The Mini Colon Model (MiCoMo): a benchtop multi-bioreactor system to investigate the gut microbiome

Zijie Jin

Department of Biomedical Engineering

McGill University, Montreal

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Abstract

The gut microbiota has been increasingly recognized for its role in human health and disease risk. *In vitro* fermentation systems allow for the investigation of gut microbial communities with precise control of various physiological parameters while decoupling confounding factors from the human host. Current systems, such as the SHIME and Robogut, are large in footprint, lack multiplexing, and have low experimental throughput. Alternatives which address these shortcomings, such as the Mini Bioreactor Array system, are often reliant on expensive specialized equipment, which hinders wide replication across labs. Here, we present the Mini Colon Model (MiCoMo), a low-cost, benchtop multi-bioreactor system that simulates the human colon environment with physiologically relevant conditions. The device consists of triplicate bioreactors working independently of an anaerobic chamber, and is maintained at a constant temperature with a water bath. Each reactor is equipped with automated pH control via two peristaltic pumps and maintained anoxic through periodic nitrogen gas sparging, all automated with two Arduino microcontrollers. By conducting 14-day experiments, we found that MiCoMo was able to support a stable complex microbiota community with a Shannon Index of 3.17 ± 0.65 from individual fecal samples after only 3-5 days of inoculation. MiCoMo also retained inter-sample microbial differences by sustaining closely-related communities unique to each donor, while maintaining both minimal variations between replicate reactors (average Bray-Curtis similarity 0.72 ± 0.13) and day-to-day variations (average Bray-Curtis similarity 0.81 ± 0.10) after this short stabilization period. Together, these results establish MiCoMo as an accessible system for studying gut microbial communities with high throughput and multiplexing capabilities. Future directions include reducing MiCoMo's footprint, which is currently limited by the lack of affordable, miniaturized pH probes, and the inclusion of host cells and host-microbiota interfaces, and thus host-microbiota interactions, while controlling environmental parameters.

Résumé

Le microbiote intestinal est de plus en plus reconnu pour son rôle dans la santé humaine et le développement de maladies. Les systèmes de fermentation *in vitro* permettent d'étudier les communautés microbiennes intestinales avec un contrôle précis de divers paramètres physiologiques tout en dissociant les facteurs associés à l'hôte humain. Les systèmes contemporains, tels que le SHIME et Robogut, sont de grandes tailles, manquent de multiplexage et ont un faible débit expérimental. Les alternatives qui comblent ces lacunes, telles que le Mini Bioreactor Array (MBRA), dépendent souvent d'équipements spécialisés coûteux, ce qui entrave la réplication à grande échelle entre les laboratoires. Ici, nous présentons le Mini Colon Model (MiCoMo), un système multi-bioréacteur de petite taille à bas prix qui simule l'environnement du côlon humain et ses conditions physiologiques. Le dispositif est composé de bioréacteurs répliqués trois fois, indépendants d'une chambre anaérobie, et maintenus à température constante grâce à un bain-marie. Chaque réacteur est équipé d'un contrôle automatisé du pH via deux pompes péristaltiques et maintenu en condition anaérobie par mixage périodique d'azote, le tout automatisé avec deux microcontrôleurs Arduino. Nous avons mené des expériences sur une période de 14 jours et avons constaté que MiCoMo est capable de soutenir une communauté de microorganismes complexe et stable, avec un indice de diversité Shannon de 3,17 ± 0,65, à partir de matières fécales après seulement 3 à 5 jours d'inoculation. MiCoMo a également permis le développement de communautés uniques étroitement liées à chaque donneur, tout en maintenant des variations minimales entre les réacteurs répliqués (similitude Bray-Curtis moyenne 0,72 ± 0,13) et une variation quotidienne (moyenne de similarité Bray-Curtis 0,81 ± 0,10). Ces résultats démontrent que MiCoMo est un système accessible pour l'étude des communautés microbiennes intestinales avec un haut débit et des capacités de multiplexage. Les prochaines orientations incluent la réduction de la taille de MiCoMo, ce qui est actuellement limité par le manque de sondes pH miniaturisées abordables et l'inclusion potentielle de cellules hôtes pour imiter l'interface hôte-microbiote, tout en contrôlant les paramètres environnementaux.

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Contribution of authors

Zijie Jin designed and performed all experiments and data analysis in this work. Drs. Andy Ng, Corinne F. Maurice and David Juncker provided guidance, insights, interpretation, suggestions and feedback on experimental design and data analysis. Zijie Jin wrote the thesis with suggestions and feedback from Drs. Andy Ng, Corinne F. Maurice and David Juncker.

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List of Abbreviations

GIT – gastrointestinal tract

WHO – World Health Organization

SCFA – short-chain fatty-acid

IBD – inflammatory bowel diseases

IBS – irritable bowel syndrome

ASD – autism spectrum disorders

OSC – oligosaccharide carbohydrates

GOS – galacto-oligosaccharides

FMT – fecal microbiota transplantation

CDI – *Clostridioides difficile* infection

TGGE – temperature gradient gel electrophoresis

NGS – next-generation sequencing

HMA – human-microbiota-associated

SHIME – Simulator for Human Intestinal Microbial Environment

MBRA – MiniBioReactorArrays

TIM – TNO Gastro-intestinal Models

MiCoMo – Mini Colon Model

PBS – phosphate buffered saline

mGAM – Modified Gifu Anaerobic Medium

ASV – amplicon sequencing variants

Chapter 1: Introduction

1.1 Relevance of the Gut microbiota for health

Human beings represent a large microbial ecosystem, where trillions of microorganisms reside[1]. Collectively referred to as the human microbiota, this microbial system is estimated to consist of 10^{13} – 10^{14} cells (with around 1:1 ratio to human cells) of interacting archaea, virus, fungi, and bacteria[1,2]. The collection of their genes, known as the microbiome, is more than an order of magnitude larger than that of human[1,2]. The human microbiota includes microorganisms inhabiting many locations of the human body[3], but more than 90% of these microorganisms reside in the gastrointestinal tract (GIT) and are referred to as the gut microbiota, with an estimated 3.8×10^{13} bacteria[4] (**Figure 1**). The gut microbiota consists of strict and facultative anaerobic bacteria that form a largely mutualistic bi-directional relationship with the mammalian host: the host provides a hospitable environment which is often characterized by low oxygen concentration and ample supply of nutrients[5], while the microbiota plays an important role in both directly and indirectly[6] training host immunity[7], reinforcing the gut barrier[8], digesting nutrients and eliminating toxins[9], modifying drug metabolism[10,11], and modulating host inflammation responses[7], thus profoundly impacting human health.

In healthy individuals, the gut microbiota consists of mainly bacteria from the phyla of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, with the first two phyla representing more than 90% of the community[12,13]. However, the gut microbiota is also characterized by high levels of inter- and intra-individual microbial differences[14–16]. The composition of the gut microbiota is heavily affected by host factors such as genetics[17], age[18], and mode of birth[19], as well as environmental factors such as life style, diet[20,21], and intake of compounds foreign to the human body (xenobiotics)[22], all of which in turn affect the microbiota's interactions with the host[23]. While no consensus definition of a "healthy" microbiota composition exists at any high taxonomic resolution due to these factors[10], it is nevertheless agreed that high taxa diversity and high microbial gene richness are indicators of a healthy gut microbial community[10,24]. Specifically, there exists a core functional microbiome

consisting of possibly conserved bacterial genes, with indispensable functionalities to human host health, such as short-chain fatty-acid (SCFA) production[25]. The gut microbiota exists in a dynamic equilibrium state with its host[26], and its disruption by perturbations due to disease or external factors such as antibiotics, can deleteriously shift the composition of the microbiota [5,10] (**Figure 2**). Such shifts may change the microbial functional composition, metabolic activities, and/or their local distribution[27–29], resulting in a loosely defined "dysbiotic" microbiota[10,27].

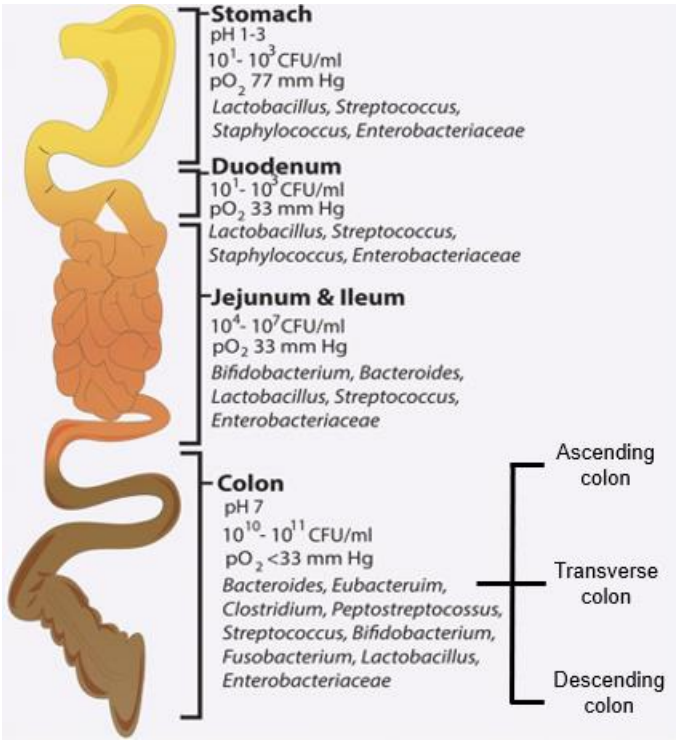


Figure 1: Environmental niches along the human GI tract.

Different conditions along the GI tract lead to non-uniform distribution and localization of microbial communities, while the colon hosts the highest density of microbes with mean pH level of 6.4 – 7 and very low oxygen concentrations (low redox potential). Adapted from Clarke et al.[30]

1.2 Microbiota-related diseases and microbiota-targeted therapies

In the gut, microbiota imbalance can be generally classified into three major categories: loss of beneficial organisms, excessive growth of potentially harmful organisms, and loss of overall microbial diversity[27]. These categories are not mutually exclusive and often occur at the same

time[27], sometimes also accompanied by a shifted host gut environment[26,31]. Many diseases have been found linked or associated to these microbial changes, including inflammatory bowel diseases (IBD)[32–34], irritable bowel syndrome (IBS)[35], obesity[11,36,37], diabetes[38–40], autism spectrum disorders (ASD)[41,42], spontaneous and autoimmune-related arthritis[43,44], as well as colorectal cancer[45,46] (**Table 1**). While it is currently unclear whether the shifted microbiota is a causal factor or result from, or both, of these disease conditions[27], the strong correlations observed led to the development of various microbiota-targeted therapies (**Table 1**, **Figure 2**).

While the consumption of fermented food has a history almost as old as that of human beings[47], the term "probiotics" was introduced in 1953 by Werner Kollath, and was only scientifically defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" in 2001 by the World Health Organization[48]. Probiotics most commonly consist of species belonging to the genera *Lactobacillus, Bifidobacterium,* and *Streptococcus*[49–51], and have been attributed to enhance immune responses, reduce serum cholesterol, and suppress the growth of potentially pathogenic microorganisms[48,52]. More recently, their pharmaceutical potential against various diseases is also being investigated, either for disease prevention or as a treatment accompanying other therapies[51,53–55] (**Table 1**).

Prebiotics, on the other hand, are defined as "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health"[56]. Undigestible by humans, these nutrients are fermented by members of the gut microbiota and selectively stimulate the growth and activity of certain members[56]. Prebiotics mostly consist of oligosaccharide carbohydrates (OSCs) such as fructan, galacto-oligosaccharides (GOS), and peptidoglycan, and often serve as possible alternatives or supplements to probiotics (in which case they are collectively referred to as "synbiotics)[56,57].

Another microbiota-targeted therapy, fecal microbiota transplantation (FMT), involves the administration of the whole gut microbiota (commonly in the form of fecal matter homogenate) from a donor into the intestinal tract of a recipient to replace the original microbiota[58–60]. Since its first modern application in 1958[61], this therapy is most notably applied in treatment of

Clostridioides difficile infection (CDI) with average 87–90% cure rate[59,60,62]. Other applications for FMT, such as in IBD[63], obesity[64] and neurological diseases[65] are also actively investigated, with mixed results (**Table 1**).

Table 1 Common microbiota-associated diseases, their impact on gut microbial composition and diversity, and corresponding attempted microbiota-targeted treatments

Disease conditions	Observed changes in microbiota	Attempted microbiota-targeted therapy
Inflammatory bowel diseases (IBD)	Decreased commensal bacteria diversity[31,34,66] Decreased Firmicutes and Bacteroidetes diversity[31,34,66], Increased Enterobacteriaceae[31,67,68]	Probiotics (mainly *Lactobacilli* spp) in mice[69–71] and human[72,73] Oligofructose and inulin (ratio 70:30%) as prebiotic in human[74] Fecal microbiota transplant[75]
Irritable bowel syndrome (IBS)	Decrease microbiota diversity and stability[76] Certain microbiota signature found to be associated with severe IBS[77]	Fructan and galactooligosaccharides as prebiotics[78,79] Various probiotics[80] Fecal microbiota transplant[81]
Obesity	Decreased fecal bacteria diversity[82] Increased Firmicutes to Bacteroidetes ratio[83,84]	Change in diet (which strongly affects microbiota)[21,85] Probiotics (mainly *Lactobacilli* spp) in mice[54,86] Prebiotics[87] Fecal microbial transplant in mice[88] and human[89]
Diabetes	Commensal microbiota signature related to disease[90,91]	Probiotics (mainly *Lactobacilli* spp and *Bifidobacterium* spp) in human[92] Prebiotics[93] Fecal microbial transplant in mice[94] and human[95]
Autism spectrum disorders (ASD)	Increased Bacteroidetes and bacteria in Clostridiales family[96,97] Decrease in Firmicutes and Bifidobacteria[96]	Probiotics (mainly *Lactobacilli* spp and *Bifidobacterium* spp)[96,97] Galactooligosaccharides as prebiotics[98] Fecal microbial transplant[99,100]
Colorectal Cancer	Increased *Akkermansia muciniphila* and *Fusobacterium nucleatum* in colorectal cancer tissue[45] Increase in Firmicutes, Bacteroidetes, Enterobacteriaceae, and Fusobacteria[101] Decrease in Proteobacteria, Bifidobacteria and Prevotella[101]	Probiotics for prevention and treatment *in vitro*[102,103] and animal models[104–106] Fecal microbial transplant for enhancing immunotherapy[55]

These novel microbiota-targeted therapies and their applications however face several limitations. For example, despite the huge market size of probiotics[48,107], the use of the term "probiotics", the evaluation of their effects and data interpretation from well-conducted clinical trials remain oftentimes unclear[48]. Not all commercially available probiotics have gone through statistically-powered double-blind clinical trials[108,109], and correlations between therapeutic effects and probiotic contributions aren't always clearly established[109]. Further, not all clinical trials report the exact composition and dosage of the probiotics evaluated[109]. Challenges have also been reported for probiotics to stably engraft a GI tract with an existing microbiota[110]. Prebiotics and FMT treatments face similar issues, as the underlying mechanisms of these therapies remain often unelucidated and show large variation between patients, clinical trials, and diseases[59,108,111]. There are also safety concerns with these treatments, such as risk of bacteremia, sepsis, malnutrition, and decreased intestinal barrier integrity leading to symptoms such as diarrhea[108,112], or even death when an FMT containing multidrug-resistant bacteria was conducted[113]. The lack of a complete understanding of the gut microbiota's involvement in disease pathology and progression makes it difficult to fully characterize potential off-target effects of such therapies and or their potential for transmitting susceptibility to chronic diseases that are hard to screen for[114].

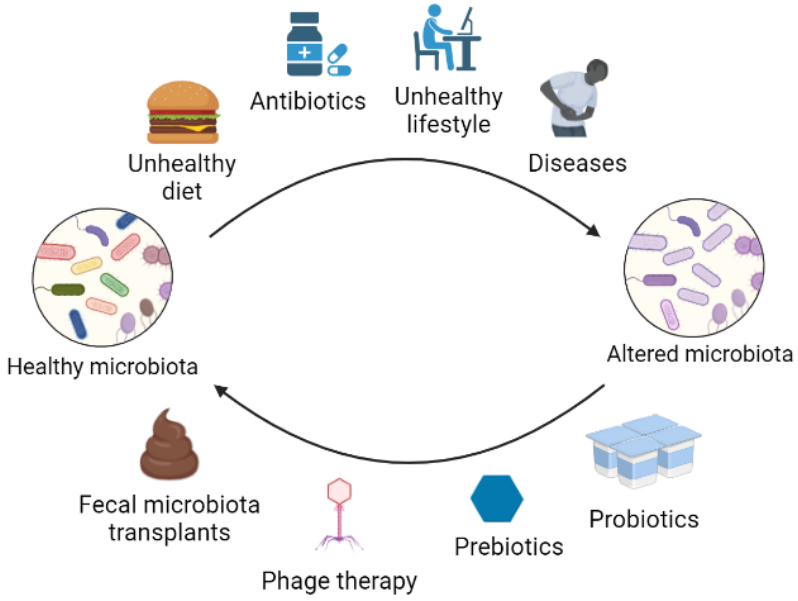


Figure 2: The gut microbiota is affected by various environmental factors.
Usage of antibiotics and disease conditions, for example, can disrupt the dynamic balance in the gut microbiota and lead to the selective loss or overgrowth of certain members. Conversely, microbiota-targeted therapies aim at re-establishing a healthy gut microbiota by

either introducing and promoting the growth of beneficial members or inhibiting growth of pathogens. Figure created with BioRender.com

1.3 Analytical methods for investigating the gut microbiota

Early microbiota research relied heavily on culturing techniques[115], dating back to as early as 1917 when Alfred Nissle showed that there exists protective bacterial strains in the human gut that prevent its colonization by potential pathogens[116]. However, this field only really took off when Robert Hungate established a roll-tube method for culturing strict anaerobes, known as the Hungate tube, which underwent many refinements during the mid-20th century and is still in use today[115–118]. At the same time, there was also the development of other modern day tools such as the glovebox and anaerobic chambers, as well as the development of culture media specific to strict anaerobes[115]. Due to limitations of culture-dependent methods, the study of the microbiota during this time was usually limited to a few bacterial strains and isolates[119,120]. Although technological advancements gradually enabled the culturing of many bacteria previously considered unculturable[121], culture-based studies still suffered from limitations such as the inability to grow a large portion of the gut microbiota and the dependence on laborious colony counting[122], and thus significantly underestimated the gut microbiota's bio-diversity[115,123]. Usage of animal models at the time on the other hand could often only establish the role of microbiota as a whole instead of mechanistically investigating the involvement of individual bacteria taxa[124].

Gut microbiota research saw a substantial development with the advancement of gene sequencing technology, namely the application of 16S rRNA sequencing for investigating microbial diversity in fecal samples for both cultivated and uncultivated bacteria by Wilson and Blitchington in 1996[123,125]. The 16S rDNA gene has since served as the most common genetic marker for studying bacteria phylogeny and taxonomy for various reasons: it is a long enough sequence (~1500 bp) present in all bacteria species with unchanged functionality over time, enabling identification of random mutations as a marker for bacterial detection[126,127]. Initially applied with technologies such as temperature gradient gel electrophoresis (TGGE)[128]

and Sanger sequencing, investigation of 16S rRNA gene benefited greatly from next-generation sequencing (NGS) technologies. NGS increased the sequencing depth with a higher throughput, decreased costs, and offered quantitative data (relative abundance) on bacteria in a complex community[123,129] at the cost of a lower read length[129]. To counter this issue, multiple hypervariable regions within the 16S rRNA gene have been identified, attempting to capture the same level of taxonomic information among members of a community with shorter gene read length[129]. The downside of identifying bacteria with variable regions of the 16S gene is low resolution of taxonomy at sub-species level, and sometimes at the species level, depending on primer choice[123,129–131].

To better capture the complexity of the gut microbiota and gain information beyond the simple taxonomical identification, metagenomics approaches, such as shotgun sequencing, have been applied on fecal samples[32,123,132]. These methods break the genome into small fragments and directly sequence all DNA fragments in the sample aiming to recover the whole microbiome, thereby revealing taxonomic information (and absolute abundance) and functional profiles (**Figure 3**). However, metagenomics approaches are significantly more expensive than 16S rRNA sequencing and require extensive computational power to assemble the acquired gene fragments with low error rates[132–134]. These approaches, along with metatranscriptomic analyses of mRNA to identify active members of the community, and metabolomic analyses of proteins to investigate microbial functional output profiles, are emerging technologies that enable us to gain a better understanding of the gut microbiota both at the compositional and functional levels[123,135,136].

1.4 *In vivo* methods for gut microbiota investigation: human cohorts and HMA mice

One major challenge faced by gut microbiota studies is accessibility of samples. Historically, microbiota studies relied heavily on fecal samples to represent the gut microbiota. The handling and storage methods of such samples often introduce bias in analyses[137,138]. It has also been found that sampling fecal matter represents better the luminal and colonic microbiota (those residing the inner section of gut), as opposed to the mucosal microbiota, which colonizes the

mucus layer above gut epithelial cells and forms a unique community[139–141]. Investigation of the mucosal microbiota, on the other hand, often relies on invasive methods such as biopsy and luminal brushing during endoscopy[142] or surgeries[140]. Regardless of sampling methods, the direct investigation of any intervention to the gut microbiota in human subjects involves high costs associated with clinical trials. Further, the cohort size is often limited by ethical concerns and causal effects are usually hard to establish due to the amount of confounding external factors[143–145].

Animal models have served as an important model to tangibly establish not only correlation, but also causality between the microbiota and various disease conditions[146]. Gnotobiotic animals are defined as any animal with a known microbial community. Such animals, usually mice, are born and raised in sterile conditions to be free of microorganisms as germ-free animals, and colonized with either a known bacterial community or isolates (gnotobiotic) or with human fecal samples (HMA mice)[146–148] (**Figure 3**). These mice have been shown to recapitulate pathophysiological features of several diseases[146], such as obesity[149], pregnancy-induced increases in adiposity[150], childhood asthma[151] and autism[152]. Gnotobiotic and HMA mice have also been used to study the gut microbiota's colonization process[148], metabolic capacity[153,154] and its interaction with the host immune system[153,155].

However these models, while cheaper and less constrained by research ethics than human trials, are subject to variations from environmental factors such as housing conditions[146,156], genetic background and diets[156,157], and require specialized animal facilities which incur additional costs. Further, a considerable portion of bacteria from the human gut microbiota fail to efficiently colonize the mouse gut[158]. After stabilization, HMA mice often establish a gut microbial community only partially resembling that of its donor[151,159], possibly due to physiological, dietary, genetic, and behavioral differences between mice and human[146]. In addition, germ-free mice have altered immune maturation compared to normal mice with their own murine microbiota due to a lack of host-microbiota co-evolution and co-adaptation[146,158]. Together, these factors might obscure or exaggerate the role of the gut microbiota in diseases and explain the low translatability of such results from mice models to clinical trials[160].

1.5 *In vitro* models of the human gastrointestinal tract

To address some of the limitations of conducting studies in human subjects and animal models, *in vitro* fermentation systems that model different sections of the human GI tract to study gut microbiota have been developed[143]. *In vitro* systems allow for the investigation of the complex interplay within the gut microbiota, between microbial communities and introduced perturbations, while decoupling confounding factors inherent to *in vivo* studies[143,161] (**Figure 3**). However, they also often suffer from reduced physiological relevance due to lack of host factors, such as the gut epithelial/mucosal layer and interplay with the immune system.

In vitro systems often take the form of a bioreactor or an array of bioreactors[161]. Loosely defined as any "apparatus for carrying out a bioprocess"[162], modern bioreactors support a precisely controlled, biologically active environment with various control strategies[163]. They enable the study of complex microbial communities, such as the gut microbiota, in a user-defined, controlled environment[161,164]. Bioreactors are versatile in controlling different physiologically-relevant parameters, such as volume, pH level, availability of nutrients, dissolved oxygen level, and residence time, thus allowing investigators to develop systems with a wide range of complexities[122,165–167]suitable for their own topic of interest. These systems differ by mode of operation, parameters controlled, equipment requirement and cost, but generally are easier to use, less time-consuming and more cost-effective than *in vivo* models[143].

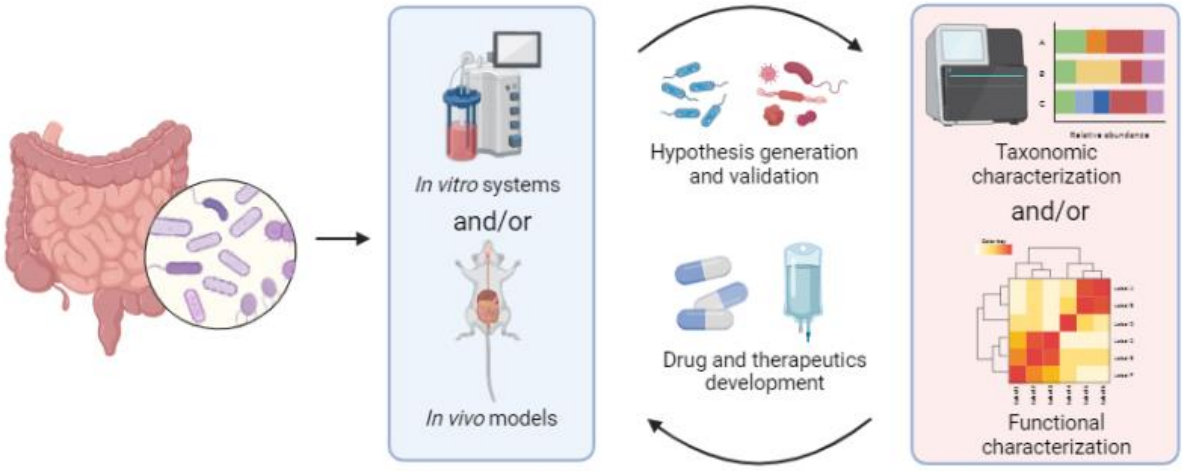


Figure 3: Gut microbiome analysis workflow for causality and enabling therapeutic development.

Microbial communities from the human gut are established in either *in vitro* systems or animal models, and subject to various perturbations. The effects of such perturbations on the microbiota are characterized computationally through the characterization of taxonomy and functional profiles, leading to new hypothesis and therapeutics development. Adapted from Young et al. [168] Figure created with BioRender.com

1.5.1 Batch models

In vitro fermentation systems can be classified based on their mode of operation as batch or continuous/semi-continuous systems[161,169]. A batch system, defined as a closed system with fixed amount of material and no additional inlet throughout the experimental period[170], is the simplest type of bioreactors. In batch bioreactors, a set volume of enriched media is prefilled before inoculation (usually fecal slurry for gut microbiota studies), and no media replenishment nor waste removal happens during the whole experimental period[143,161]. Due to limitation in nutrient supply and waste build-up, these systems are usually limited to short-term studies up to 72 hours, and are used in applications focusing on analysis of fermentation end-products from the gut microbiota rather than gut microbiota itself[161,171,172]. Thanks to their simplicity, numerous batch bioreactors have been developed and many studies have applied them to evaluate the fermentation of food components, such as dietary fibres[173,174] and short carbohydrates[175] by the gut microbiota, as well as investigating its SCFA, carboxylic acids, and

gas production[144,172,176]. Ease of operation and availability of batch bioreactors also often lead to their application as preliminary screening tools before conducting more lengthy and complicated studies in other systems[143].

1.5.2 Chemostat models

Continuous bioreactors, also known as chemostats[177], are more sophisticated and physiologically relevant systems equipped with a continuous supply of fresh culture medium and removal of waste. A variant of chemostats operates in a semi-continuous manner, providing timed influx of nutrients and efflux of reactor content, to more closely mimic the physiological conditions of human food intake and processing[178]. These systems are usually equipped with online monitoring and control systems for parameters such as pH, liquid levels, temperature, and dissolved oxygen levels at specific setpoints, thus allowing constant experimental conditions for extended periods, up to months[161,179]. Chemostats can either operate as a single unit (single stage) or be connected in series with other units to form a multi-stage bioreactor system.

Single stage chemostats typically have a rather simple setup and operation protocol, although more complicated than those of batch systems, exemplified by a twin-vessel single-stage chemostat model named "RoboGut"[180,181]. This system has a pair of identical, independent reactor vessels with 400 mL working volume, allowing for experiments to run with a control vessel running in parallel. This system is equipped with fully automated control over physiological parameters and runs in a continuous manner (**Figure 4**). With a good reproducibility and reliability, RoboGut has been used for the investigation of gut bacteriophage communities and, more importantly, for the development of controlled complex microbial consortia as a safer approach to FMT therapy to treat *C. difficile* infections[182,183]. A downside of this system is the ~35 days stabilization time it requires for fecal communities before experiments can be initiated[181].

More complex chemostats operate as multi-stage systems to achieve higher physiological relevance, and many established *in vitro* systems for studying the gut microbiota belong to this

group. The Simulator for Human Intestinal Microbial Environment (SHIME) system[184–186], which is probably the most well-known example, consists of 5-stage chemostat reactors mimicking different sections of the human GI tract, from stomach and small intestine to ascending, transverse, and descending colon, with volumes ranging from 500 mL to 800 mL. This system includes physiologically relevant pH values for each compartment, growth media supplemented with human enzymes and bile acids in the stomach and small intestine reactors, and operates semi-continuously to mimic the human eating and digestion processes[187,188]. The three colon reactors, inoculated with human fecal samples, have been shown to develop distinctive microbial communities, possibly reflecting the *in vivo* spatial difference of gut microbiota composition[184,189] (**Figure 4**). The SHIME model is able to maintain a stable microbial community after around two weeks of stabilization[189,190], and has been used in various applications including investigating gut microbiota composition[184], metabolism of various compounds[191–193], as well as the effects of prebiotics[194] and probiotics[195].

Another established multi-stage chemostat system by the company TNO is the TNO Gastro-intestinal Models (TIM)[122,196], includes two models together mimicking the whole human GI tract. Unlike the SHIME system, these systems are separated into the TIM-1 system[196] which simulates the upper GI tract from stomach to small intestine, and the TIM-2 system[122] which simulates the colon environment including the microbiota. Designed as a simulator for GI tract and digestion processes, TIM-2 is equipped with a peristaltic mixing system by changing water pressure, mimicking the gut peristalsis observed *in vivo*[197], as well as a dialysis system going through the reactors to remove accumulated waste [198]. This system includes 4 compartments with 70 mL volume, each connected in series to mimic different sections of the colon with fully automated control on pH, temperature, and fluidics transfer, and runs in a continuous manner instead of having a feed schedule like SHIME, followed by a fed-batch mode after some time (**Figure 4**). However, the pH level is constant across its sections, and it has not been shown whether microbial communities in these compartments are distinctive from each other. While this system requires significantly less time for fecal inoculum to stabilize (12 hours to a day) compared to SHIME, experiments conducted in it are also shorter, typically lasting 3 days. Similar to the SHIME, this model has been shown to maintain a stable microbiota community, and demonstrated good reproducibility and translational potential in various applications[199–202].

While the above-mentioned fermentation systems have all demonstrated good reproducibility, relevance to clinical observations, and are validated with various types of samples, they suffer from some common drawbacks, especially the large footprints, long stabilization times required, and relatively high costs of acquisition and operation. Due to usage of liter-sized reactors, these fermentation systems often lack multiplex capability and require multiple runs in sequel if an experiment needs to be repeated. They often also have low experimental throughput and require a long fecal inoculum stabilization period before experiments can be initiated (2 weeks for SHIME and almost a month for RoboGut), resulting in relatively limited replicates done for many studies[180,203]. Lastly, the large footprint of these systems requires dedicated lab space and considerable peripheral equipment, along with extra personnel to operate and maintain[182,184]. While efforts have been made to increase the multiplex capabilities and throughput of these systems, such as the twin-vessel of RoboGut and a similar effort to run 2 SHIME systems in parallel (the TWIN-SHIME system)[184], these solutions put more demands on resources of the operating lab, thus severely limiting their accessibility worldwide[204].

1.5.3 Miniaturized systems

To address some of the limitations of large-sized fermentation systems, several groups have developed miniaturized bioreactor systems to mimic the GI tract environment and to study the gut microbiota. These systems are usually designed to have high experimental throughput and/or high multiplex capacity with reduced footprint, thus requiring less resources. Similar to their larger counterparts, miniaturized fermentation systems have been developed with various complexities and modes of operation.

Some miniature gut models try to keep a high physiological relevance while reducing the footprint. One such attempt is the CoMiniGut system[205], a set of 5 parallel, independent batch reactors with 5 mL working volume, in a custom climate box to keep anaerobiosis and constant temperature. Each reactor is independently pH controlled, and has three 8-hour pH modulation sessions, gradually increasing the pH to mimic the change of environment along the GI tract as food travels. Similar to bigger batch systems, CoMiniGut is mainly used to study the

fermentation of prebiotics and human milk by the gut microbiota over a short time period[205]. It is proposed as a screening tool with high throughput and reproducibility, while its miniaturized volume enable reduced experimental costs with rare and expensive compounds. A more complex miniaturized system, the EnteroMix model[206], consists of 4 bioreactors connected in series to mimic different sections of colon. Each reactor is set at a different pH level and has less than 10 mL working volume, allowing for four systems to be run in parallel. This system runs on a semi-continuous feeding schedule similar to that of larger system SHIME, and only requires 24 hours stabilization time after fecal inoculation. However, it is not reported whether bacterial communities reached a stable state during this period, and fecal samples from different donors resulted in similar functionality in the system, indicating some potential drawbacks of the system[206], and limited its applications to relatively short time periods[207].

Other miniature gut models developed focused on maximizing experimental throughput. One such example is the batch culture model MiPro, which is based on a 96 deep-well plate, shaken in an anaerobic chamber[166]. This system trades control of physiological parameters for higher multiplex potential: it has no control over pH nor fluidics, but allows for 96 replicate experiments to run in parallel. Focusing mostly on proteomic profiling of the gut microbiota, this system is proposed to be a scalable tool for high-throughput screening of drug-microbiota interactions, demonstrated by seeding the system with fecal inoculum and conducting experiments over 24-hour periods[166,208]. MiPro is also shown to maintain stable microbial taxonomical and functional profiles over 5 days, allowing for slow-acting xenobiotics to take effect[166].

O'Donnell et al. reported a similar batch model adapted from a commercially available micro-Matrix cassette fermentation machine[209]. This model has 6 mL working volume, is equipped with automated dissolved oxygen level and pH control, and supports 24 experiments to run in parallel. While a major shift in microbial composition from a fecal inoculum over the 24-hour experimental period is observed in this model, it is confirmed to have good reproducibility across replicate reactors. Thus, the developers emphasized the capability and potential of this system for high-throughput investigation of antibiotics and therapies treatment effects[209,210]. Continuous fermentation systems have also been developed to maximize multiplex, notably

the MiniBioReactorArrays (MBRA) system[165,211]. The MBRA can run 24 different experiments in parallel with a working volume of 15 mL and takes 7 days for fecal inocula to stabilize[165]. This system shows comparable between-replicate variation with that observed in animal models and supports a stable microbial community similar to the original sample[165]. It has been used to study the effect of various emulsifiers on gut microbial diversity and composition[212]. However, this system relies on an anaerobic chamber for anoxic conditions and temperature control. In addition, it lacks pH control and relies on expensive multi-channel pumps and multi-point stir plates for fluidic transfer and mixing (**Figure 4**).

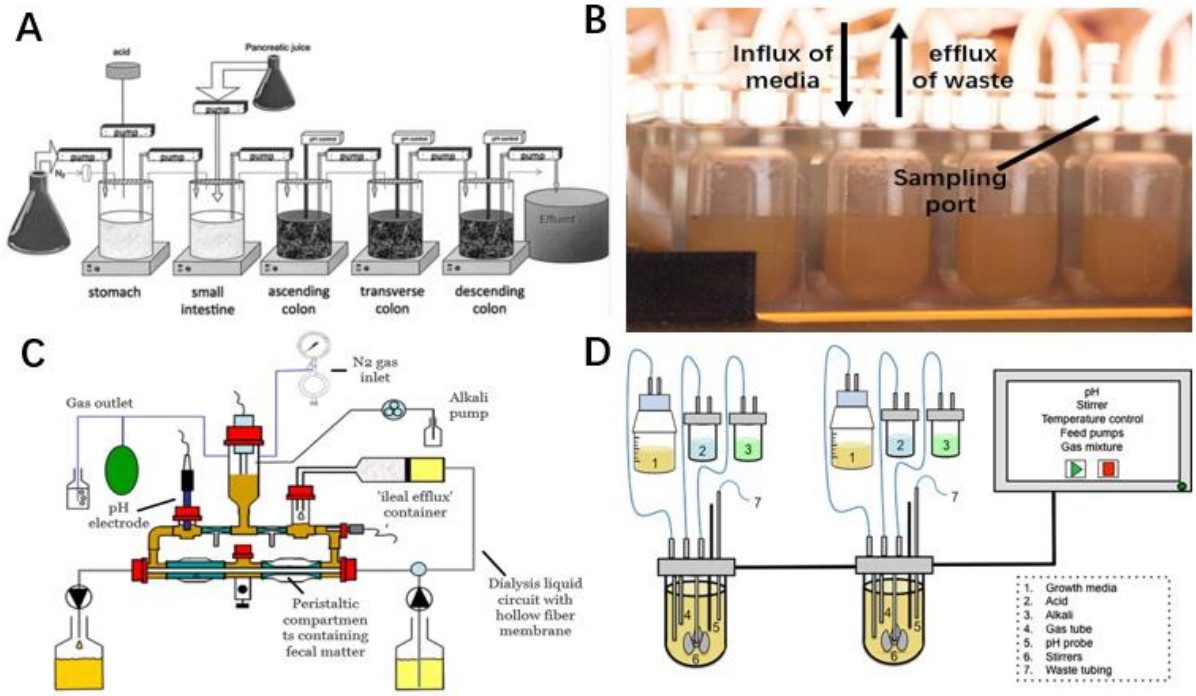


Figure 4: Schematics and pictures of selected *in vitro* systems for studying gut microbiota discussed in this work

A) Schematics of the semi-continuous SHIME system with 5-stage reactors equipped with pH control, mimicking the whole GI tract[185]. B) Picture of the continuous MBRA system sitting on a multi-point stir plate for mixing in an anaerobic chamber. The system is not equipped with pH control. Adapted from Naimi et al.[165,212]. C) Schematic of the TIM-2 system which operates in a mixed mode of continuous and fed-batch, equipped with peristaltic compartments and dialysis liquid circuit to mimic absorption. Adapted from Rehman et al. [202] D) Schematic of the Robogut system, a continuous, single-stage system with two parallel reactors equipped with automatic pH, temperature and fluidic control[161].

1.6 Rationale of this work

Table 2 and **Table 3** summarize the engineering and biological parameters of the various *in vitro* fermentation systems for the study of the gut microbiota discussed above. Considering the advantages and limitations of these existing fermentation systems for studying the gut microbiota, there is a need for novel models that fill the gap between large-sized, complicated systems with high degrees of control but low multiplex and throughput, and miniaturized systems with high multiplex and throughput but less physiological relevance. Further, decreased dependence on specialized equipment and dedicated lab space is preferred for the novel device to address limitations on accessibility and reproducibility across different labs.

Table 2 Engineering parameters of some *in vitro* fermentation systems for gut microbiota discussed in this work

System	Operation Mode	Stages and volume	pH control and setpoint†	Multiplex	Anaerobic conditions	Mixing
Single batch fermentation system[213]	Batch	Single stage with 100 mL working volume	6.5	8 parallel and independent vessels	Flush with N_2/CO_2	Constant magnetic stirring
CoMiniGut[205]	Batch	Single stage with 5 mL working volume	Increase from 5.7 to 6.0 during the first 8 h of fermentation followed by an 8 h pH increment from pH 6.0 to 6.5 pH, then 8 h increment from 6.5 to 6.9 to mimic different section of colon[205]	5 parallel reactor units	Kept in a climate box	Constant magnetic stirring
Micro-Matrix device[209]	Batch	Single stage with 6 mL initial volume	7	24 parallel reactor units (wells of cassette)	Flush with N_2/CO_2	None
Mipro[166]	Batch	Single stage with 1.2 mL working volume	Not controlled	96 parallel units (wells)	Kept in anaerobic chamber	Constant shaking on shaker
SHIME[184,186,214]	Semi-continuous	5 stages simulating whole GI tract, with 500 mL working volume for ascending colon, 800 mL for transcending colon and 600 mL for descending colon	2 for stomach reactor, 6.6 for small intestine reactor, 5.6 and 5.9, 6.15 – 6.4, and 6.6 – 6.9 for the colon reactors respectively	Maximum 2 parallel system sets[184]	N_2 flushing	Constant magnetic stirring
EnteroMix model[206]	Semi-continuous	4 stages simulating different section of colon, working volumes of 3,5,7 and 9 mL respectively	5.5, 6.0, 6.5, and 7.0 for each stage respectively	4 parallel system sets[143]	N_2 flushing	Mixing exists, but exact method not reported
TNO Gastro-Intestinal Model (TIM)[122,215]	Continuous followed by fed-batch	4 stages together simulating colon with absorption, maximum 70 mL each[122,196]	6.5 for experiment with fresh fecal inoculum 5.8 for experiment with pre-fermented fecal sample	None	N_2 flushing	Peristaltic mixing by changing water pressure surrounding reactor
Robogut[181]	Continuous	Single stage, 400 mL working volume	6.9-7.0	2 parallel reactor units	N_2 bubbling in reactor	Unreported, presumably through N_2 bubbling and stirring
MBRA[165]	Continuous	Single stage, 15 mL working volume	Not controlled	Up to 48 parallel reactor units	Kept in anaerobic chamber	Constant magnetic stirring

†In most cases, bioreactors are equipped with pH control systems adjustable to experimental design. The pH setpoint reported here is just the most commonly done/reported value for each system

Table 3 Biological parameters of some in vitro fermentation systems for gut microbiota discussed in this work

System	Residence time†	Microbial Stabilization time	Media	Inoculum	Application
Single batch fermentation system[213]	24 hours	None, experiments start immediately after inoculation	Gifu anaerobic medium	Fecal sample diluted in PBS Final concentration 10% w/v	Effects of prebiotics[213]
CoMiniGut[205]	24 hours	None, experiments start immediately after inoculation	Basal medium	Fecal sample frozen with glycerol and thawed in buffer Final concentration 1% v/v	Effects of prebiotics and human milk oligosaccharides[205]
micro-Matrix[209,210]	24 hours	None, experiments start immediately after inoculation	Modified previously developed custom medium[216]	Fecal sample frozen with glycerol and thawed in buffer Final concentration 5% w/v	Evaluating anti-*Listeria* activity of pediocin PA-1 derivative[210]
MiPro[166,217]	24 hours	None, experiments start immediately after inoculation	Modified previously developed custom medium[218]	Fecal sample diluted in PBS Final concentration 2% w/v	Investigating gut microbiota response to drugs[217]
SHIME[184,186,214]	24 – 72 hours	10 - 20 days	SHIME medium[186]	Varies depending on experiment[185]	Evaluating gut metabolism[193] Effects and survival of probiotics[195,219] Effects of prebiotics[220]
EnteroMix system[206]	48 hours	24 hours before experiments start; community stabilization unreported	SHIME medium[186]	Fecal sample diluted in medium Final concentration 25% w/w	Effects of polydextrose on gut microbiota and mucosal functions[206]
TNO Gastro-Intestinal Model (TIM)[122,215]	Normally 72 hours[198]	12 - 16 hours	Previously developed custom medium[221]	Fecal sample pre-fermented in a fed-batch reactor or fresh fecal sample diluted with 1:1 buffer	Effects of prebiotics[222] Probiotics survival[223] Gut microbiota SCFA production[122]
Robogut[181,224]	24 hours	At most 34 days	Modified previously developed custom medium[167,181,221]	Fecal sample diluted in medium Final concentration 1.25% w/v	Analysis and development of defined mixed community comparing to fecal community[224]
MBRA[165,211]	21 days	7 days	Modified previously developed custom medium [211]	Fecal sample frozen with glycerol and thawed in buffer Final concentration 5% w/v	Effects of dietary emulsifiers on gut microbiota[212]

† Residence time applies for systems operating in batch mode. For continuous and semi-continuous systems, the retention time is reported.

Preface to chapter 2

Chapter 2 consists of a manuscript ready to be submitted to the peer-reviewed journal Microbiome.

Chapter 2

The Mini Colon Model: a benchtop multi-bioreactor system to investigate the gut microbiome

Zijie Jin[1,2], Andy Ng[1,2], Corinne F. Maurice[3], and David Juncker[1,2]

[1]McGill Genome Centre, McGill University, 740 Doctor Penfield Avenue, Montreal, QC, H3A 0G1 Canada [2]Department of Biomedical Engineering, McGill University, Montreal, Quebec H3A 2B4, Canada [3]Department of Microbiology and Immunology, McGill University, Montreal, QC H3A2B4, Canada

Abstract

In vitro fermentation systems allow for the investigation of gut microbial communities with precise control of various physiological parameters while decoupling confounding factors from the human host. Current systems, such as the SHIME and Robogut, are large in footprint, lack multiplexing, and have low experimental throughput. Alternatives which address these shortcomings, such as the Mini Bioreactor Array system, are often reliant on expensive specialized equipment, which hinders wide replication across labs. Here, we present the Mini Colon Model (MiCoMo), a low-cost, benchtop multi-bioreactor system that simulates the human colon environment with physiologically relevant conditions. The device consists of triplicate bioreactors working independently of an anaerobic chamber and equipped with automated pH, temperature, and fluidic control. We conducted 14-day experiments and found that MiCoMo was able to support a stable complex microbiota community with a Shannon Index of 3.17 ± 0.65, from individual fecal samples after only 3-5 days of inoculation. MiCoMo also retained inter-sample microbial differences by developing closely-related communities unique to each donor, while maintaining both minimal variations between replicate reactors (average Bray-Curtis similarity 0.72 ± 0.13) and day-to-day variations (average Bray-Curtis similarity 0.81 ± 0.10) after this short stabilization period. Together, these results establish MiCoMo as an accessible system for studying gut microbial communities with high throughput and multiplexing capabilities.

Key words

Microbiota; Gut model; 16S rRNA gene; Bioreactor; Microbiome

Introduction

The gut microbiota has been increasingly recognized in its role for human health and disease[1]. The trillions of microorganisms residing in the human gut respond to environmental factors such as diet and compounds foreign to the human body (xenobiotics), and have been found to profoundly impact human mental and physical condition, in addition to modulating disease progression and drug metabolism[2]. The gut microbiota is characterized by high levels of inter- and intra-individual differences[3–5]. As such, the study of individualized responses of the gut microbiota to perturbations often prove difficult, and various experimental technologies have been developed to limit or control these variables.

For example, gnotobiotic mice and human-microbiota-associated (HMA) mice are powerful models to study the response of either a defined microbial community or a complex one directly transplanted from human fecal samples. Such *in vivo* approaches, integrated with host interactions and immune responses, offer highly physiologically relevant experimental conditions. However, these systems are also expensive to use, requiring specialized animal facilities, and are limited by the inherent biological variability and animal housing conditions[6,7]. Further, the incorporation of host interactions can confound the specific response of the microbiota from that of the host [8].

In vitro fermentation systems that model various sections of the human gastrointestinal (GI) tract, on the other hand, allow for the investigation of microbial communities with precise control of various physiological parameters, such as nutrient availability and pH levels, while decoupling interference from the human host[9]. Various systems with a range of complexity have been developed and implemented in the rapidly growing field of gut microbiome research. The Simulator for Human Intestinal Microbial Environment (SHIME) system[10], which is probably the most well-known example, consists of 5-stage chemostat reactors mimicking different sections of the human GI tract. Similar systems include the TNO models[11,12] and

Robogut[13], all equipped with automatic control for physiological conditions and which can be set up as single or multi-stage reactors. While validated with several types of samples, these *in vitro* systems are large in footprint with liter-sized reactors, thereby limiting multiplex capabilities[9,10] and experimental throughput, as the reactors typically require a few weeks for microbiota stabilization [9,14]. The footprint also means that labs using these systems need to be well-equipped with dedicated spaces for running them.

Several teams have taken another approach, attempting to miniaturize these bioreactors, notably the Mini Bioreactor Arrays (MBRA)[15,16] and the Mipro[17] systems. The MBRA can run 24 different experiments in parallel with a working volume of 15 ml and has been used to study the effect of various emulsifiers on gut microbial diversity and composition[18]. However, this system still relies on an anaerobic chamber for anoxic conditions and temperature control. In addition, it lacks pH control and relies on expensive multi-channel pumps and multi-point stir plates for fluidic transfer and mixing. In contrast, Mipro operates in batch mode and relies on manual sampling and refilling of bacterial media. Excelling at multiplexing (96 different experiments can run in parallel), this system is more suitable for quick and large-scale initial screening within 24 – 48 hours instead of time series experiments. However, Mipro isn't equipped with a mixing system and similar to the MBRA, it requires an anaerobic chamber for anoxic conditions[17].

Considering the above, there is a need for *in vitro* systems that fill the gap between existing systems in terms of footprint, physiological parameters controlled, stabilization time prior to experimental time, cost, and circumventing the need for an anaerobic chamber. Here, we present the Mini Colon Model (MiCoMo), a low-cost, miniaturized multi-bioreactor system that simulates the human colon with the capacity to change culture conditions to match physiological conditions or specific experimental needs. Consisting of triplicate 30-ml working volume reactors, MiCoMo allows for automatic and user-adjustable control of physiological conditions such as pH, temperature, anoxia, and media feeding schedule. The system has a small footprint thanks to the small working volume and operates independent of an anaerobic chamber. Fabricated without specialized material or parts, MiCoMo uses common disposable labware that can be acquired easily. The cost of it thus can be limited to a fraction of the

currently available systems. We validated MiCoMo's performance by investigating the growth of strict anaerobes, before monitoring the development and stabilization of microbial communities obtained from fecal samples of several healthy unrelated volunteers. We find that MiCoMo allowed for fast stabilization of complex microbial communities (< 5 days), while sustaining microbial diversity from individual donors over the course of 14 day-experiments. Given the low cost and ease of operation, we believe MiCoMo is a suitable and accessible tool to conduct individualized human gut microbiota studies.

Methods

Media Preparation

Modified Gifu Anaerobic Medium (mGAM) (Hyserve, Germany) was chosen as the media for MiCoMo according to previously published studies[19]. The medium was prepared by dissolving 41.7 g powder in 1 L distilled water and sterilized by autoclaving at 121 °C for 30 min. 0.01% Antifoam 204 (Sigma, Canada)[20] was added to the media to minimize foam formation during the experiment.

Validation with growth of strict anaerobes

For inoculating strict anaerobes, mGAM was pre-reduced in an anaerobic chamber (COY laboratory, functioning with 5%H_2, 20% CO_2, and balance N_2) 24 h before usage. *Clostridium beijerinckii* (ATCC 51743) and *Bacteroides fragilis* (32-6-I 11 MRS AN) were each seeded in 5 mL pre-reduced media and left overnight at 37 °C in an anaerobic chamber. Three ml of overnight culture of each bacterium was then inoculated in individual MiCoMo reactors with 27 mL media supplemented with 0.4 g/L L-cysteine. In addition, 1ml of overnight culture was serially diluted and seeded on pre-reduced mGAM agar plates. Colony counting was performed 48 h after incubation of agar plates in an anaerobic chamber at 37 °C. OD600 vs. $CFU{\cdot}mL^{-1}$ curves were generated for each bacterial isolate by measuring the OD of samples of known concentrations obtained from colony counting. The final seeding density in MiCoMo was $1.76x10^8$ $CFU{\cdot}mL^{-1}$ for *B. fragilis* and $1.10x10^6$ $CFU{\cdot}mL^{-1}$ for *C. beijerinckii.* One sample was

taken immediately after inoculation for OD600 measurement at time 0 (T0). At specific time points, output pumps were manually turned on to collect 0.1 mL of sample, and the OD600 of samples were measured with a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, USA). The collection schedule was every hour until T7 for *B. fragilis* and every two hours until T8 for *C. beijerinckii*, then at T24 and T48 for both isolates. In these experiments, the MiCoMo automatic pH and fluidic adjustments were disabled, and 3 ml of fresh media were added every 24 h to compensate for evaporation.

Fecal sample collection and preparation

This study was conducted following McGill University's approved ethics protocol A04M2715B. Fecal samples from four anonymized healthy unrelated volunteers without history of antibiotic usage within 6 months prior to participation were collected, weighed, and aliquoted in sterile 50 mL Falcon tubes within 15 minutes of collection. The aliquoted samples were then stored in -80 °C until use.

Prior to inoculation, fecal samples were resuspended in phosphate buffered saline pre-reduced with 4 g/L L-cysteine at 20% w/v concentration (rPBS, with pH = 7). The fecal slurry was centrifuged at 200g for 3 minutes to remove large cellular debris. Three ml of supernatant were then inoculated in individual MiCoMo reactors with 27 mL L-cysteine-supplemented media (see below), resulting in a final fecal sample concentration of 2% w/v, for each sample.

MiCoMo operation and sampling

The day prior to fecal inoculation, MiCoMo reactors were sterilized by incubating in 70% ethanol for 1 h. The system was then assembled in a biosafety cabinet and 10% bleach was run through all the piping connections and reactors for 30 min. Sterile MilliQ water was then flushed in the system for 5 min. pH probes were sterilized by soaking in 10% bleach for 1 h, followed by rinsing with sterile MilliQ water.

Upon fecal inoculation, 1 mL of 40 $g \cdot L^{-1}$ L-cysteine (C1276-50G, SIGMA) solution was added to 99 ml of sterile mGAM with antifoam to enhance the oxygen scavenging and establishment of anoxia. This additional L-cysteine supplementation was only added to media for initial

inoculum and not applied to media feed during the rest of experiment. The supplemented mGAM medium was then incubated in a water bath at 100 °C for 30 min to remove dissolved oxygen, and the container overhead was flushed with sterile nitrogen gas before being left to cool down to room temperature. Anoxic media was then added into individual reactors in a water bath maintained at 37 °C. The nitrogen flushing and pH control system were subsequently initiated. The system was adjusted to a pH setpoint and maintained for at least 30 min prior to the inoculation with fecal matter via the seeding port of the reactors. For all experiments with fecal samples, the pH was maintained at 6.7 with +/- 0.1 tolerance.

For practical experimental purposes, we started 4-hour feed cycles immediately after inoculation. At the end of each cycle, 4 mL (13%) of reactor content was removed from each reactor and 4.5 ml of fresh media was added. The excess media was necessary to compensate for liquid loss due to evaporation (which resulted in ~3ml loss per day). Once every 24 h, 4 mL of removed content (one cycle) was collected for each reactor and immediately centrifuged at 14,000g to precipitate the bacteria. The supernatant and pellets were then stored at -80 °C. In each experiment, the feed cycles were maintained for 14 days.

DNA extraction and sequencing

DNA in fecal samples and from the daily reactor samples were extracted with QIAamp PowerFecal Pro DNA Kit (51804, Qiagen, Germany) and DNeasy UltraClean Microbial Kit (12224-50, Qiagen, Germany), respectively. DNA extraction and purification followed the supplier's protocols. DNA amplification and amplicon library preparation/sequencing were performed by the UQAM genomics platform (CERMO-FC genomic platform, Department of biological sciences, Université du Québec à Montréal). Briefly, extracted DNA samples were amplified with primer pairs specific to the V4-V5 region of bacterial 16S rRNA (515F/926R) and sequenced with Miseq V3 kit. The forward and reverse primer sequences were: 5' – GTGYCAGCMGCCGCGGTAA – 3' and 5' - CCGYCAATTYMTTTRAGTTT - 3', respectively[21]. Sequencing reactions were performed on a Miseq using MiSeq reagent kit v3 (600-cycles; Illumina). The reads were 2 × 300 bp with an average depth of ~30,000 reads per sample[19]. Adapters were trimmed after sequencing and raw reads were demultiplexed with

Local Run Manager.

Sequencing data analysis

FASTQ data of paired end sequences were denoised using the DADA2 plugin of QIIME2, version 2020.11[22]. All sequences were trimmed at 25 bp for both forward and reverse reads to remove primer pairs and further truncated at 260 bp for forward reads and 230 bp for reverse reads. All other settings of DADA2 remained as default. Amplicon sequencing variants (ASVs) were further analyzed with diversity plugins of QIIME2 with a sampling depth of 4,100 to include all samples while ensuring taxonomic recovery and recapturing sample diversity patterns[23]. Taxonomy was assigned to the ASVs by a Naïve-Bayes classifier pre-trained with SILVA rRNA database[24] using only regions specified by the primer used in these experiments. Alpha and beta diversity analyses and PERMANOVA analyzes were done with the *diversity* plugin of QIIME 2. Graphs were plotted with either QIIME 2 *emperor* plugin or GraphPad Prism 9. Statistical tests were conducted with GraphPad Prism 9.

Results

Design, fabrication and components of MiCoMo

A schematic and images of MiCoMo are shown in Figure 1. MiCoMo consists of 3 single stage reactors with 55 mL capacity and 30 mL working volume. Each individual reactor is equipped with acid/base adjustment and fluidic transfer tubing with Luer-lock connectors, as well as a gas sparging line for N_2 flushing to keep reactors anoxic. During the operation of MiCoMo, the anaerobic reactors, maintained at 37 °C in a water bath, can be contained in a biosafety cabinet to avoid any potential contamination. MiCoMo operates on a 4-hour feed cycle for all experiments in this study. The feed cycle leads to an overall reactor turnover time of 30 hours.

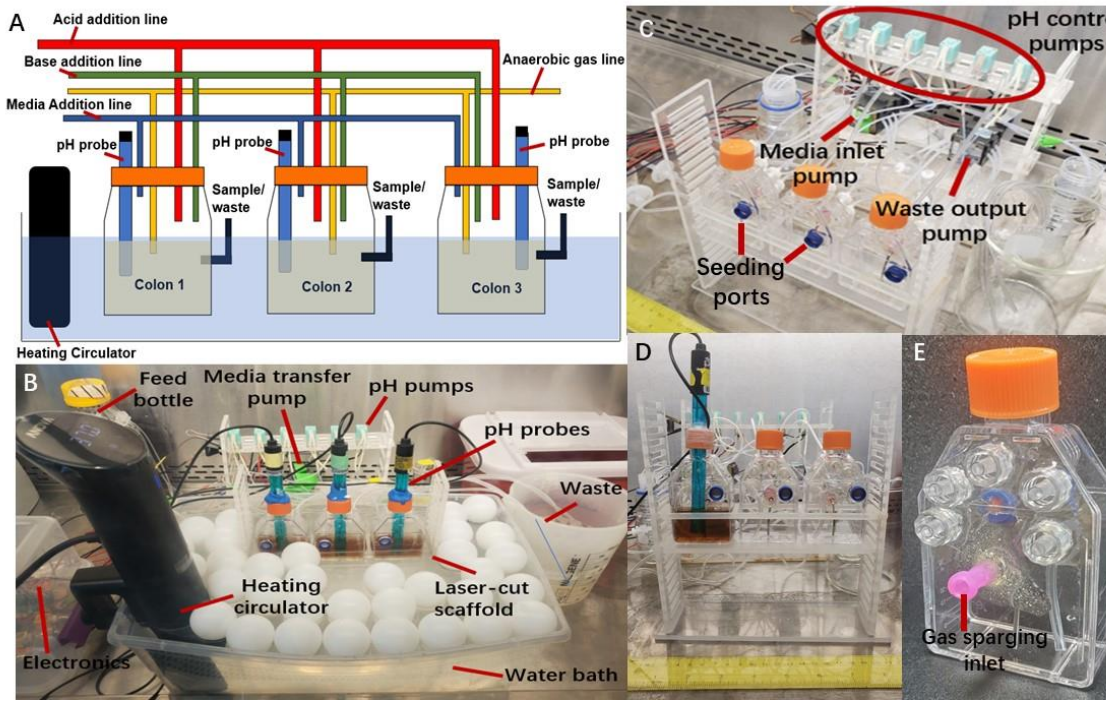


Figure 1: A) Schematic and B) operating photograph of MiCoMo showing major components.
The reactors are kept in water bath during operation with ping-pong balls to minimize evaporation. The whole device is kept in BSC to minimize risk of contamination. C) Major pumps and tubing connection of MiCoMo. Two multi-channel pumps transfer media in and remove waste from all reactors, while two single channel pump control acid and base addition for each reactor through connection ports at the back of reactors. At beginning of each experiment fecal slurry is seeded into each reactor manually through seeding port at the front of reactor.) Front view of MiCoMo, with one reactor with media and pH probe. E) Picture of individual MiCoMo reactor. Luer-lock ports connect media inlet, water removal, gas vent, gas sparge inlet and acid/base addition, respectively.

Validation of MiCoMo operations

We first validated MiCoMo's maintenance of anoxic condition with strict anaerobic bacterial isolates. We maintained pre-reduced PBS with 1 $mg{\cdot}L^{-1}$ resazurin in MiCoMo for 24 hours and confirmed no color change. Next, we grew the following strict anaerobes in the system: *Clostridium beijerinckii* (Gram +) and *Bacteroides fragilis* (Gram -). We observed the

expansion of both strict anaerobes in MiCoMo reactors operating in batch mode. Within 48 hours' experimental time, *B. fragilis* grew from $1.76\pm0.61\times10^{8}$ CFU·mL^{-1} to $4.32\pm2.41\times10^{9}$ CFU·mL^{-1} and *C. beijerinckii* grew from $1.10\pm0.55\times10^{6}$ CFU·mL^{-1} to $1.49\pm0.15\times10^{7}$ CFU·mL^{-1} (Figure 2A).

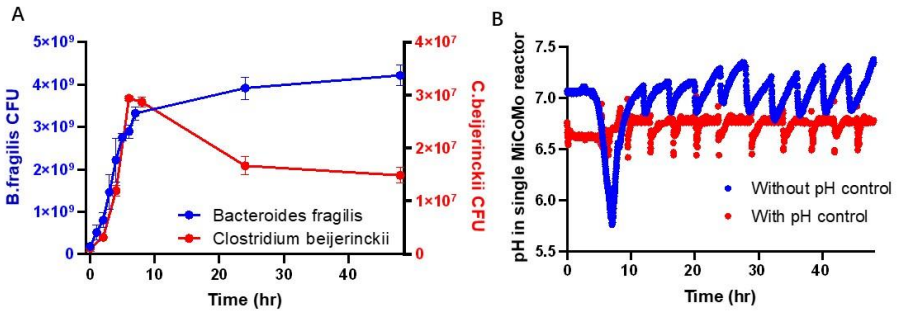


Figure 2: Validation of MiCoMo control system

A) Growth curves of strict anaerobes in MiCoMo operating in batch mode B) Log of pH in MiCoMo seeded with fecal sample operating on automatic feeding cycle, with and without active pH control

Next, we evaluated the effect of pH control on MiCoMo community dynamics by seeding MiCoMo with fecal samples from one volunteer and compared pH dynamics in the reactors with and without pH control (three technical repeats for each condition). We found that without pH control, the pH of MiCoMo quickly reduced from 7.0 to ~5.7 within the first 8 hours (2 feed cycles). The pH then increased back to ~7.0 within the next 4-5 hours, suggesting a swift growth of bacteria depleting the initially available nutrients. Interestingly, MiCoMo underwent cyclic pH fluctuations corresponding to the feed cycles from that point on. In contrast, with pH control, the pH of MiCoMo was kept at the setpoint pH of 6.7 with $\pm$ 0.1 tolerance (values automatically adjusted within 10 s); (Figure 2B)

Stabilization of complex microbial communities derived from fecal samples

We inoculated MiCoMo with fecal samples from healthy volunteers to determine whether MiCoMo can sustain the growth of complex microbial communities. Four unrelated healthy volunteers were included in this study. A fifth sample was created by pooling fecal matter from two individuals at 1:1 weight ratio to explore the effects of pooling donor samples, a common approach for inoculating germ-free mice[8,25,26].

We adopted a previously published approach[27] for examining the stability of microbial cultures[15] based on a beta diversity metric, the Bray-Curtis similarity (1 – Bray-Curtis distance). We computed the Bray-Curtis similarity of each daily sample with the average of all other daily samples, termed averaged similarity thereafter, to gauge the long-term community stabilization (Figure 3A). Furthermore, we also evaluated the daily Bray-Curtis similarity between consecutive days, termed daily similarity thereafter, (Figure 3B), and computed its daily rate of change by using a 3-point moving window slope to evaluate short-term community dynamics (Supplementary Figure S4)

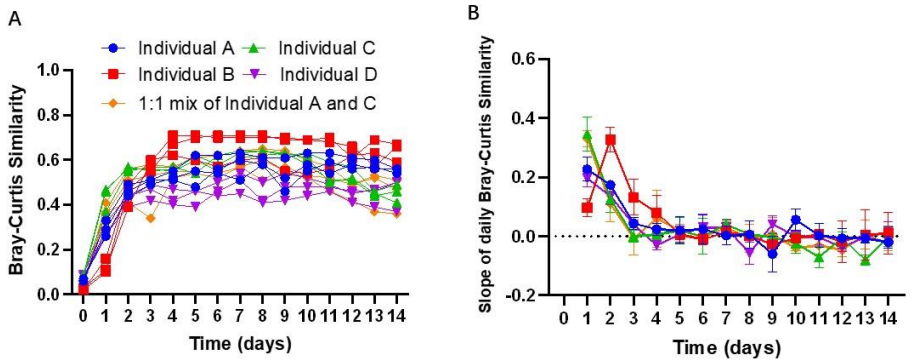


Figure 3: Stabilization of microbial communities derived from fecal samples in MiCoMo.

A) Average Bray-Curtis similarity between each daily reactor sample and samples of all other days in that reactor. Day 0 indicates the original fecal sample. Each line indicates an individual reactor. B) Rate of change of Bray-Curtis similarity between consecutive days,

evaluated by 3-point moving window average. Each line is average across 3 technical repeats of the same individual donor, error bars indicate standard deviations.

For all volunteers, except individual B, we found that microbial communities changed rapidly over the first 24 hours of inoculation, with averaged similarity quickly increasing from <0.1 to around 0.5. The rate of change of community structure quickly decreased after this initial transition period, but the overall community underwent another 24 h of transition before reaching a relatively stable state: we observed a rate of change of 0.28 ± 0.07 during the 24-h transition period (Day 1) and 0.14 ± 0.03 within the next 24 hours (Day 2). The community then transitioned to a stable state with rates of change less than ± 0.025. During this period the averaged similarity reached a plateau between 0.45 to 0.55, depending on the individual, and a mean daily similarity of 0.80 ± 0.09 (all volunteers average, except individual B).

Meanwhile, the fecal inoculum from individual B showed different stabilization patterns: the major transition period for individual B occurred on Day 2 instead of Day 1, with a rate of change of 0.33 ± 0.04 and 0.10 ± 0.03, respectively, while the averaged similarity showed similar behavior as the other individuals. Further, the microbial community from individual B took additional time to reach stability with slopes of 0.13 ± 0.01 and 0.08 ± 0.06 on Day 3 and 4, respectively. Starting from Day 5, this community entered a stabilized state similar to that from the other volunteers, indicated by the plateauing of averaged similarity and characterized by a high daily similarity of 0.87 ± 0.08 with minimal fluctuations for the rest of the 14-day period. Interestingly, following this longer transition period, the microbial community developed into a stable state with similar day-to-day variations, but overall higher averaged similarity, around 0.6, relative to the other individuals.

After analyzing the temporal stability of MiCoMo inoculated with complex microbial communities, we then compared the replicate reactors within each MiCoMo run from the same original donor to gauge the consistency of these technical replicates. We found that MiCoMo can maintain highly consistent technical replicates: the between-replicate similarity ranged from 0.41 to 0.9, with an average of 0.72 ± 0.13 and a median of 0.74. We found that the mixed fecal matter inoculum has a lower between-replicate similarity (0.63 ± 0.12) comparing to the

other donors (0.74 ± 0.13), although this difference is not statistically significant ($p>0.05$, unpaired T-test). This could be due to the development of distinctive stochastic community structures in the replicate reactors.

Diversity dynamics and structure of microbial communities in MiCoMo

Having established that MiCoMo can lead to stabilized microbial communities in 3 - 5 days, we examined the alpha diversity and structure of these communities to evaluate how representative they are relative to the original fecal inocula.

Here, we adopted one commonly applied metric for alpha diversity, the Shannon index. We found that fecal samples included in this study have a Shannon index between 4.62 to 5.73, and that the transfer and growth of fecal samples in MiCoMo lead to a slight decrease in Shannon index for all individuals. This decrease was most significant during the first 24 hours, with the average Shannon index decreasing from 5.21 ± 0.43 to 3.13 ± 0.60. During the rest of the experimental period, the Shannon index in MiCoMo gradually decreased for a few additional days before stabilizing at 3.17 ± 0.65 (average of all volunteers, Day 5 – 14). As expected, these transition patterns mirrored the similarity patterns discussed above. We also calculated the amount of observed ASVs within each MiCoMo sample (Figure 4B). We found the observed ASVs follow a similar trend as alpha diversity, and that stabilized ASV counts are typically ~50% of that observed from the original fecal sample.

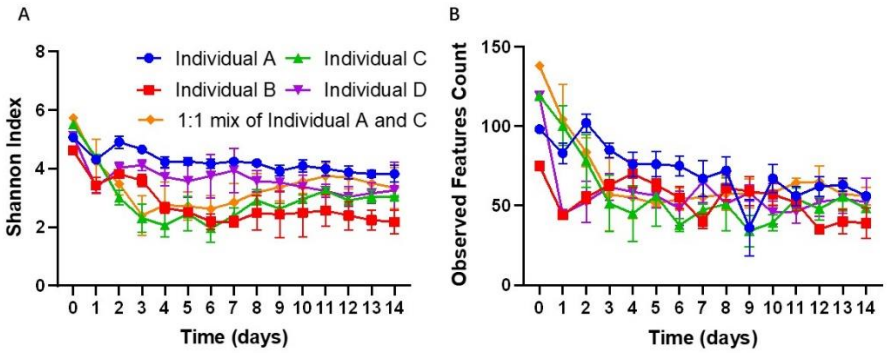


Figure 4: Alpha diversity of microbial communities derived from fecal samples in

MiCoMo.

A) Alpha diversity measured by Shannon Index. B) Observed features (ASVs) count. Each line is average across 3 technical repeats of the same individual donor, error bars indicate standard deviations.

Next, we evaluated differences between MiCoMo communities and their respective original fecal sample. We computed the distance matrix between MiCoMo samples from different volunteers for two metrics, the Bray-Curtis similarity and Jaccard similarity (1 – Jaccard distance), which accounts only for the presence/absence of members within a community, as opposed to Bray-Curtis which also considers evenness. Acknowledging the fact that MiCoMo culture led to the loss of some bacterial taxa and an inevitable transition of microbial community structure, we were interested in whether communities from different individuals would develop and significantly cluster away from each other. To this end, we applied a permutational multivariate analysis of variance (PERMANOVA)[28] on MiCoMo samples from each volunteer and analyzed the distance matrices by principal component analysis (Figure 5A, B). There is a clustering of the original fecal samples distinct from the grown MiCoMo communities, indicating the transition of microbial communities in MiCoMo, yet the cultured samples were non-overlapping and could be easily traced back to their corresponding original donor.

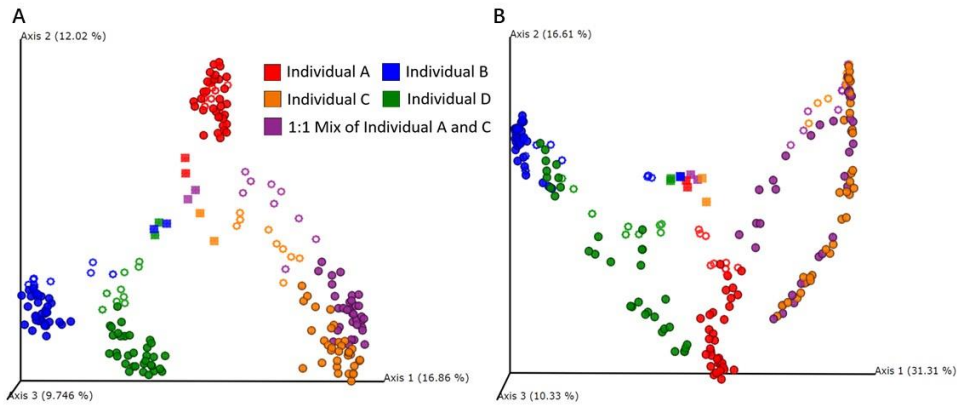


Figure 5: Principal component analysis on diversity and structure of microbial

communities derived from fecal samples in MiCoMo by A) Jaccard Distance B) Bray-Curtis Distance.

Color: samples from individual donors. Square: original fecal sample. Rings: samples from individual replicate reactors in Day 1 - 3. Circles: samples from individual replicate reactors in Day 4-14.

This was confirmed with a PERMANOVA analysis (Supplementary Table 1). Between each pair of communities from independent volunteers, PERMANOVA resulted in a pseudo-F test score of >30 and a p-value of less than 0.001 after 999 permutations. When comparing between the mixed community obtained from a mixed inoculum (individuals A + C) to the respective inocula, the pseudo-F score was lower, especially for individual C, which was in line with the PCoA plots.

Last, we assigned taxonomy to MiCoMo-developed communities to explore the compositional dynamics over 14 days. We analyzed the overall community taxonomy at the family level and investigated the composition of individual genera within each phylum (Figure 6 and Supplementary Figure S1 – S3).

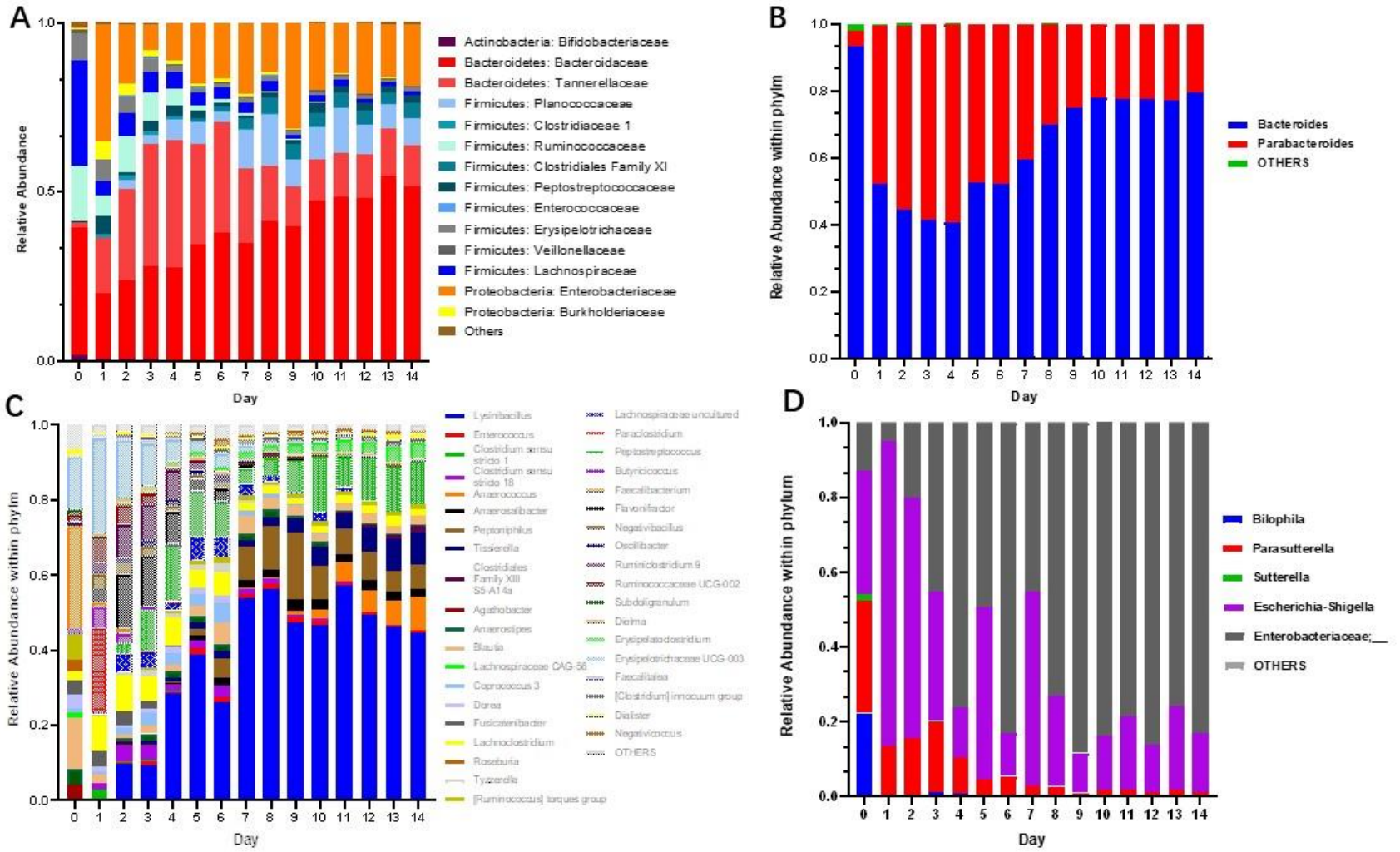
As detailed above, one important feature of MiCoMo is its ability to develop individual-specific microbial communities, leading to individual-specific temporal dynamics. Nevertheless, some general trends could be identified among the commonly found gut microbiota phyla. In all individuals, we observed an expansion of Bacteroidetes from 0.31% - 37.6% in the inocula to 30.0% - 67.2% in stabilized complex cultures. This expansion was largely contributed by *Bacteroidaceae* and *Tannerellaceae* families, most notably bacteria from the *Bacteroides* and *Parabacteroides* genera, both common members of the human gut microbiota[29,30]. This increase was accompanied by an overall loss of Firmicutes, especially those of Clostridiales order, which exhibited ~5-fold decrease in abundance for multiple volunteers. These decreases were most significant among the families of *Lachnospiraceae* and *Ruminococcaceae*. Notably, *Faecalibacterium prausnitzii*, a common commensal gut microbiota[31], which consisted of 15% - 30% of abundance in the original fecal samples in this study, did not manage to maintain a niche in MiCoMo.

The phylum of Actinobacteria, although a common member of human gut microbiota, is usually not found in high prevalence[32]. Indeed, for most volunteers, we found Actinobacteria consisting less than 2% of ASVs, with the exception of individual B with 20% of ASVs assigned to Actinobacteria, the majority of which belong to the *Bifidobacteriaceae* family. This family was generally not supported by MiCoMo, and gradually decreased in abundance over the first few days of culture.

We did not observe any sustained expansion of facultative aerobic bacterial taxa belonging to the Proteobacteria phylum in MiCoMo, which remained less than 10% of the overall community; except for individual A, where Proteobacteria consisted of ~20% of community once stabilized. Indeed, we observed a short expansion of Proteobacteria during the first few days of culture, whereby they could occasionally make up as much as 20 – 25% of the ASVs. Such expansion was however typically suppressed after 3-4 days of culture, and the relative abundances of Proteobacteria were maintained at low levels after this stabilization period.

Figure 6: Representative dynamics of bacterial taxonomy in MiCoMo over 14 days culture for Individual A.

Day 0 indicates original fecal sample, Day 1 – 14 indicate cultured samples from MiCoMo, averaged over 3 technical replicates. ASVs that didn't account for at least 1% of total abundance in any day were grouped in Others. A) Family-level taxonomy of the whole community B) Genus-level taxonomy within the Bacteroides phylum C) Genus-level taxonomy within the Firmicutes phylum D) Genus-level taxonomy within the Proteobacteria phylum


A
Relative Abundance
Day
Actinobacteria: Bifidobacteriaceae
Bacteroidetes: Bacteroidaceae
Bacteroidetes: Tannerellaceae
Firmicutes: Planococcaceae
Firmicutes: Clostridiaceae 1
Firmicutes: Ruminococcaceae
Firmicutes: Clostridiales Family XI
Firmicutes: Peptostreptococcaceae
Firmicutes: Enterococcaceae
Firmicutes: Erysipelotrichaceae
Firmicutes: Veillonellaceae
Firmicutes: Lachnospiraceae
Proteobacteria: Enterobacteriaceae
Proteobacteria: Burkholderiaceae
Others
B
Relative Abundance within phylm
Day
Bacteroides
Parabacteroides
OTHERS
C
Relative Abundance within phylm
Day
Lysinibacillus
Enterococcus
Clostridium sensu stricto 1
Clostridium sensu stricto 18
Anaerococcus
Anaerosalibacter
Peptoniphilus
Tissierella
Clostridiales Family XIII S5-A14a
Agathobacter
Anaerostipes
Blautia
Lachnospiraceae CAG-56
Coprococcus 3
Dorea
Fusicatenibacter
Lachnoclostridium
Roseburia
Tyzzerella
[Ruminococcus] torques group
Lachnospiraceae uncultured
Paraclostridium
Peptostreptococcus
Butyricicoccus
Faecalibacterium
Flavonifractor
Negativibacillus
Oscillibacter
Ruminiclostridium 9
Ruminococcaceae UCG-002
Subdoligranulum
Dielma
Erysipelatoclostridium
Erysipelotrichaceae UCG-003
Faecalitalea
[Clostridium] innocuum group
Dialister
Negativicoccus
OTHERS
D
Relative Abundance within phylum
Day
Bilophila
Parasutterella
Sutterella
Escherichia-Shigella
Enterobacteriaceae;__
OTHERS

Discussion

Given the increased attention to the vital role the gut microbiota plays in human health, *in vitro* systems for controlled experimental investigation have been extensively developed and implemented. Most notable among them are bioreactors for propagating and maintaining microbial communities derived directly from human fecal samples. However, despite their versatility and functionalities, one common limitation is that these systems are typically dependent on additional specialized expensive lab equipment and setup, such as anaerobic chambers or multi-channel pumps[15]. Despite the commercialization of several models, most systems' applications are limited within the lab of creation and the accessibility world-wide is usually restrained[33]. As such, one of our primary foci for MiCoMo design was to ensure the system is low-cost and can be easily established by most labs. The whole system costs ~$1,500 CAD and can be assembled by personnel with limited engineering experience with ease (A list of components and price can be found in Supplementary Information). The small working volume of reactors (30 ml) and the compact design also reduce the system footprint, allowing the whole MiCoMo to fit on a typical lab bench or within a biosafety cabinet. Compared to other small scale-systems, MiCoMo operates independently of anaerobic chambers, which are usually expensive and cumbersome to setup and maintain. Notably, MiCoMo is also equipped with pH control, which also makes it suitable for mimicking physiological conditions leading to a pH shift in the GI tract. Together, these features allow for easy replication across laboratories, as well as multiplexing capabilities by establishing multiple sets of MiCoMo systems in parallel within relatively small spaces, if desired.

Our validation experiments demonstrated that MiCoMo can maintain anoxic conditions at specific pH levels, leading to suitable growth conditions for two strict anaerobes and allowing for investigators to adjust the pH according to their own experimental needs. At a more fundamental level, the triplicate reactors of MiCoMo can be easily reconfigured to connect to each other in series instead of in parallel. Individually equipped with pH control system, these reactors could, when connected in series, mimic the human GI tract from stomach to colon by adjusting the pH setpoint and inoculating with different samples. This setup would enable the investigation of how the gut microbiota responds to perturbations along the GI tract.

When analyzing complex microbial community stability and structure, we were most interested in whether our system achieved a performance comparable to the currently available *in vitro* systems and animal models. Our observed Shannon index from fecal samples is comparable to previously published values that typically range from 4-6.5 for healthy individuals[34]. The decrease in alpha diversity and in observed ASVs for microbial communities grown in MiCoMo likely reflects a selection process by the specific growth conditions used (media, retention time, etc.) as well as the initial composition of the fecal inoculum. Notably, due to lack of incubation time (feed cycles were immediately started after inoculation), some slow-growing bacteria might have been washed off during the initial transition period before being able to adapt to the new *ex vivo* conditions. Such selection processes were commonly observed in other *in vitro* system as well[15,35]. Importantly, despite the decrease, MiCoMo-grown communities demonstrated an alpha diversity similar to that observed in previously reported *in vitro* systems after stabilization [15,34], indicating that MiCoMO was able to support growth of complex and diverse communities from a variety of fecal samples.

A big challenge for assessing stability of microbial communities in *in vitro* systems lies in the lack of a clear consensus for defining community stability and distinguishing natural variations within communities from major community shifts. Here, by adopting previously published analyzes and diversity metrics, we are able to directly compare our system to a previously validated *in vitro* system, such as the MBRA[15]. Notably, Auchtung et al. not only reported stability metrics of their *in vitro* system, but also analyzed and compared these metrics to those observed in mouse models[36]. It was reported that the six weaned mice with stable microbial communities analyzed by Auchtung et al. demonstrate a day-to-day variation in Bray-Curtis similarity (daily similarity) of 0.79 ± 0.06, and a between-replicate similarity of 0.71 ± 0.05. Meanwhile, the MBRA system had a daily similarity of 0.74 ± 0.05 and a between-replicate similarity of 0.54 ± 0.07 to 0.61 ± 0.08 during stable operations, depending on the volunteer. The MiCoMo system, with a daily similarity of 0.81 ± 0.07 during stable operation (all volunteers included; Day 5 – Day 14 for individual B and Day 3 – Day 14 for all other individuals) and a between-replicate similarity of 0.72 ± 0.13, thus exhibited similar performance (no significance difference between MiCoMo and mice, unpaired t-test with $p >$

0.5 for both categories). This demonstrates that MiCoMo is able to support stable microbial community growth, with variations comparable with an *in vivo* mouse model and a previously reported *in vitro* systems, from various fecal inocula, after a timeframe of 3-5 days.

When analyzing the principal component analysis plots, we observed that the communities developed from the pooled sample (Individuals A and C) interestingly clustered closely and almost exclusively with one of its source donors, individual C, by Jaccard distance for all three replicates; whereas this was not the case for the Bray-Curtis distance. The difference between replicate reactors from the same pooled fecal sample emphasizes the need for technical replicates. Further, this distinction hints at the importance of using the number of individual human donor samples as the statistical inference unit when conducting large-scale perturbation analysis, as suggested by Walter et al.[8], as opposed to only using the number of technical replicates (replicate mice or bioreactors with same inoculum).

Looking at the taxonomy of MiCoMo-grown microbial communities, except for a selected few known members of the gut microbiota, we limited the taxonomic assignment to the genus level, as there is extensive literature discussing the limitations of 16S rRNA sequencing with selected variable regions to reach species-level identification[37,38].

We first report an overall decrease in the relative abundance of Firmicutes, likely due to their extreme intolerance to oxygen (loss of cultivability after < 2 min of oxygen exposure for some species has been reported[39]), in addition to possible nutrient preferences. Other validated *in vitro* systems have reported similar observations, with either a decrease in abundance or a complete loss of members of this phylum[15,35]. In addition, the expansion of facultative bacterial species belonging to the Proteobacteria phylum has been observed in various *in vitro* fermentation systems[15,35], likely due to their high resilience to oxygen exposure and short doubling time[40–42]. Interestingly, we did not observe this phenomenon for most volunteers in MiCoMo after the first few days of inoculation. Rather, we observed an expansion of several known members of the gut microbiota, such as *Bacteroides uniformis* and *B. thetaiotaomicron*, both species being strict gut anaerobes[29,43,44]. These observations indicate that some of the underlying microbial interactions known to take place in the gut could also be occurring in MiCoMo, such as limiting the expansion of Proteobacteria. Importantly, MiCoMo does not

seem to select for the most adaptable and aero-tolerant species, although these may establish their niche early on during the stabilization period.

In this paper, we demonstrate that MiCoMo is able to support stable and distinct microbial communities from different volunteers, using a previously validated culture medium, as a first proof-of-functionality of MiCoMo. However, the strength of the MiCoMo system lies in its versatility: with user-customizable pH setpoint, gas sparging and feed schedules, one can easily adjust the MiCoMo environment to better accommodate individual-specific gut conditions. For instance, the pH setpoint can be decreased along with a gas sparging with increased interval in order to mimic the gut environment of IBD patients with reduced pH and increased oxygen concentration[45,46]. In order to better support a mucosal microbial communities, mucin could also be supplemented into the system, as previously done in the SHIME system[47].

Conclusions

In conclusion, we developed MiCoMo, an optimized, fully controllable miniaturized, pH-controlled, and anerobic bioreactor system for simulating the gut environment. We showed that it allows for fast stabilization of complex microbial communities (approx. 3 – 5 days), while sustaining microbial diversity from individual volunteers. We believe its small footprint, low cost, and ease of fabrication will allow for easy replication across labs studying the effect of various perturbations on individual gut microbial communities with high throughput. We expect future developments of MiCoMo to focus on two aspects: further miniaturization and increased multiplexing capacity along with compartmentalization of the human GI tract. The size of the pH probes currently dictates the size of the bioreactors, and can be replaced by miniaturized pH probes (more expensive) or probes with minimal footprint and low cost[48]. Then, due to its inherent modular nature, MiCoMo can be modified to integrate the biologic compartmentalization of the human GI tract by introducing a small intestine chamber inoculated with small intestine microbiota samples, and/or a stomach chamber for mimicking food digestion. These modifications are also compatible with increased multiplexing capacity. Finally, future development might make it possible to optionally incorporate host cells into MiCoMo, thereby incorporating back host-microbiota interactions while controlling

environmental parameters.

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Supplementary Information

Supplementary Tables

Table S1 PERMANOVA analysis between each pair of MiCoMo-grown communities from independent volunteers for Jaccard and Bray-Curtis indices

Bray-Curtis						
Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
A	B	88	999	84.62	0.001	0.0011
A	C	88	999	60.86	0.001	0.0011
A	D	88	999	44.27	0.001	0.0011
A	Mixed	88	999	44.67	0.001	0.0011
B	C	88	999	111.32	0.001	0.0011
B	D	88	999	37.12	0.001	0.0011
B	Mixed	88	999	87.10	0.001	0.0011
C	D	88	999	61.25	0.001	0.0011
C	Mixed	88	999	4.24	0.007	0.0070
D	Mixed	88	999	46.98	0.001	0.0011
Jaccard						
Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
A	B	88	999	44.32	0.001	0.0010
A	C	88	999	34.62	0.001	0.0010
A	D	88	999	40.94	0.001	0.0010
A	Mixed	88	999	28.78	0.001	0.0010
B	C	88	999	38.15	0.001	0.0010
B	D	88	999	34.29	0.001	0.0010
B	Mixed	88	999	43.00	0.001	0.0010
C	D	88	999	32.11	0.001	0.0010
C	Mixed	88	999	7.89	0.001	0.0010
D	Mixed	88	999	33.63	0.001	0.0010

Table S2 MiCoMo components list

Category	Item name	Catalogue number	Supplier	# of items for 1 set of 3 reactors	Unit price (USD)
Water bath	Anova Sous vide	AN400-US00	Amazon	1	$120
Water bath	plastic box		Dollarama	1	$2
Water bath	ping-pong balls		Dollarama	1	/
Pumps (fluidics)	6-Channel Pump	RP-6R01S-3P6A-DC10VS	Takasago	2 (1 could also work but adds uncertainty)	$234.92 (from 26180 JPY)
Pumps (pH)	RP-Q Series (0.2ml/min)	RP-Q1.2N-P20Z-DC3V pump	Takasago	6	$54.56 (from 6080 JPY)
Solenoid Valves (gas sparging)	Diaphragm Valve - KV series	KV-2-NCG	Takasago	3	$60.03 (from 6690 JPY)
Scaffold (Stand)	Clear Scratch- and UV-Resistant Cast Acrylic Sheet, 6" x 12" x 3/16"	8560K163	Mcmaster Carr	6"X12"	$6.76
Scaffold (base)	Clear Scratch- and UV-Resistant Cast Acrylic Sheet, 6" x 12" x 1/4"	8560K163	Mcmaster Carr	6"X12"	$12.35
Tubing (fluidic)	High-Temperature Abrasion-Resistant Soft Silicone Tubing for Food, Beverage and Dairy, 1 mm ID, 2 mm OD	3184K284	Mcmaster Carr	~10 ft for 1 assembly	$0.35
Tubing (pH)	Extreme-Temperature Teflon® PTFE Semi-Clear Tubing for Chemicals, 1/32" ID, 1/16" OD	5239K23	Mcmaster Carr	~5ft for 1 assembly	$1
Tubing (gas and feed bottle) (also used in reactor assembly)	Clear Masterkleer Soft PVC Plastic Tubing for Air and Water, 1/16" ID, 1/8" OD	5233K51	Mcmaster Carr	~10ft for 1 assembly	$0.2
Tubing (connectors)	Plastic Quick-Turn Tube Coupling, Plugs, for 1/16" Barbed Tube ID, Polycarbonate	51525K271	Mcmaster Carr	~20	$0.52
Tubing (connectors) (Included reactor connectors)	Plastic Quick-Turn Tube Coupling, Sockets, for 1/16" Barbed Tube ID, Polycarbonate	51525K281	Mcmaster Carr	~20	$0.46
Reactor (pH probe)	ASP200-2-1M-BNC pH Lab Electrode	3550_0	Phidget	3	$25
Reactor (body)	Corning 430639 cell culture flask, 25 cm2, canted neck, vented cap, sterile	UZ-01936-02	Corning	3	$3
Reactor (gas needle)	Stainless Steel Dispensing Needle with Luer Lock Connection, 1-1/2" Needle Length, 16 Gauge	75165A753	Mcmaster Carr	3	$0.3
Reactor (seeding port)	PK100 BLUE CAP,9MM, BONDED PRE-SLIT	29320-U	Superlco	3	$0.5
Electronics (Microcontroller)	ARD-MEGA Arduino Mega2560 Compatible Development Board	ARD-MEGA	Abra Electronics	2	$28
Electronics (pH module)	pH/ORP Adapter	1130_0B	Phidget	3	$30

Electronics (jumper wires)	TUOFENG 22 awg Solid Wire-Solid Wire Kit-6 Different Colored 30 Feet/9M spools 22 Gauge Jumper Wire-Hook up Wire Kit	/	Amazon	1 set is enough for like forever	$20
Electronics (transistors)	TIP120 Transistor Darlington Power NPN 5A 60V	TIP120	Abra Electronics	11	$0.7
Electronics (terminal blocks)	2492P Screw Terminal Block: 3-Pin, 0.1″ Pitch, Side Entry (3-Pack)	2492P	Abra Electronics	11	$1.2
Others (glue)	Norland Optical Adhesive 81	NOA81	Norland Adhesives		

Supplementary Figures

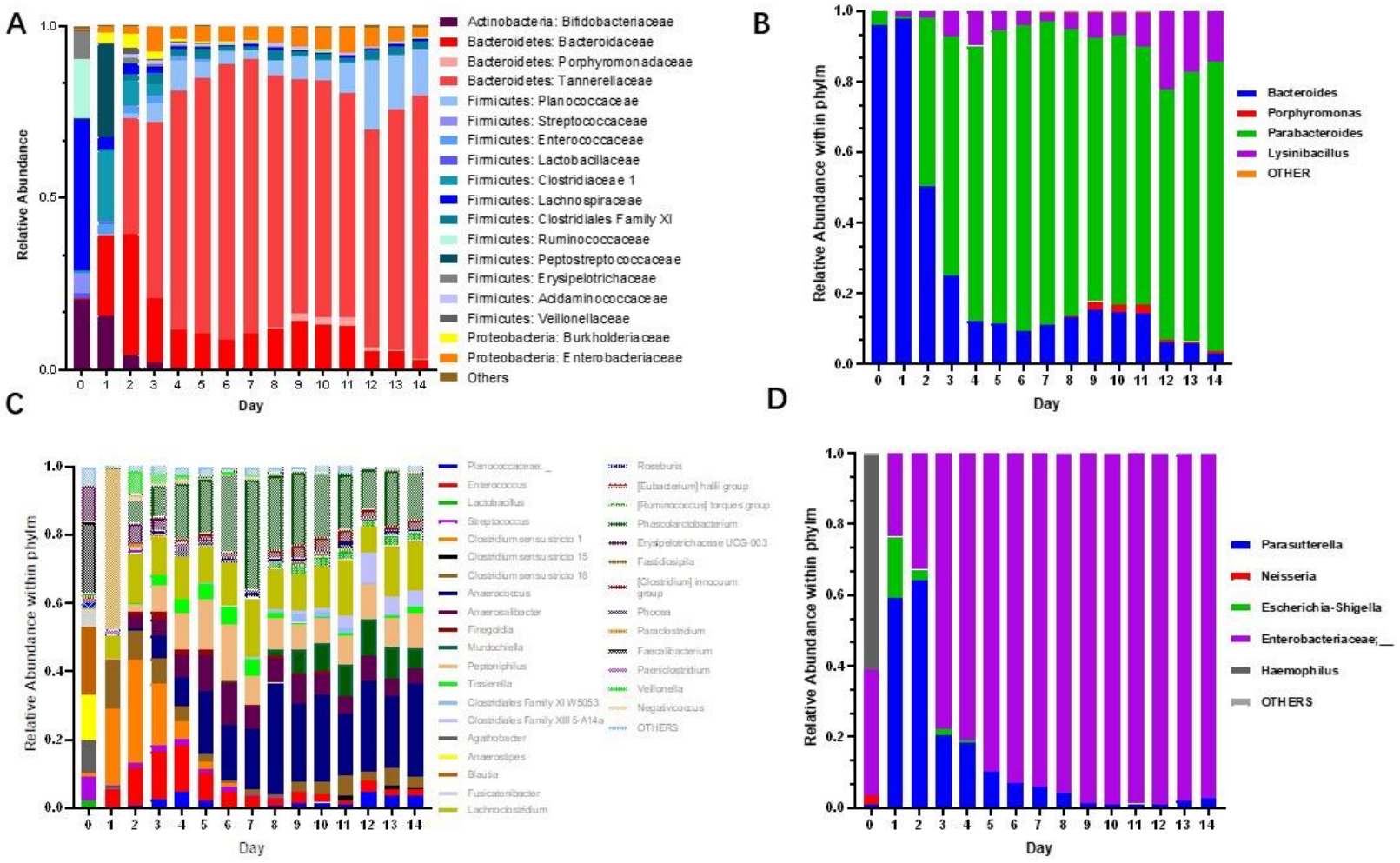
A
Relative Abundance
Day
Actinobacteria: Bifidobacteriaceae
Bacteroidetes: Bacteroidaceae
Bacteroidetes: Porphyromonadaceae
Bacteroidetes: Tannerellaceae
Firmicutes: Planococcaceae
Firmicutes: Streptococcaceae
Firmicutes: Enterococcaceae
Firmicutes: Lactobacillaceae
Firmicutes: Clostridiaceae 1
Firmicutes: Lachnospiraceae
Firmicutes: Clostridiales Family XI
Firmicutes: Ruminococcaceae
Firmicutes: Peptostreptococcaceae
Firmicutes: Erysipelotrichaceae
Firmicutes: Acidaminococcaceae
Firmicutes: Veillonellaceae
Proteobacteria: Burkholderiaceae
Proteobacteria: Enterobacteriaceae
Others
B
Relative Abundance within phylm
Day
Bacteroides
Porphyromonas
Parabacteroides
Lysinibacillus
OTHER
C
Relative Abundance within phylm
Day
Planococcaceae; _
Enterococcus
Lactobacillus
Streptococcus
Clostridium sensu stricto 1
Clostridium sensu stricto 15
Clostridium sensu stricto 18
Anaerococcus
Anaerosalibacter
Finegoldia
Murdochiella
Peptoniphilus
Tissierella
Clostridiales Family XI W5053
Clostridiales Family XIII 5-A14a
Agathobacter
Anaerostipes
Blautia
Fusicatenibacter
Lachnoclostridium
Roseburia
[Eubacterium] hallii group
[Ruminococcus] torques group
Phascolarctobacterium
Erysipelotrichaceae UCG-003
Fastidiosipila
[Clostridium] innocuum group
Phocea
Paraclostridium
Faecalibacterium
Paeniclostridium
Veillonella
Negativicoccus
OTHERS
D
Relative Abundance within phylm
Day
Parasutterella
Neisseria
Escherichia-Shigella
Enterobacteriaceae;__
Haemophilus
OTHERS

Figure S1: Dynamics of bacterial taxonomy in MiCoMo over 14 days culture for Individual B.

Day 0 indicates original fecal sample, Day 1 – 14 indicate cultured samples from MiCoMo, averaged over 3 technical replicates. ASVs that didn't account for at least 1% of total abundance in any day were grouped in Others. A) Family-level taxonomy of the whole community B) Genus-level taxonomy within the Bacteroides phylum C) Genus-level taxonomy within the Firmicutes phylum D) Genus-level taxonomy within the Proteobacteria phylum

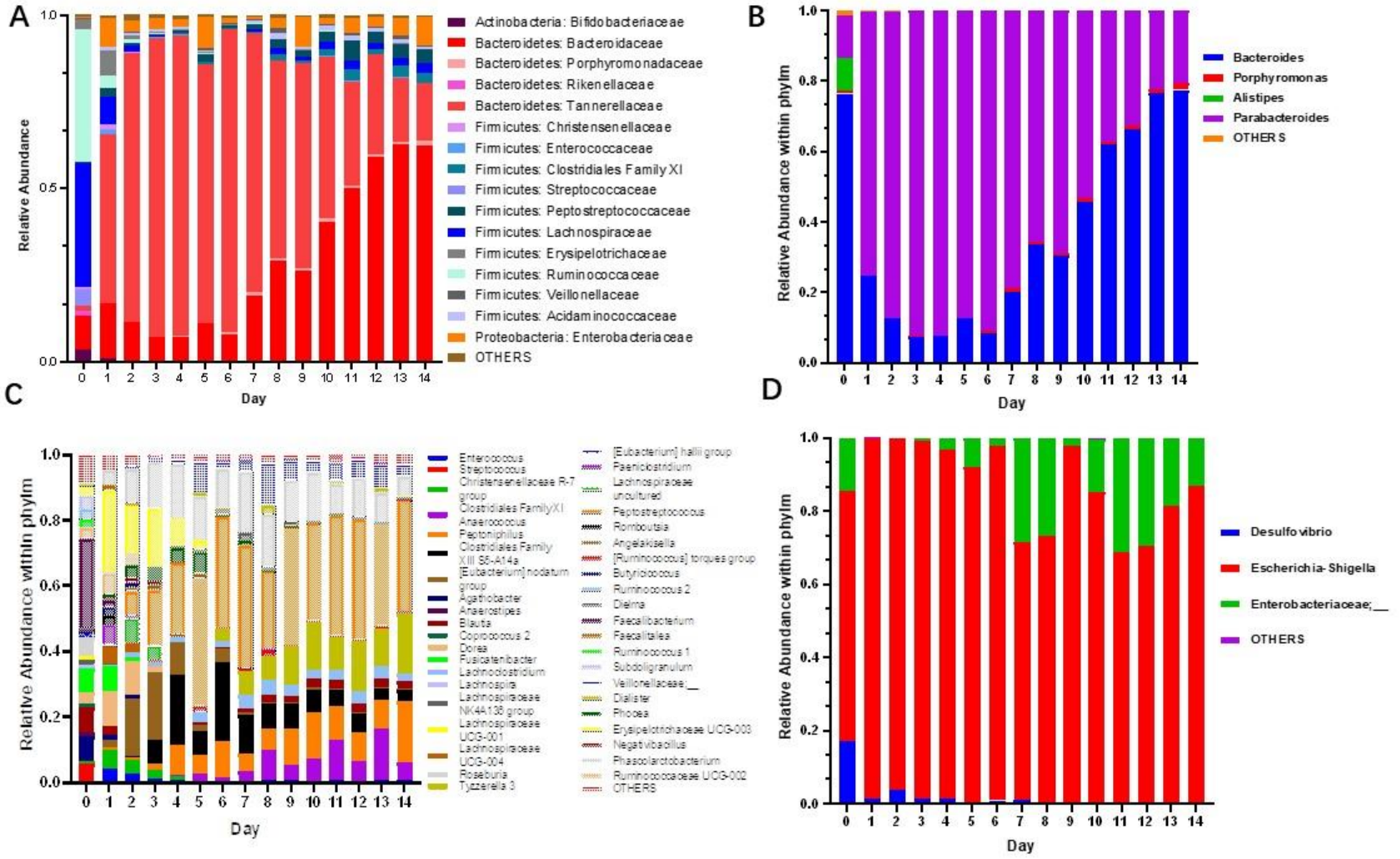
A
Relative Abundance
Day
Actinobacteria: Bifidobacteriaceae
Bacteroidetes: Bacteroidaceae
Bacteroidetes: Porphyromonadaceae
Bacteroidetes: Rikenellaceae
Bacteroidetes: Tannerellaceae
Firmicutes: Christensenellaceae
Firmicutes: Enterococcaceae
Firmicutes: Clostridiales Family XI
Firmicutes: Streptococcaceae
Firmicutes: Peptostreptococcaceae
Firmicutes: Lachnospiraceae
Firmicutes: Erysipelotrichaceae
Firmicutes: Ruminococcaceae
Firmicutes: Veillonellaceae
Firmicutes: Acidaminococcaceae
Proteobacteria: Enterobacteriaceae
OTHERS
B
Relative Abundance within phylm
Day
Bacteroides
Porphyromonas
Alistipes
Parabacteroides
OTHERS
C
Relative Abundance within phylm
Day
Enterococcus
Streptococcus
Christensenellaceae R-7 group
Clostridiales FamilyXI
Anaerococcus
Peptoniphilus
Clostridiales Family XIII S5-A14a
[Eubacterium] nodatum group
Agathobacter
Anaerostipes
Blautia
Coprococcus 2
Dorea
Fusicatenibacter
Lachnoclostridium
Lachnospira
Lachnospiraceae NK4A136 group
Lachnospiraceae UCG-001
Lachnospiraceae UCG-004
Roseburia
Tyzzerella 3
[Eubacterium] hallii group
Paeniclostridium
Lachnospiraceae uncultured
Peptostreptococcus
Romboutsia
Angelakisella
[Ruminococcus] torques group
Butyricicoccus
Ruminococcus 2
Dielma
Faecalibacterium
Faecalitalea
Ruminococcus 1
Subdoligranulum
Veillonellaceae;__
Dialister
Phocea
Erysipelotrichaceae UCG-003
Negativibacillus
Phascolarctobacterium
Ruminococcaceae UCG-002
OTHERS
D
Relative Abundance within phylm
Day
Desulfovibrio
Escherichia-Shigella
Enterobacteriaceae;__
OTHERS

Figure S2: Dynamics of bacterial taxonomy in MiCoMo over 14 days culture for Individual C.

Day 0 indicates original fecal sample, Day 1 – 14 indicate cultured samples from MiCoMo, averaged over 3 technical replicates. ASVs that didn't account for at least 1% of total abundance in any day were grouped in Others. A) Family-level taxonomy of the whole community B) Genus-level taxonomy within the Bacteroides phylum C) Genus-level taxonomy within the Firmicutes phylum D) Genus-level taxonomy within the Proteobacteria phylum

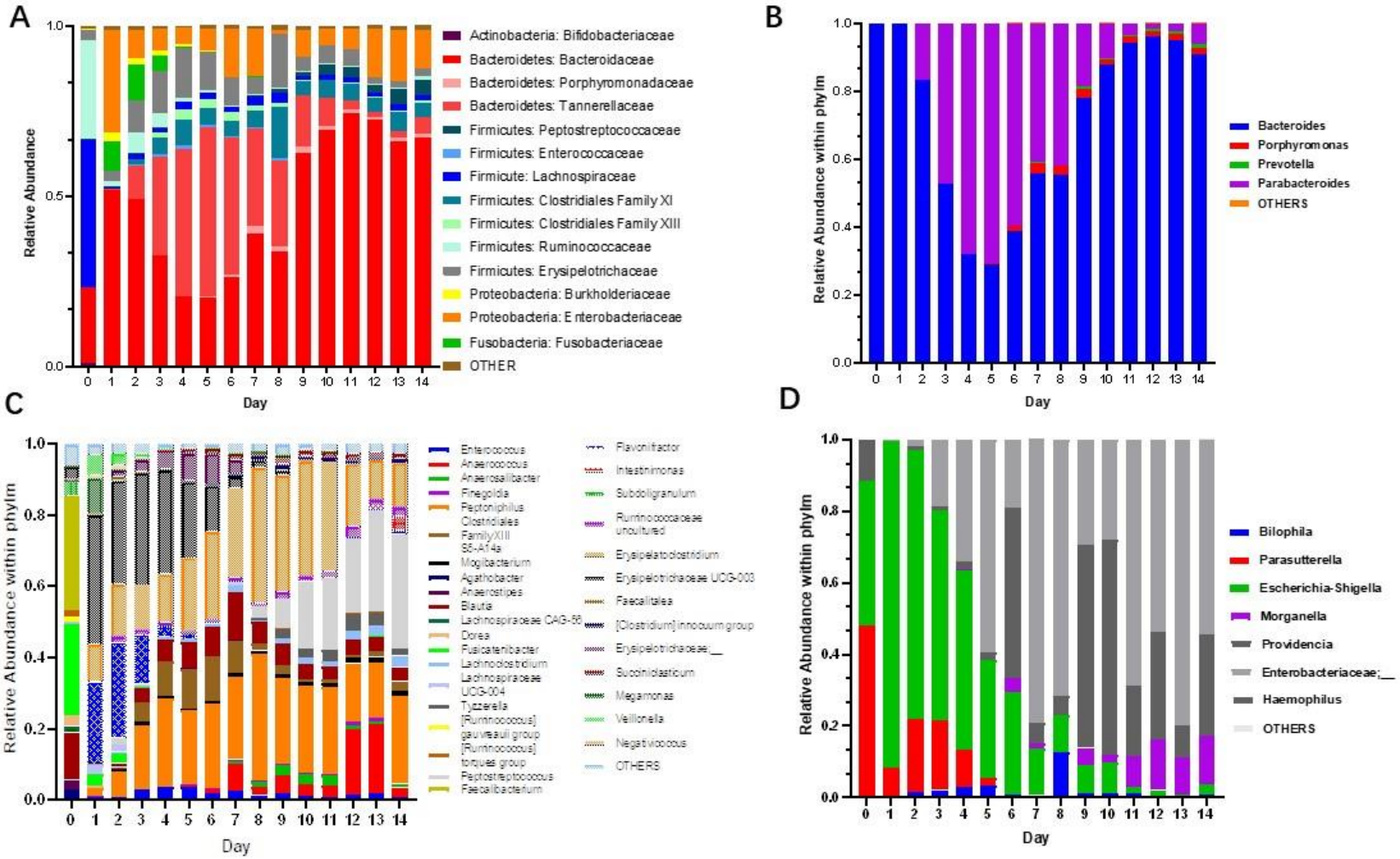
A
Relative Abundance
Day
Actinobacteria: Bifidobacteriaceae
Bacteroidetes: Bacteroidaceae
Bacteroidetes: Porphyromonadaceae
Bacteroidetes: Tannerellaceae
Firmicutes: Peptostreptococcaceae
Firmicutes: Enterococcaceae
Firmicute: Lachnospiraceae
Firmicutes: Clostridiales Family XI
Firmicutes: Clostridiales Family XIII
Firmicutes: Ruminococcaceae
Firmicutes: Erysipelotrichaceae
Proteobacteria: Burkholderiaceae
Proteobacteria: Enterobacteriaceae
Fusobacteria: Fusobacteriaceae
OTHER
B
Relative Abundance within phylm
Day
Bacteroides
Porphyromonas
Prevotella
Parabacteroides
OTHERS
C
Relative Abundance within phylm
Day
Enterococcus
Anaerococcus
Anaerosalibacter
Finegoldia
Peptoniphilus
Clostridiales Family XIII SS-A14a
Mogibacterium
Agatholbacter
Anaerostipes
Blautia
Lachnospiraceae CAG-56
Dorea
Fusicatenibacter
Lachnoclostridium
Lachnospiraceae UCG-004
Tyzzerella
[Ruminococcus] gauvreauii group
[Ruminococcus] torques group
Peptostreptococcus
Faecalibacterium
Flavonifractor
Intestinimonas
Subdoligranulum
Ruminococcaceae uncultured
Erysipelatoclostridium
Erysipelotrichaceae UCG-003
Faecalitalea
[Clostridium] innocuum group
Erysipelotrichaceae;__
Succiniclasticum
Megamonas
Veillonella
Negativicoccus
OTHERS
D
Relative Abundance within phylm
Day
Bilophila
Parasutterella
Escherichia-Shigella
Morganella
Providencia
Enterobacteriaceae;__
Haemophilus
OTHERS

Figure S3: Dynamics of bacterial taxonomy in MiCoMo over 14 days culture for Individual D.

Day 0 indicates original fecal sample, Day 1 – 14 indicate cultured samples from MiCoMo, averaged over 3 technical replicates. ASVs that didn't account for at least 1% of total abundance in any day were grouped in Others. A) Family-level taxonomy of the whole community B) Genus-level taxonomy within the Bacteroides phylum C) Genus-level taxonomy within the Firmicutes phylum D) Genus-level taxonomy within the Proteobacteria phylum

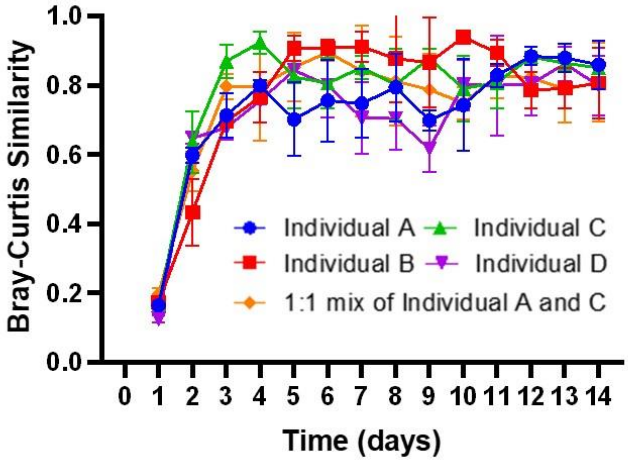

Figure S4: Bray-Curtis similarity between each daily reactor sample and the previous day.

Each line is average across 3 technical repeats of the same donor, error bars indicate standard deviations.

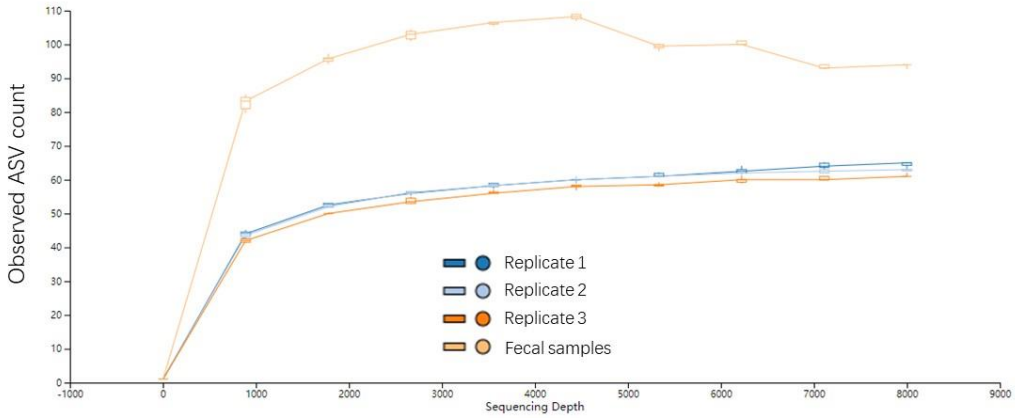

Figure S5: Alpha rarefaction curves for all replicates included in this work.

Each line is average of samples from one replicate reactor or from fecal sample, from all individuals

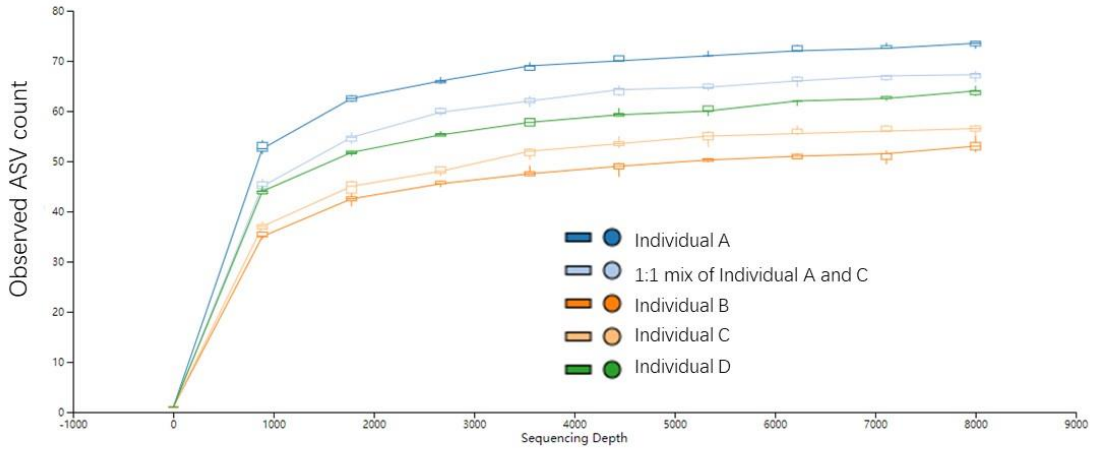

Figure S6: Alpha rarefaction curves for all individuals included in this work.

Each line is average of all replicate reactor samples and fecal sample from one individual

Chapter 3: Conclusion and future aspects

3.1 Conclusion

In this work, we present the Mini Colon Model (MiCoMo), an optimized, fully controllable, miniaturized, pH-controlled, and anaerobic bioreactor system for simulating the gut environment. We believe that MiCoMo fills the gap between existing large-scale fermentation systems and miniaturized systems and combines the advantages of both types to support a controlled environment for studying gut microbiota. **Table** briefly summarizes the characteristics of MiCoMo compared to existing large-scale and miniaturized *in vitro* systems. We show that MiCoMo establishes an *in vitro* environment with faster stabilization of complex microbial communities (approx. 3 – 5 days) relative to the large-scale systems (which take several weeks) while controlling physiologically relevant parameters, possibly thanks to its smaller working volume. Constructed from low-cost and easy-to-acquire components, MiCoMo is easy to operate and is able to maintain a stable microbial community with variations comparable to that of existing systems and animal models. We further demonstrate that MiCoMo's capability to sustain high microbial diversity from fecal inoculum and keep distinctive community features from individual donors. We believe its small footprint, low cost, and ease of fabrication will allow for easy replication across labs studying the effect of various perturbations on individual gut microbial communities with high throughput.

Table 1 Generalized comparison of MiCoMo with existing *in vitro* systems

System	Working volume	Max experimental period supported	Microbial stabilization time	Multiplex	Control of physiological parameters
Large-scale systems	100 mL – 2 L	Several days – several months	A few weeks	Very limited (maximum 2 for most systems)	High
Miniaturized systems	< 1 mL – 10 mL	Typically, < 1 week	Often not reported/established	High (up to 96-plex)	Limited
MiCoMo (this work)	30 mL	> 2 weeks	3 – 5 days	3-plex	High

Future improvements for MiCoMo include reducing its footprint currently limited by the lack of affordable, miniaturized pH probes, thus further increasing its multiplex capability. To

improve physiological relevance, additional compartments representing upper GI tract can be developed and interfaced with MiCoMo, which will allow investigation of interaction between gut microbiota and chemicals which are processed and altered in the upper GI tract, such as dietary components. Inclusion of host cells and host-microbiota interfaces, mimicking host-microbiota interactions in a controlled manner is another potential direction for future development. Meanwhile, several design and biological considerations for application of *in vitro* systems to study gut microbiota are yet unclear and unaddressed by the current systems including MiCoMo. Such considerations are discussed in this section in hope of assisting development of next generation *in vitro* systems.

3.2 Biological considerations for *in vitro* systems

In vitro systems are subject to contaminations, which need to be taken into consideration when evaluating them. This risk could come from both the maintenance required during operation and the difficulty to sterilize and assemble the system before start of an experiment. While many bioreactors are based on lab-scale apparatus and are autoclavable[165,185], the need for sterilizing different parts of whole fermentation system separately and assembling afterwards still carries a risk of contamination. Single-stage and batch systems are sometimes designed to be autoclavable with all tubing assemblies pre-connected to migrate this risk[181,225,226], but for bigger, multi-stage systems this option is not always available. Meanwhile, complex systems usually require more maintenance, resulting in higher risks of contamination during the process. To this end, batch culture systems requiring no input after initial inoculation offer the highest safety[161], while systems with larger footprint, such as the SHIME, which need to operate outside of environments with guaranteed sterility, are more subject to contamination[227]. It should be noted that currently it is not yet clear to what extent the potential environmental contaminations might affect a microbial community as complex as the gut microbiota.

Medium selection is another noteworthy consideration when interpreting gut microbiota data from *in vitro* systems. As demonstrated by many studies, diet contributes majorly to shaping the gut microbiota *in vivo* and can shift an established gut microbiota rapidly[20,21]. Similarly, choice of growth medium play an important role in the capability of any *in vitro* fermentation

system to maintain a stable and physiologically relevant microbial community. To this end, many recipes have been developed, and modern fermentation systems typically employ variations of enriched media[165,184,213], sometimes further supplemented with gut-specific nutrients such as mucin[186] and bile salts[166]. Such recipes are often home-made to be compatible with one specific *in vitro* system, making cross-platform comparisons difficult. Although the impact of medium selection on gut microbiota diversity *in vitro*[6] has been investigated, current efforts are limited to simple batch culture models likely due to their high throughput. More studies are therefore needed to evaluate the impacts of baseline media on more complicated digestion models. Further, the gut microbiota is characterized by high inter-individual difference, and so even if the fecal inocula came from a cohort with a similar life style and diet[25,228], such differences can still lead to differential response to the same controlled environmental setting and media.

3.3 Design considerations for *in vitro* systems to study gut microbiota

3.3.1 Caveats of *in vitro* systems

One major design consideration for *in vitro* fermentation systems is the trade-off between attempting higher physiological relevance by controlling more parameters and having higher multiplex potential by minimizing the footprint and operational complexity of the system[143,161]. Nevertheless, it is generally understood that *in vitro* systems are only able to simulate a fraction of *in vivo* conditions, and the inoculated gut microbiota undergoes some shifts in even the most complex systems[184,198]. However, *in vitro* systems excel in the reproducibility of experimental conditions over mouse models and human trials. As a result, the focus of such *in vitro* systems is often to support a stable microbial community as opposed to one that is necessarily the most reflective of the original fecal sample[122,165]. Further, while *in vitro* systems enable more direct and mechanistic analysis of the gut microbiota's response to xenobiotics and change of conditions[161], the complex interactions within the microbial communities often remain obscure. As an example, the developers of the MBRA system reported *Akkermansia muciniphila*, a known mucin degrader[229], among the most abundant bacterial species in their model, which operates without mucin nor mucus layer[165]. The exact reason why this bacteria's growth is

supported in the model thus remains unclear and likely relies on metabolic products of other community members.

3.3.2 Factor involved in design of *in vitro* systems

While many design factors, such as pH[230], temperature, compartmentalization[185], residence time[231] and waste removal[198], have all been shown to significantly impact the microbiota composition in fermentation systems, in most cases these findings remain limited to the specific study and model tested. It is not always clear whether, or to what extent, these impacts are reflective of clinical scenarios due to difficulty of conducting human trials and probing the gut microbiota in different sections of the gut *in vivo*. For example, individual bioreactors of *in vitro* gut models have volumes ranging from a few milliliters to several liters and are equipped with various mixing systems. However, it is unclear how this factor relates to the grown microbial community: while one can argue that larger reactor size is more physiologically relevant to the human colon (volume of 500 mL to 1L)[232], this volume is also highly variable depending on fasting stage[232]. On the other hand, lab mice, with on average 1 – 1.5 mL of colon volume closer to that of miniaturized systems, have also long been applied as models for microbiota studies[155,157]. Similarly, most *in vitro* systems are equipped with constant mixing through either magnetic stirring or gas bubbling, which is a common practice for bioreactors to ensure sufficient nutrient transfer and create a uniform environment[233]. However, the human gut is known to achieve movement and mixing of content through peristalsis[197], which likely doesn't achieve thorough mixing and leads to local spatial microbiota variation. In an *in vitro* setting however, lack of thorough mixing could lead to sedimentation of reactor content, which is particularly problematic for investigation of some common prebiotics[56,57]. These facts highlight the challenges for defining accurately what a "physiologically relevant" environment is for simulating the gut microbiota.

3.4 Emerging and future directions of *in vitro* systems

Despite the variety of available fermentation systems simulating different gut environments, most of them are designed with specific functionality in mind. While this contributes to the high specificity and design needs of such systems, it also incurs a high cost for switching

research questions with different environmental requirements. For example, if a compound is found to shift the microbiota composition in a batch reactor model, it would then be beneficial to further test and confirm such behavior in a more complex digestion model. A more customizable design, then, might be favorable in such cases and will enable labs to explore multiple degrees of topic. Such systems are recently being explored. For example, Habib et al. proposed a modularized system consisting of one or multiple unit bioreactors with variable volume and configurable to operate in both single and multi-stage manner[234]. We also aimed for this goal in developing MiCoMo, which is modular by nature and easily modifiable to integrate the biologic compartmentalization of the human GI tract, by adding additional bioreactor units or reconfiguring existing ones at the cost of reducing multiplex capabilities.

The incorporation of the eukaryotic interface in a controlled manner is another desirable feature for *in vitro* systems for gut microbiota studies[235]. The host-microbiota interaction is usually modelled *in vitro* with a permeable interface between microbial community and a layer of epithelial cells, such as seen in the Transwell inserts[236]. Application of such systems in gut microbiota study is however challenging as host cells require aerobic conditions, whereas the gut microbiota requires anaerobic ones. Nevertheless, several systems have been developed to simulate the specific interactions between gut epithelial cells and gut bacteria, such as the HMI model[237], the HoxBan model[238] and the HuMiX model[239]. These systems are typically miniaturized, exploiting the capability of combining aerobic and anaerobic streams in one microfluidic device. They however are still limited in their physiological relevance as they typically use cancer cell lines as a representation of the host cells, lack a mucus layer seen *in vivo,* and often only show co-culturing of host cells with one or several bacterial strains as opposed to a complex microbial community. A gut-on-chip model has recently been developed to support prolonged co-culture between primary human gut tissue and gut microbiota from fecal samples[240], but the gut microbiota community nevertheless suffers from a big loss of diversity. Together, these systems emphasize the high potential of incorporating host-microbiota interactions in *in vitro* gut simulator designs, but more work is needed to adapt such interfaces to fermentation devices while keeping advantages from both.

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