pharmaceuticals, antibiotics, or host-secreted compounds (Koppel et al., 2017; Maier et al., 2018b) using individual members of communities comprised of microbes from culture collections, mutant libraries, other human body sites, or environmental systems. The ability of MicDrop to screen clonal populations could be particularly useful for assays characterizing the behavior of isolates free from the effects of inter-species interactions like competition or facilitation (Desai et al., 2016; Maier et al., 2018b).

Yet, we acknowledge MicDrop still has some limitations. We rely on 16S rRNA as a molecular barcode for droplets sharing the same bacterial SV, meaning that the platform is sensitive to similar challenges due to inter-species rRNA copy number variation

confronting 16S rRNA microbiota surveys (Kembel et al., 2012); and MicDrop cannot detect differences in growth originating from distinct clones of the same SV. For precise growth assays targeting bacteria from a limited number of taxa, traditional culture methods could be better suited. An additional limitation of MicDrop in its current form is the time and manual effort needed to setup individual droplet generation experiments and ensure accurate Poisson dilution of bacterial cells. Experimental effort could be reduced and reproducibility enhanced by automating sample switching. Last, we focused here on culture in liquid media using soluble substrates; future extensions of MicDrop that provide solid physical surfaces to colonize (Datta et al., 2016; Macfarlane et al., 2005) or insoluble substrates like mucin will require developing techniques to avoid the clogging of microfluidic channels.

3. Growth rate contribution to community composition in an artificial gut system.

Artificial gut models provide unique opportunities to study human-associated microbiota. Still, microbiota cultured in these models over long periods of time often do not precisely recapitulate inoculating microbial communities. This discrepancy may be due to multiple *in vitro* limitations, one of which is the degree to which gut microbes are suited to growing in artificial gut media. Testing though how previously uncultured commensal microbes grow in a medium is laborious and challenging to perform at scale.

To address these obstacles, we developed a technique to isolate bacteria from mixed communities into microfluidic droplets and simultaneously measure their growth. We then applied our microfluidic technique to the same community of human stool bacteria and media formulation that was used to inoculate an artificial gut. Our microfluidic technique enabled us to measure growth rates for 94 bacterial sequence variants in parallel. We found that growth rates of individual isolates significantly correlated with these isolates' persistence in an artificial gut after 14 days of continuous culture.

Our findings suggest that media formulations for artificial gut systems can be optimized by testing how individual gut bacterial isolates grow on a given medium. More

broadly, our results show that gut microbiota community structure reflects, at least in part, the fitness of individual bacterial taxa in a given environment. Lastly, our microfluidic technique may be of general utility for high-throughput screening of gut microbial growth and phenotypes.

3.1 Introduction

Artificial gut models replicate components of the human gastrointestinal tract and are used to culture enteric microbial communities (Gibson and Fuller, 2000; Kettle et al., 2015; Macfarlane and Macfarlane, 2007; Macfarlane et al., 1998; McDonald et al., 2013; Rajilic-Stojanovic et al., 2010; Van den Abbeele et al., 2010b; von Martels et al., 2017). Gut models have multiple advantages, including: environments that can be controlled (*e.g.* temperature, pH), arbitrary sampling frequencies, and reduced ethical concerns. Furthermore, continuous flow *in vitro* models of the large intestine seeded with fecal bacteria support stable and reproducible gut microbial communities (Auchtung et al., 2015; McDonald et al., 2013; Van den Abbeele et al., 2010b). Given these advantages, artificial gut models have been used to carry out novel studies involving complex perturbations or and diverse settings where *in vivo* studies are impractical, such as the measuring the effects of nutritional supplements to the infant gut (Gamage et al., 2017),

the systematic testing of the effects of antibiotics on a gut microbial community (Newton et al., 2013), and measuring the protective effects of probiotics on chemotherapy and antibiotic induced dysbioses (Ichim et al., 2018).

Yet, despite their advantages, a recurring challenge of artificial gut studies involves the degree to which they recapitulate the gut microbiota *in vivo*. After inoculation, artificial gut models often suffer a drop-in diversity within 24 hours, followed by a more secular drift away from inoculating community structure over subsequent weeks (Rajilic-Stojanovic et al., 2010). Some reports show that as little as 15% of the starting community is present at after 8 days in an artificial gut model (Auchtung et al., 2015). The loss of diversity and dissimilarity between the fecal inoculum and the stable community cultured in artificial guts raises questions about these models' applicability to the *in vivo* gut community.

The choice of media is likely to be one important factor in how well artificial gut models can culture gut microbial communities. Of course, gut models can be deficient at simulating multiple *in vivo* phenomena such as strictly anaerobic conditions, lack of mucosal adhesion sites, lack of a host immune system (both innate and adaptive,) and other host contributions such as retention time and changes in pH. (Child et al., 2006; Leitch et al., 2007; Macfarlane et al., 1998; Rajilic-Stojanovic et al., 2010; Van den Abbeele

et al., 2010a; Vandeputte et al., 2016). Still, prior experiments suggest that media selection is likely to be among the most critical aspects of model fidelity. Complex and undefined media have been previously used to culture more fastidious microbes and maintain diversity after cultivation (Browne et al., 2016; Goodman et al., 2011), while more recent evidence shows the importance of defined media and its ability to sustain specific gut bacteria (Tramontano et al., 2018). Media choice is the most obvious consideration when culturing a complex community.

Typical media components in artificial gut studies commonly include simple and/or complex carbohydrates (such as glucose and inulin), an undefined source of vitamins, minerals, and nucleic acids (such as meat or yeast extract), reducing agents (sodium thioglycollate and L-Cysteine,) as well as hemin and Vitamin K as growth factors for members of the genus *Bacteroides* (Gibbons and Macdonald, 1960). Prior studies suggest varying these media compositions will vary the populations of bacteria cultured in artificial gut systems (Freeman et al., 2003; Macfarlane et al., 1998). Additional evidence supporting the importance of media choice emerges from both computational and experimental studies showing that addition or subtraction of nutrients, including trace minerals and vitamins, have differential impact on the ability of individual isolates to grow in culture (Oberhardt et al., 2015). Together, these observations suggest the

hypothesis that the ability of artificial gut systems to culture microbial communities is shaped by the ability of individual microbial taxa to grow on a chosen gut media.

Testing this hypothesis has been challenging due to the diversity of gut microbial communities and limitations of traditional bacterial culture techniques. Unrelated individuals harbor hundreds of unique bacterial strains (Faith et al., 2013), meaning that optimizing media across a range of stool donors could involve testing thousands of strains. Retrieving such diversity from strain repositories is expensive and restricts experiments to only those strains in culture. Bacteria may also be directly isolated from human samples of interest, but because most taxa are rare, exhaustive capture of bacterial species requires spotting thousands of bacterial colonies, and limits isolation to colony-forming bacteria (Goodman et al., 2011). The emphasis on cultivation has led to novel culturing approaches that have been successful in isolating previously uncultured microbes from the human gut, but these labor-intensive techniques involve over 200 different culture conditions and isolation methods (Lagier et al., 2012). To sidestep the time-consuming nature of bacterial culture at the scale of hundreds to thousands of isolates, cultivation-free computational growth measurement techniques have been recently developed (Brown et al., 2016; Korem et al., 2015; Oberhardt et al., 2015; Vieira-

Silva and Rocha, 2010). However, these methods are not suited for previously uncultured microbes or microbial genomes without a closed sequence.

An alternative approach that could be used to measure how gut isolate growth on media at scale involves microfluidic techniques (Kaminski et al., 2016). Microfluidic approaches utilize very small volumes of liquids (nano- and picoliters) to culture bacteria. Microfluidic chips have been used to cultivate previously uncultured bacteria from the gut (Ma et al., 2014) and soil (Nichols et al., 2010) in chambers. Alternatively, droplet microfluidics can encapsulate microbes in distinct droplets of media that are dozens to hundreds of microns in diameter and separated by oil. These methods were initially introduced to isolate microbes from seawater communities (Zengler et al., 2002). Droplet microfluidics has also been used for phenotyping cross-feeding in synthetic microbial communities (Park et al., 2011), as well as screening previously isolated co-cultures of bacteria for inhibitory growth. Still, microfluidic droplet techniques have yet to be applied to gut microbial communities for screening growth on different media types.

Here, we used a droplet microfluidic technique (MicDrop) to isolate gut bacteria from mixed communities in droplets and measure their growth rates. We then applied this technique to a human gut microbial community that was also used to seed an artificial gut experiment. We found that microfluidic droplets could be used to measure the growth

of 94 sequence variants, representing 76% of taxa present in the original stool sample. Growth kinetics of taxa cultured in droplets also predicted the growth of taxa in the artificial gut, supporting the hypothesis that the ability of artificial guts to culture gut microbial communities is shaped by the ability of individual microbial taxa to grow on a chosen gut media.

3.2 Results

Establishing a dataset of bacterial dynamics in an artificial gut model

To test the hypothesis that the growth of individual microbial taxa in artificial gut media predicts how an overall community will grow in an artificial gut, we next used a fecal community from a healthy volunteer to carry out microfluidics and artificial gut experiments in parallel (Figure 16). For the artificial gut experiment, we used this stool to inoculate a continuous flow bioreactor system that has been used extensively in the past to culture human gut microbiota (Guzman-Rodriguez et al., 2018; McDonald et al., 2013; Reese et al., 2018; Silverman et al., 2018) (Figure 17). The artificial gut was fed modified Gifu Anaerobic Medium (Rettedal et al., 2014), a complex medium with a variety of carbon and nitrogen sources, such as peptone, yeast extract, meat extract, and liver extract, as

well as extra amino acids, vitamin K, and hemin. We chose mGAM because it enables a wide growth of mammalian gut bacteria (Rettedal et al., 2014; Tramontano et al., 2018).

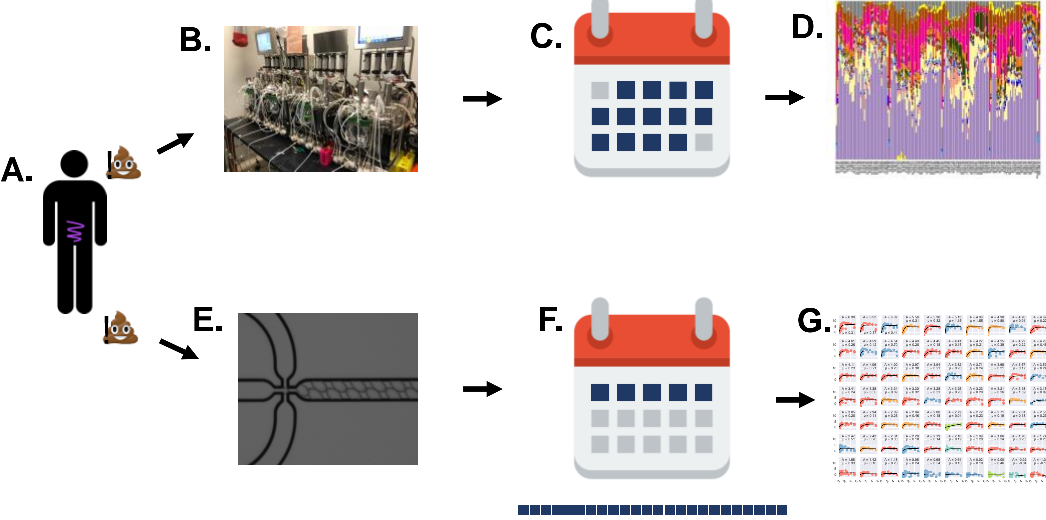

Figure 16. Experimental scheme for measuring growth rate contribution to bioreactor community. (A) Stool from a healthy donor was obtained and used to seed (B) an artificial gut continuous flow system. (C) Daily samples were taken for 30 days. (D) Community composition was determined by 16S sequencing. (E) A separate stool sample from the same donor was isolated into droplets as described in the methods. (F) We sampled droplets hourly for the first 24 hours and then daily for five days after. (G We then generated growth curves as described previously.

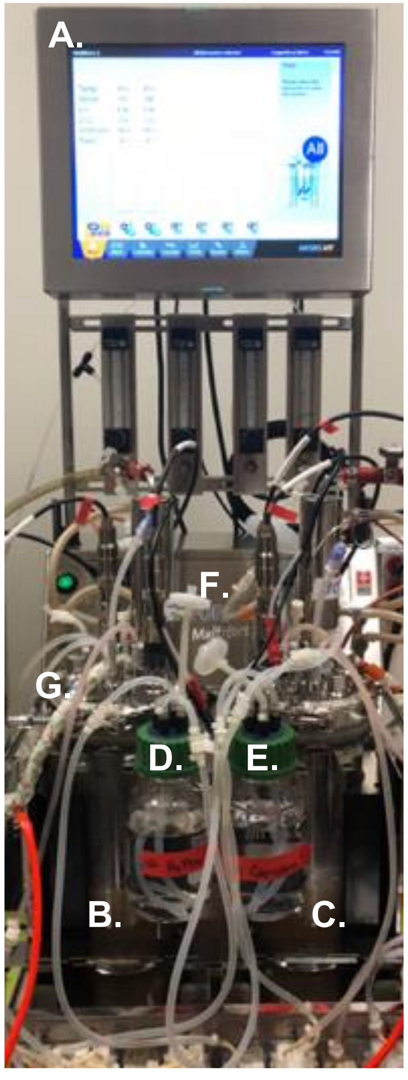

Figure 17. Annotated picture of the artificial gut system. A) Computer control of environmental conditions (oxygen, pH, temperature, mixing). B & C) Artificial gut vessels. D & E) Acid and base vessels for pH control. F) Sample ports. G) Media and waste lines.

Microbiota dynamics in the artificial gut exhibited the same trends away from starting inocula observed in prior studies (Auchtung et al., 2015; Rajilic-Stojanovic et al., 2010). At the time of inoculation, all of the phyla and 79% of the sequence variants (SVs) present in the inoculation stool could be detected in the artificial gut and, there was a significant correlation between community composition between the inoculum and artificial gut communities. Yet, over the course of two weeks, overall bacterial diversity declined and community dissimilarity increased relative to the starting fecal inoculum over the first ten days of the experiment (Figure 18A,B**).** After ten days, there were no longer significant correlations between the inoculum and artificial gut community structure ($p>0.05$; Spearman). At the end of four weeks, only 14% of inoculum SVs were still detected in the artificial gut (Table 2).

Table 2. Total and culturable bioreactor community. We analyzed the fraction of SVs present at each taxonomic level compared with the total SVs present in the stool sample used to inoculate.

	Total in stool	Day 0	Day 1	Day 7	Day 14
Phylum	4	1	1	1	1
Class	10	1	0.9	0.9	0.8
Order	10	1	0.9	0.9	0.8
Family	16	0.94	0.88	0.81	0.63
Genus	59	0.92	0.88	0.4	0.23
SV	112	0.79	0.78	0.29	0.18

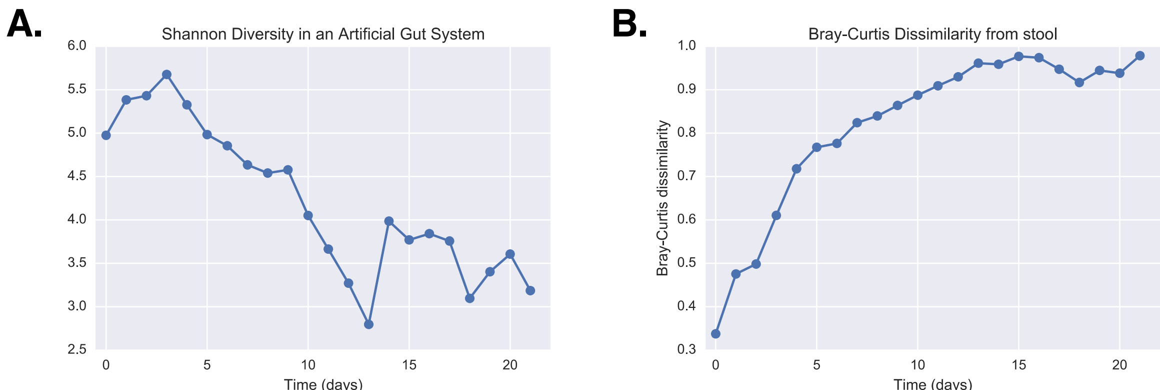

Figure 18. Alpha and beta diversity metrics over time in our artificial gut system. We calculated the Shannon diversity index (a) and Bray Curtis dissimilarity (b) at the SV level over time in our artificial gut system.

Applying droplet microfluidics to microbiota inoculating the artificial gut

Stool samples from the same donor taken 3 months apart were used to inoculate the artificial gut and used to inoculate a droplet microfluidics experiment. While these

stool samples were taken at different time points, they shared 65 SVs and showed a Bray-Curtis Dissimilarity of 0.5 (Figure 21). We isolated and cultured bacteria from this sample in droplets loaded with mGIFU media over the course of five days. We were able to fit growth curves for a total of 94 SVs (Figure 19, described in Chapter 2). We captured 76% of the bacteria found in stool in droplets. These taxa included representatives from major human gut bacterial phyla (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria; Table 3) and represented 76% of the inoculum's SVs, a proportion approaching prior culture efforts using mGAM medium (Rettedal et al., 2014).

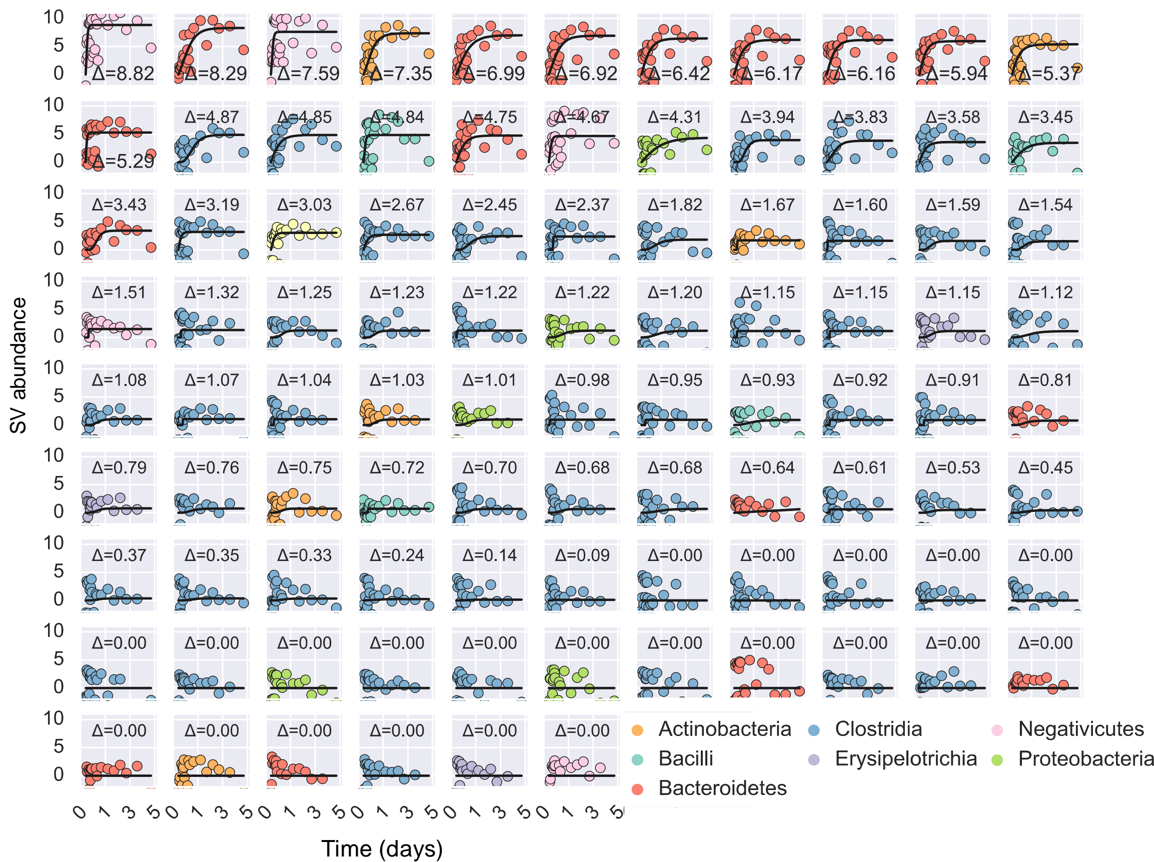

Figure 19. All detected SVs in MicDrop experiment using a human stool sample. Modified Gompertz growth curves are fit to time-series. SVs are colored by taxonomy and sorted according to total growth (curve asymptote height; indicated by Δ), which is denoted on each sub-plot. To ease viewing, curves are shifted vertically so y-intercepts are at the origin.

Table 3. Number and fraction of microbes from a human stool sample cultured by MicDrop in mGAM medium. SVs were considered as 'detected' if present in more than five longitudinal measurement. 'Growth' was defined by an inferred number of doublings equal or greate

Taxonomic level	Taxa in inoculum	Taxa detected in droplets	Taxa in inoculum & detected in droplets	Fraction of inoculum detected in droplets	Taxa grew in droplets	Taxa in inoculum & grew in droplets	Fraction of inoculum that grew in droplets
Phylum	4	5	4	1.00	5	4	1.00
Class	10	11	10	1.00	7	5	0.50
Order	10	14	10	1.00	8	5	0.50
Family	17	21	16	0.94	13	7	0.41
Genus	56	53	40	0.71	20	13	0.23
Sequence Variant	89	94	68	0.76	34	22	0.25

Of the detectable SVs, we then measured how many exhibited evidence for growth in droplets. We defined a cut-off for growth as inferred doublings of at least 2.14 times (Δ ln(SV DNA abundance) $\geq$ 1.48) based on our antibiotic-based control experiments (Chapter 2). A total of 34 SVs were defined as growing (Figure 20A), which accounted for 25% of the inoculum's SVs. Of the SVs with positive growth, 12 SVs were not

detected by sequencing in the inoculum, which suggests they could be laboratory contaminants. Still, these SVs resemble known gut bacteria and may alternatively represent rare microbes that require culture to be detected, which is a previously reported phenomenon (Lagier et al., 2012).

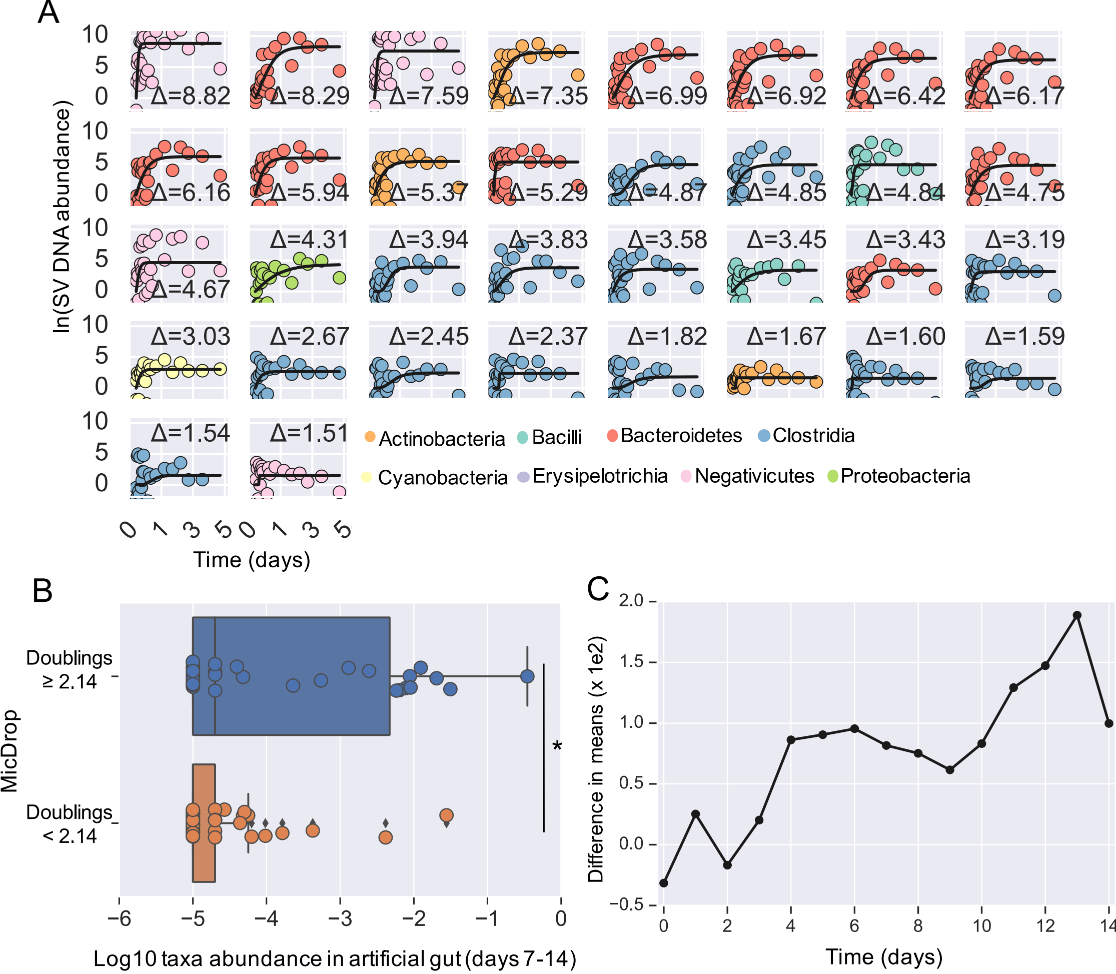

Figure 20. Comparison of SV growth kinetics and persistence in an artificial gut. (A) Abundance over time of SVs in MicDrop from a fresh human fecal sample. Modified Gompertz growth curves are fit to time-series. SVs are colored by taxonomy and sorted according to total growth (curve asymptote height; indicated by Δ), which is denoted on each sub-plot. Only SVs inferred to double at least 2.14 times were considering growing and are shown (ln(Δ SV DNA abundance) ≥1.48; threshold determined using control experiments in Figure S8). To ease viewing, curves are shifted vertically so y-intercepts are at the origin. (B) Long-term abundance of SVs in an artificial gut

(grouped across days 7-14) grouped by whether SVs were identified by MicDrop as growing (doubling ≥ 2.14 times) or non-growing doubling < 2.14 times) (Mann-Whitney U, $p < 0.02$). (C) Differences in mean abundances of growing and non-growing SVs increased over time in an artificial gut system ($\varrho=0.80$, $p < 0.0004$, Spearman correlation).

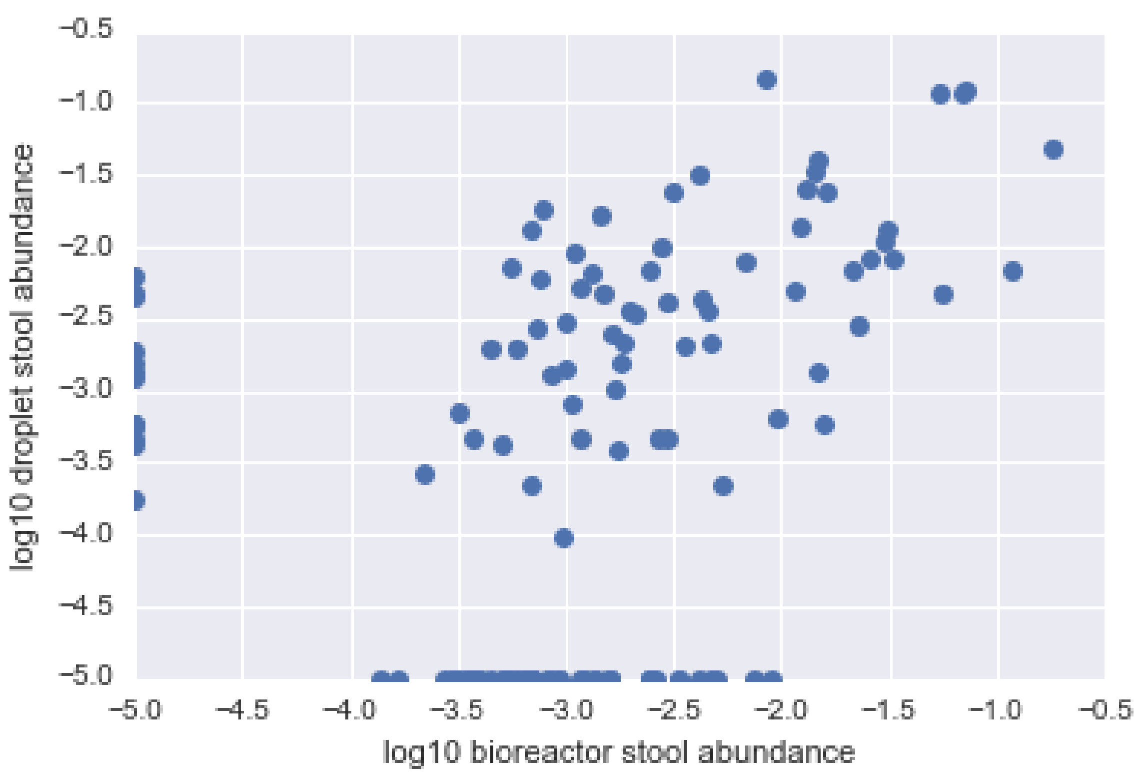

Figure 21. Shared SVs between stool samples used for droplets (y-axis) and seeding the artificial gut system (x-axis). 65 SVs were shared between samples. Spearman ϱ = 0.44 p = 3.7e-7. Bray-Curtis Dissimilarity = 0.5.

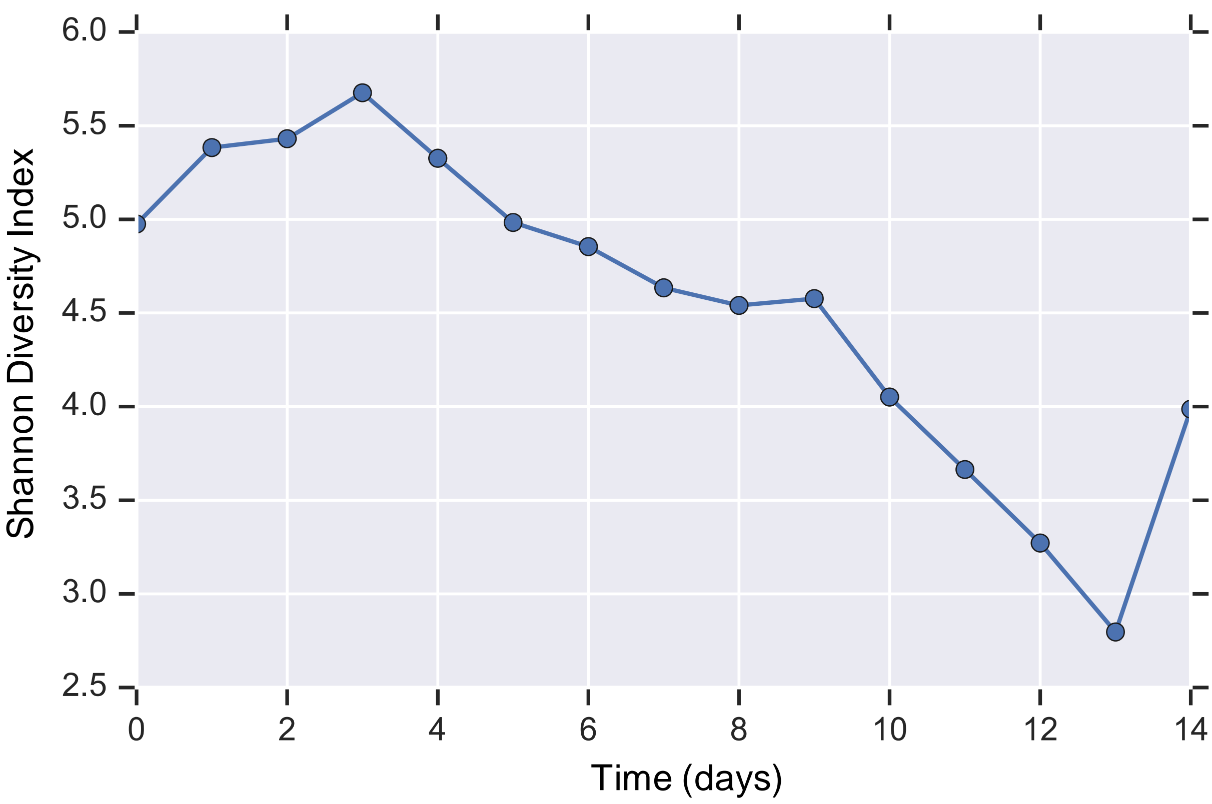

Figure 22. Microbiota diversity (Shannon index) over time in the artificial gut system. (ρ = -0.91, p = 3.06e-6, Spearman correlation)

Of the SVs with positive growth, the median growth rate across all SVs was 0.30 hr^{-1} (doubling time = 2.3 hours). All of the SVs showing positive growth had growth rates of > 0.03 hr^{-1}. This indicates a doubling time of > 24 hours, which is the most common retention time among the general population (Heaton et al., 1992) as well as the time of

turnover used in our artificial gut system. While colonic transit time a key determinant in community composition (Vandeputte et al., 2016), our droplet growth assay is analogous to batch culture, which would allow us to see growth rates that exceed colonic transit time.

The growth of SVs measured with MicDrop were ultimately associated with SV dynamics in the artificial gut. Such associations were not apparent on short-time scales (i.e. 1-5 days after inoculation), which is consistent with the notion that non-growing SVs require several days to wash out of an artificial gut after inoculation. However, from day 7-14 of the artificial gut experiment, we observed elevated abundances among artificial gut SVs that grew in the MicDrop platform (inferred doublings ≥ 2.14) relative to ones that did not (SV doublings < 2.14) ($p < 0.02$, Mann-Whitney U test) (Figure 20B). This difference in abundance increased over time in the artificial gut system ($\rho = 0.80$, $p < 1e\text{-}4$, Spearman correlation) (Figure 20C). Still, some SVs grew well in droplets, but did not persist in the artificial gut (left-most points of upper bar in Figure 20B); or, by contrast, did not grow in droplets, but were relatively abundant in the artificial gut (right-most points of lower bar in Figure 20B). The former may represent examples of SVs that are outcompeted in mixed culture, while the latter may be examples of SVs that depend on inter-species interactions to persist.

3.3 Discussion

Here, we developed a droplet microfluidic technique to isolate bacteria from mixed communities in droplets and measure their growth. We then showed this technique could be used to culture and assay the kinetics of growth of individual microbes from a mixed human gut bacterial community isolated from stool. These kinetics were predictive of overall dynamics of the community grown in artificial gut. The positive correlation that we observed between growth and relative abundance in the artificial gut system, the initial drop in diversity implies that our artificial gut system is selecting for growth ability in our medium of choice. Furthermore we saw a negative correlation with growth rate and starting fecal inoculum ($\varrho = -0.29$, $p < 0.005$, Spearman correlation)(Figure 23). There are two implications to these findings: 1) that the growth medium commonly chosen for artificial gut systems is too rich, and 2) that the constant influx of nutrient rich media plays an important role in community assembly.

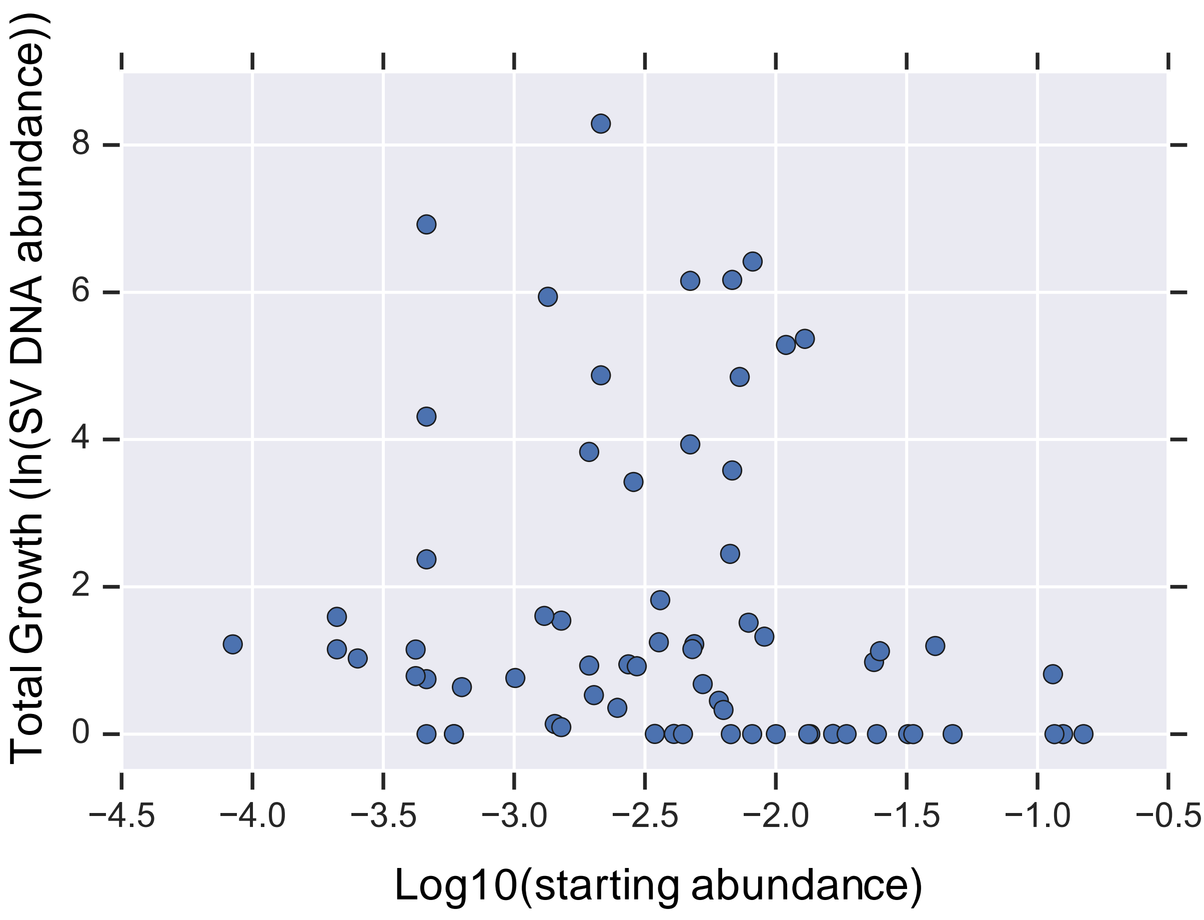

Figure 23. Relationship between total growth of SVs in microfluidic droplets and SV abundance in the inoculating stool sample (Spearman ϱ = -0.29, p < 0.005).

Nutrient limited media has been shown to support more diverse communities, by the mechanism that this limits fast-growers who commonly out-compete other members of the community (Fierer et al., 2007; Mello et al., 2016). Further, minimal media is needed

to identify metabolic interactions between microbes (Freilich et al., 2011; Wintermute and Silver, 2010). The drop in diversity commonly seen in these artificial gut systems is commonly attributed to the unsuitability of the medium, and our results of the positive contribution of growth to the final community composition suggest that the nutrients available in our medium is supporting the growth of certain species. More diluted, or more varied sources of carbohydrates might be successful in mitigating this selection pressure. The richness of the medium may also mask metabolic interactions that could enable a more diverse community. While community dynamics in the gut may be shaped by inter-species interactions, community dynamics are also likely to be governed by the fitness of component members within a given environment.

An additional selective pressure on growth in our artificial gut system is retention time. Our artificial gut system turns over once every twenty-four hours (modeling a 24-h retention time.) This constant influx of nutrient rich media to the artificial gut system might also play a role in community assembly. Stool consistency, which is a documented proxy for transit time in the gut, has been shown to correlated with species diversity, with interpreted slower transit times harboring more diversity than faster ones and changing retention time in an artificial gut system from 20-h to 60-h also resulted in different communities (Vandeputte et al., 2016) (Child et al., 2006). Of our isolates, there were 34

SVs with growth rates less than 0.03 hr^{-1} (doubling time > 24 hours. Of these SVs with doubling times of > 24 hours 26 of the 34 (76%) were from the phylum Firmicutes. One SV, *Coprococcus comes* has been reported to grow with mucin as a main carbon source, which is lacking in our media (Tramontano et al., 2018), as well as has been shown to grow in the mucosal lining of the large intestine (Nava et al., 2011), which could explain an observed slower growth rate. Moreover, with a doubling time of over 24 hours, *C. comes* could not maintain a population in a system that turns over faster than its rate of growth. Lowering the retention time of our artificial gut system serves two-fold purposes: slower waste removal, which may remove some selection pressures on the faster growers, as well as reduced nutritional availability, which in turn would also reduce selection of faster growers. Changing retention time may impact the specific contribution of growth rate on final community composition.

More broadly, our results suggest that the individual growth characteristics of microbial community members can be predictive of overall community dynamics. The complex and dynamic nature of the individual human gut microbiome, combined with the presence of high inter-individual diversity, has implied that the microbiome is an emergent system dependent on lots of interactions (Faust and Raes, 2012; Stein et al., 2013). Our findings that bacterial SV growth in isolation is associated with persistence in

an artificial gut supports the ecological hypotheses that intrinsic lifestyle characteristics of bacteria shape overall community dynamics. The notable minority of SVs present at high abundance that showed low growth as an isolate indicates that in our artificial gut system, we do not see growth in the community dependent on cooperative interactions. Instead, this observation implies that in our artificial gut system, fitness, as measured by growth rate, is a dominant contributor to community composition, not cooperation.

Our findings suggest an efficient approach for optimizing the relevance of artificial gut models for the culture of host associated microbial communities. As the community in our artificial gut system drifted away from the starting fecal community, growth rate in the artificial gut media became predictive of final community composition. This association, and lack of association between growth rate and the starting fecal community, implies that the chosen media, while shown to support diverse growth of gut microbes (Rettedal et al., 2014), does not accurately reflect the conditions in the lumen of the large intestine. Media choice is acknowledged to be an important part of determining the final community in these artificial gut systems (Auchtung et al., 2015; Freeman et al., 2003; Kettle et al., 2015; Rajilic-Stojanovic et al., 2010), and we have shown the contribution of growth rate in a given medium and its impact on final community composition.

Specifically, our results imply that media optimization could be approached by creating a panel of reference microbial isolates and testing their individual growth rates in candidate media. This approach would reduce the number of media experiments to be performed in the artificial gut itself, which tends to be more labor- and time-intensive than typical *in vitro* methods for culturing single bacterial isolates. To date, several efforts have already pursued artificial gut media optimization using panels of single isolates (Lagier et al., 2012; Li et al., 2018; Oberhardt et al., 2015; Tramontano et al., 2018); identifying specific media components could allow for culturing of targeted microbes of interest. One example is *Faecalibacterium prausnitzii*, which composed 18% of the fecal inoculum, yet showed no growth in our droplet assay or in our artificial gut system. While *F. prausnitzii* is a common part of the human microbiome, the inability to culture in artificial gut systems has been previously observed and attributed to unsuitable media choices (Auchtung et al., 2015). Choice of gut medium is important when considering individual microbes, as well as overall similarity to the fecal inoculum

Lastly, our droplet microfluidic approach likely has applications beyond the optimization of artificial gut systems. Our droplet methods could be useful in settings where the ability to isolate and assay the growth of individual microbes from complex communities is needed, such as in diseased or novel environments. Additionally, our

ability to capture 76% of the fecal inoculum suggests that droplet microfluidics offers a way to assay the growth of rare microbes that may otherwise be undetected in batch culture experiments. Potential applications include measuring carbohydrate utilization, drug metabolism, and antibiotic susceptibility of large panels of microbes from diverse sources (Maier et al., 2018a; Maurice et al., 2013; Tramontano et al., 2018). Still, potential drawbacks and existing limitations of our method include lack of a physical substrate for adherence, what media components can be put into droplets, and lack of host factors. Future experiments are needed to tackle these experimental limitations and will help define the technical limits of microfluidic droplet culture for microbiota research.

4. Conclusions and future directions

In this document, I present a novel technique of isolating and measuring the growth of previously uncultured microbes from the human gut. Using droplet microfluidic techniques for isolation and culture combined with molecular techniques such as NGS and qPCR, we were able to quantify growth of individual isolates in the presence of antibiotics, as well as in a common media used to culture gut microbes.

We showed that this technique is relevant to assay growth of bacteria from mixed communities. This is especially valuable as we recognize the diverse microbial communities that are relevant both to humans and the environment. As the relevance of personalized medicine is recognized, there is a pressing need for high-throughput functional assays of the microbiome. This method for assaying growth can be relevant for screening diverse microbiomes against a wide range of compounds, not limited to nutritional components. This method could have relevance in identifying interactions between drugs and the microbiome, or a way to identify antibiotic susceptibility in different patients. Additionally, it could be used to identify different functional elements of a diseased versus a healthy microbiome.

We used this droplet method to identify the role of growth rate in final community composition in an artificial gut system. This has direct relevance as the need for in vitro models of complex communities are being developed. While nutritional components of media is intuitively known to effect the composition of communities, e showed that an individual microbes' growth in isolation is correlated with relative abundance in the artificial gut system. This is important as it implies that individual fitness, in addition to interactions drive community composition in a complex system. This finding has major implications on the development of more optimal media for artificial gut systems. If there is a target microbe of interest to study within a community, MicDrop would likely be an appropriate platform to test for growth ability before transitioning to an artificial gut system, which are cumbersome to set up and maintain.

The difficulties in accurately and efficiently isolating and phenotyping microbes from the gut has been previously pointed out in this document. This represents a large challenge in identifying putative microbes for both therapeutics and in disease. A high-throughput method of growing diverse microbes would allow for major advances in identifying new microbes as well as testing existing ones against conditions relevant to human health.

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Biography

Rachael Jin Bloom was born and raised in Chapel Hill, North Carolina. She received her Bachelor of Arts degree in Biology from the University of North Carolina at Chapel Hill, with minors in Chinese and Creative Writing in 2009. While raising a daughter and after working as a technician in the lab of Dr. Patrick Sullivan at UNC-Chapel Hill, she began the PhD program in Genetics and Genomics at Duke University in 2013. She is a Marcy Speer Fellow and a National Science Foundation Graduate Research Fellow.