Gastrointestinal Tract-in-a-Box

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Abstract

The human gastrointestinal tract (GIT) hosts a diverse and complex community of microorganisms, including bacteria, archaea, viruses, and eukaryotes, which is referred to as the GIT microbiota. Recently, there has been a considerable amount of studies pertaining to the influence the GIT microbiota has on human health and disease, showing that microbial dysbiosis has been linked to numerous diseases such as diabetes, autoimmune diseases, cancer, and neurodegenerative diseases. However, the findings are still preliminary and limited, and many microbial functions and features remain unknown due to the inaccessibility of the human GIT for sampling, limiting systematic experimentation. Studying the GIT microbiota using *in vitro* GIT systems represents an important approach that enables systematic experimentation and sampling, and efficient control and manipulation of specific microbial compositions and responses. Current chemostat-based systems however are large (> 500 mL), making them costly and slow, and their throughput is limited (21-35 days stabilization) with often only one test performed. While miniaturized chip-based systems can offer faster throughput and modularity in studying microbiota, they are simplified models with one chamber (< 1 mL), lack physiological conditions, and support only one microbe, and thus do not capture the physiological and microbial complexities of the GIT. Here, we present a miniaturized, milliliter-sized, GIT system that is enclosed in a box (GITBox), which is comprised of three 40 mL mini-bioreactors that simulate the stomach, small intestine, and colon, and recreates physiological conditions suitable for microbial growth, including controlling pH to *in vivo* ranges, mixing, and establishing anaerobiosis and temperature. The growth of single strain microbial species was first demonstrated over a period of 180 h. Based on a cyclic 4 h feeding procedure that mimics the human digestive process, complex microbiota derived from a human fecal inoculum was

cultured, whereby microbial stabilization was achieved within five days and the original fecal composition was retained for up to 12 days. In addition, the capabilities of the GITBox for studying the response to external stimuli were demonstrated by challenging the stabilized microbial culture with antibiotics, followed by probiotics, in 14-day trials. By tracking the response of each targeted microbial community, we observed quick (< 24 h) microbial collapse and recovery responses upon each treatment. The GITBox is versatile, enables a representative simulation of the GIT microbiota, and allows the systematic control and testing of numerous key parameters for advanced microbiota studies pertaining to human health and disease.

Résumé

Le tractus gastro-intestinal (TGI) humain héberge une communauté de microorganismes diversifiée et complexe, y compris les bactéries, les archées, les virus et les eucaryotes, ce que l'on appelle le microbiote TGI. Récemment, il y a eu une quantité considérable d'études concernant l'influence du microbiote TGI sur la santé et la maladie humaine, montrant que la dysbiose microbienne est liée à de nombreuses maladies comme le diabète, les maladies auto-immunes, le cancer et les maladies neurodégénératives. Cependant, les résultats sont encore préliminaires et limités, et de nombreuses fonctions et caractéristiques microbiennes restent inconnues en raison de l'inaccessibilité du TGI human pour l'échantillonnage, ce qui limite les tests systématiques. L'étude du microbiote TGI utilisant de systèmes *in vitro* représente une approche importante qui permet l'expérience et l'échantillonnage systématiques, ainsi que le contrôle et la manipulation efficaces de compositions et de réponses microbiennes. Cependant, les systèmes actuels basés sur les chémostats sont grands (> 500 mL), ce qui les rend coûteux et lents, et leur débit est limité (stabilisation de 21-35 jours) avec souvent un seul test effectué. Bien que les systèmes miniaturisés, basés sur les « chip », peuvent offrir la modularité et un débit plus rapide dans l'étude du microbiote, ils sont des modèles simplifiés avec un chambre (< 1 mL), manquent de conditions physiologiques et ne supportent qu'un seul microbe, et donc ne captent pas les complexités physiologiques et microbiennes du TGI. Ici, nous présentons un système miniaturisé (d'une taille de l'ordre du millilitre) confiné dans une boîte (GITBox), composé de trois mini-bioréacteurs de 40 mL qui simulent l'estomac, l'intestin grêle et le côlon, et recrée des conditions physiologiques adaptées à la croissance microbienne, y compris le contrôle du pH à des intervalles *in vivo*, le mélange et l'établissement de l'anaérobiose et de la température. La croissance d'espèces microbiennes à une seule souche a été démontrée sur une période de 180 h.

En nous basant sur une procédure d'alimentation cyclique de 4 h reconstituant le processus digestif humain, nous avons cultivé un microbiote complexe dérivé d'un inoculum fécal humain; la stabilisation microbienne a été atteinte en cinq jours et la composition microbienne de l'échantillon fécal original a été conservée jusqu'à 12 jours. Les capacités du GITBox pour étudier la réaction aux stimuli externes sont démontrées en effectuant des essais de 14 jours au cours desquels des antibiotiques, puis des probiotiques, sont utilisés pour perturber la culture microbienne préalablement stabilisée. En suivant la réaction de chaque communauté microbienne ciblée, nous avons observé des réactions microbiennes rapides (< 24 h) en ce qui concerne l'effondrement et le rétablissement du microbiote à la suite de chaque traitement. Le GITBox est polyvalent, permet une simulation du microbiote TGI de manière représentative, de même que de contrôler et expérimenter avec de nombreux paramètres d'intérêt dans le cadre d'expériences avancées et systématiques se rapportant au rôle du microbiote dans la santé et la maladie humaines.

Acknowledgements

I would like to thank my supervisor Dr. David Juncker for his guidance, mentorship, and support throughout the project. In addition, I would like to thank my colleagues at the lab for providing a friendly and supportive environment. In particular, Dr. Andy Ng for making a considerable amount of time to discuss and work with me on the majority of the project, and as such, the project would not have been as successful without his endless contributions. I would also like to thank Ayokunle Olanrewaju, Arya Tavakoli, Philip Zimny, Frédéric Normandeau, Edward Zhang, and Grace Oh for their friendship and humour within these past few years. Personally, I would like to thank my family for their support, and my friends, in particular Lilly Gates, for her endless encouragement, inspiration, and smiles. Finally, I wish to thank the Department of Biomedical Engineering at McGill University and the Fonds de Recherche Nature et Technologies for their financial support.

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1 Project description

1.1 Motivation and rationale

The microbiota of the gastrointestinal tract (GIT) has emerged as a major contributor to human health and disease. However, many of their functions, features, and roles underlying the ever-growing list of health conditions have yet to be discovered or understood fully due to the inaccessibility in sampling the human GIT, which has largely limited systematic experimentation. The majority of studies have been conducted with animal models, notably germ-free mice, which are administered with external microbes to simulate the GIT microbiota, however, these models differ substantially from humans in terms of physiology, anatomy, and genetics, and as such their results are not clearly translatable to humans.

Simulating the GIT microbiota *in vitro* enables the study and testing of many microbial parameters in a controlled and systematic manner. There have been several GIT systems to date, including chemostat-based systems and miniaturized organ-on-a-chip systems. However, chemostat-based systems are large in size (> 500 mL vessels), which makes them costly and slow. In addition, their throughput is limited, taking between 21-35 days before any testing can be done with many trials performed only once without replicates, while maintaining a microbial fecal culture that is not fully representative of the *in vivo* composition of the original fecal sample.

In contrast, miniaturized GIT systems, such as microfluidic organ-on-a-chip devices, can offer faster throughput experimentation and modularity in systematically studying microbiota, with several systems focusing on the microbial-epithelial interface of the GIT. However, these systems have only shown support for a single microbe and also use a one chamber (< 1 mL) design with a simple flow through-based approach, which do not capture critical parameters and

conditions that define the GIT such as the large microbial complexity, distinct regions of the GIT and their specific transit times and pH conditions, supply of digestive enzymes, and mixing.

Hence, the motivation for this work is to develop a GIT system that is miniaturized, with a size that is able to support numerous crucial parameters that define the GIT such as different regions, a physiological GIT environment, and more importantly, a complex microbiota community. Here, a size in the milliliter range, with 40 mL mini-bioreactors, is investigated to meet the requirements listed above. In addition, the system should address several key factors, including accelerating the microbial stabilization time, preservation of the original fecal microbiota composition *in vitro*, and tracking of rapid microbial responses when perturbed by external stimuli. To this end, the development of such a system can enable a more representative simulation of the GIT microbiota and open up numerous avenues for advanced and systematic experimentation on the GIT microbiota.

1.2 Project objectives

The three objectives for this project are (i) design and develop a miniaturized GIT system that can mimic various physiological regions and conditions of the GIT, (ii) characterize the support, stabilization time, and composition of a complex microbiota community in the system, and (iii) examine the microbial responses to external stimuli such as antibiotics and probiotics, which would validate the testing capabilities of the system.

1.3 Manuscript-based thesis

This thesis will be presented in manuscript form. The introduction section will provide a detailed scope of the background information, while the body of the work will be presented as a

manuscript, which will be submitted to a peer reviewed journal for publication. Lastly, the general conclusion section will summarize this project as a whole, and provide further insights on the results along with future directions of this project.

1.4 Contribution of authors

For the manuscript, Sa Xiao developed the miniaturized GIT system and performed all the experiments, data analyses, figure preparations, and writing. Susan Westfall aided in preparing the culture materials, which included bacteria and fecal samples, food media, and digestive enzyme solutions, as well as conducting colony counting of the samples on exclusion agar plates. Dr. Ng provided his expertise and suggestions for all experiments and results, and helped to review and edit the manuscript. Dr. Prakash, the collaborator on this project, provided many resources from his lab as well as support and suggestions on this project. Dr. Juncker supervised this project and continuously offered support, ideas, and feedback regarding all aspects of the project. This thesis was written and prepared by Sa Xiao, with help on reviewing and editing from Drs. Ng and Juncker.

1.5 Declaration of novelty

This project represents one of the first GIT systems that is miniaturized and milliliter-sized (40 mL reactors), can mimic numerous physiological conditions of the GIT, and capable of supporting complex microbiota that is rapidly stabilized and representative of *in vivo* fecal compositions. In addition, the system can facilitate rapid microbial responses when perturbed by external stimuli such as drugs and supplements, which illustrates the abilities of the system to conduct systematic studies on GIT microbiota.

2 Introduction

In this chapter, the role of the GIT microbiota and the methods to study it will be reviewed. An overview of the human GIT microbiota will first be provided, including microbial colonization and development in the GIT, and the contributions of the GIT microbiota to human health and disease. Next, the existing methods in studying the GIT microbiota will be described, including *in vivo* animal models and *in vitro* GIT systems that can simulate the GIT microbiota. Experimental *in vitro* GIT systems will be described with a particular focus on their capabilities and advantages, and more importantly, limitations in studying and testing GIT microbiota. Collectively, these sections will provide the necessary background for the work presented in this thesis.

2.1 The GIT as a microbial reservoir

There are approximately 10^{14} microbial cells, or microbes, in and on the human body [1]. These microbes, made up of bacteria, archaea, viruses, and eukaryotes, are found everywhere, colonizing in places such as the respiratory tract, skin, reproductive tract, and the gastrointestinal tract (GIT). More importantly, the majority of microbes reside in the GIT, which collectively make up a diverse and complex microbiota, referred to as the GIT microbiota [2]. The GIT, which is a complex digestive organ that inherently contributes to the digestion and absorption of nutrients in an individual, represents a highly attractive site for microbiota due to its nutrient rich environment and large size. The GIT is comprised of numerous regions and is categorized into upper and lower portions. The upper regions include the mouth, esophagus, stomach, and small intestine, while the lower regions include the colon, rectum, and anus. The stomach, small intestine, and colon are the regions that contribute the most to nutrient storage, digestion, and

microbial colonization (Fig. 1a) [3]. The complete GIT has an average estimated surface area of 300 m^2 and resting volumes of 30-100 mL in regions such as the small intestine and colon, while able to store up to 1 L in the stomach [2, 4], and thus is an ideal reservoir for microbiota.

The established GIT microbiota in adults hosts a myriad of microbes, with over 99% of them being anaerobes [5]. As we move down the GIT from the stomach to the colon, the composition and complexity of the microbiota increases (Fig. 1a). The stomach hosts a small population of microbiota (10^1 microbes/g). The microbial populations and diversity in this region are low due to the stomach's gastric secretions during digestion, which creates a highly acidic environment (< pH 2) [6]. This region is mostly composed of the *Lactobacillus*, *Helicobacter*, and *Veillonella* genera, which are species capable of colonizing in acidic conditions [7]. The microbial populations and diversity steadily increase in the small intestine (10^4-10^7 microbes/g). The small intestine secretes digestive enzymes, which is critical for digestion and absorption of nutrients in the GIT, and establishes an aerobic environment. Due to low nutrient availability and the presence of oxygen, however, only a moderate amount of facultative anaerobes are able to colonize [6, 8]. The small intestine is largely composed of the *Lactobacillus*, *Streptococcus*, and *Enterococcus* genera, which are dominant facultative anaerobic species [6, 7]. The colon represents the most diverse and populated region (10^{12} microbes/g), with suitable microbial growth conditions such as anaerobiosis, high nutrient availability, and pH (pH 5.5-7.0). This region thus enables the growth of a dense amount of both facultative and obligate anaerobes [6, 8]. The colon is composed of major species such as the *Bacteroides* and *Clostridium* genera, as well as the *Proteobacteria* and *Actinobacteria* phyla [7, 9].

The microbial diversity varies across the GIT, going from the epithelial surface to the intestinal lumen (Fig. 1b). The intestinal epithelium is lined by a mucus layer, which protects the

host from potential microbial overgrowth and translocation to systemic sites, and thus the diversity and population are low in these regions. In contrast, the intestinal lumen, which is the first contact for microbiota colonized within each region, harbors an abundant and diverse microbial community [2, 8]. Altogether, studies have shown that the established GIT is inhabited by over 1000 different microbial species along and across the GIT, with the majority of these species falling under four dominating phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* [1, 8].

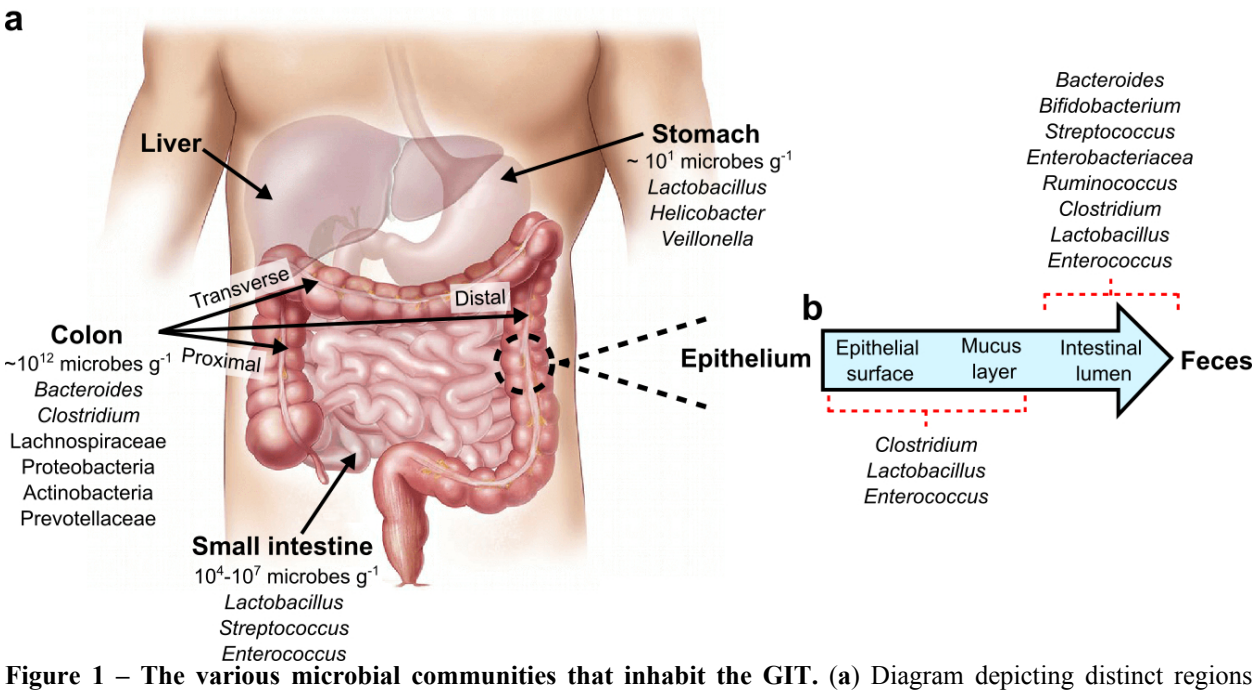

Figure 1 – The various microbial communities that inhabit the GIT. (**a**) Diagram depicting distinct regions along the GIT with estimated microbial numbers per gram as well as specific communities found in each region. (**b**) Schematic showing the variations of microbial species across the GIT from the epithelium to the intestinal lumen. Image adapted from Sekirov *et al.* [2].

2.2 The development and stabilization of the GIT microbiota

The development of the GIT microbiota begins immediately at birth, with the GIT of infants rapidly colonized by microbes in their surrounding environment [10]. The first microbes they encounter are usually dependent on the mode of delivery, either from the mother's vagina (natural birth) or the external environment (Caesarian section) [10, 11]. The initial composition and diversity of microbiota in an infant are low and steadily increases through the early development of the infant. By age three, the microbiota stabilizes and resembles that of an adult, and remains stable throughout adulthood [1, 8, 10, 11]. The microbial compositions vary across each individual, providing a distinct microbial profile specific to each person. There are many factors governing the distinct microbial compositions of each person, with the most studied upon thus far including the mode of delivery, diet, and the use of therapeutics, while further studies are aiming to also look at the effects of genetics, lifestyle, and geographical environment on the microbial development (Fig. 2) [10].

The mode of delivery has shown varying effects on the initial microbiota composition [12, 13]. Infants delivered vaginally showed microbiota that were highly reflective to those of the mother, including an abundance of the *Lactobacillus*, *Prevotella*, and *Sneathia* genera, which are species that inhabit the female reproductive tract [13]. On the other hand, infants delivered by C-section showed microbiota that were more similar to those found on the skin or in the immediate environment (*e.g.* hospital), including the *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, and *Clostridium* genera [13, 14].

During the time infants mature into adulthood, as well as during adulthood, the microbiota diversify based on the foods that they consume. Infant feeding methods, notably breast milk, are a major source in the early composition of the GIT. The breast milk contains a rich amount of

microbiota, with over 600 different species that include large genera such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*, and thus breast-fed infants are exposed to an abundant and diverse amount of microbiota through breast milk [11, 15]. Studies have also shown the effects of different sources of proteins, fats, and carbohydrates on the developing and stable microbiota [16, 17]. For example, comparisons between plant- and animal-based proteins show that plant protein promotes more *Bifidobacterium* and *Lactobacillus* while decreasing *Bacteroides* and *Clostridium*, and animal protein promotes more *Bacteroides*, *Alistipes*, *Bilophila*, and *Ruminococcus* while decreasing *Bifidobacterium* [16, 17]. Similarly, comparisons between the consumptions of different fats, such as saturated and unsaturated fats, and of different carbohydrates, including digestible (*e.g.* starch and sugars) and non-digestible (*e.g.* fiber), show various effects on the composition levels of each species [16]. In addition, studies have also shown the influences of specific dietary regimes on microbiota [18], with diets such as Western, gluten-free, and Mediterranean. For instance, a Western diet has shown decreases of *Bifidobacterium*, *Lactobacillus*, and *Eubacterium*, which can be contributed to the high animal fat and protein foods within these diets [19]. A Mediterranean diet, which is composed of foods with high fiber, antioxidant, unsaturated fat, and plant protein, as well as low glycemic carbohydrates, has shown large increases in *Bacteroides*, *Bifidobacterium*, *Lactobacillus,* and *Prevotella* [20].

While the microbiota composition adapts with various food sources and diets, therapeutics such as drugs for the treatment of diseases have also shown profound and immediate effects on the microbial composition during the development stage and well within the stable adulthood stage. These primarily involve the use of antibiotics, which effectively perturbs the microbiota composition [10, 21]. In infants, antibiotics have shown to shift the composition of the GIT

microbiota toward a high abundance of *Proteobacteria* populations, which include potentially pathogenic species, and a low abundance of *Actinobacteria* and *Bacteroidetes* populations, such as *Bifidobacterium* and *Bacteroides*, which are beneficial species [11, 21]. In addition, antibiotics have also shown to reduce the overall microbial diversity of the infant, while selecting for drug-resistant species to thrive [22]. Antibiotics affect the microbial composition even during the stable adulthood stage, with perturbation leading to decreased microbial diversity in the short-term [23]. Moreover, increases in antibiotic-resistant species can be observed, either due to susceptible species becoming resistant over frequent usage of antibiotics or the takeover of already-present resistant species [23]. In the long-term after treatment, the microbial composition does eventually recover and re-stabilize, however, there is the likelihood that the newly stable composition has shifted from the original with different populations dominating the GIT microbiota [21, 23].

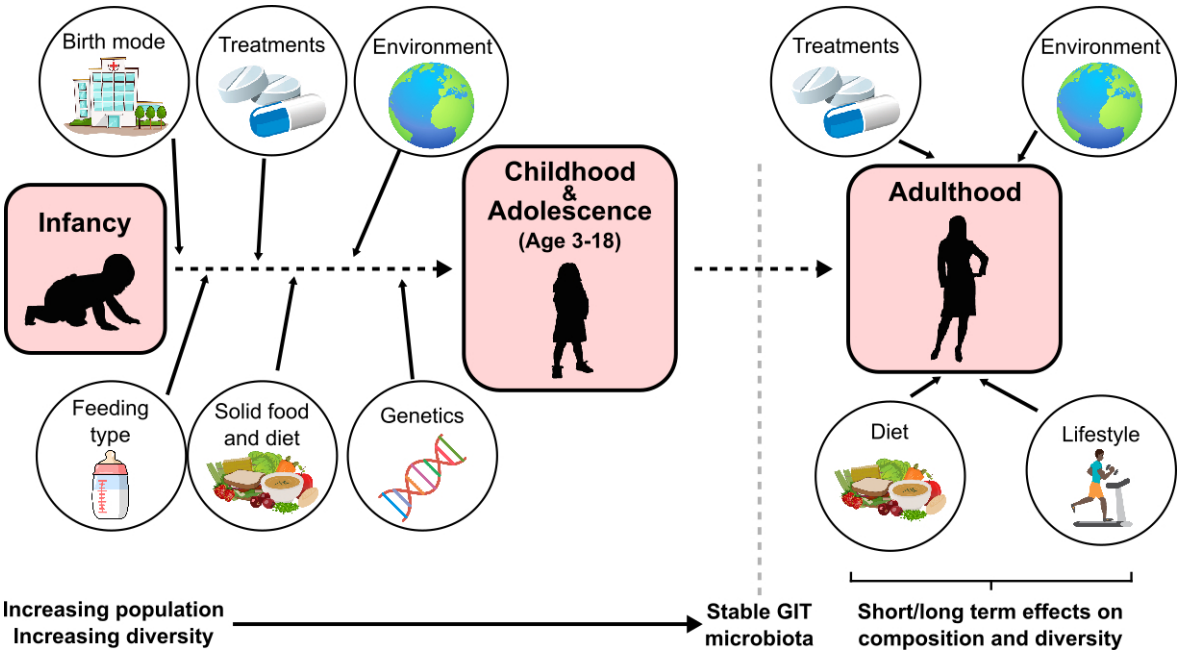

Figure 2 – Different factors that affect the development and stability of the GIT microbiota. During the development stage, the GIT microbiota of an individual is established based on the birth mode, infant feeding type, treatments such as antibiotics, diet, environmental surroundings, and genetics. The stabilized GIT microbiota encounters short and long term changes in composition based on diet, lifestyle, treatments, and environment in adulthood.

2.3 The contributions of the GIT microbiota to health and disease

The GIT microbiota has emerged as a large contributor to human health and disease, which is indicative of the considerable increase in published GIT microbiota topics in recent years (Fig. 3). In terms of supporting the health of the host, studies have shown that these microbes provide benefits such as maintaining gut integrity as well as shaping the structure of the intestinal epithelium [24], digesting nutrients and harvesting energy [25], protecting against pathogenic species [26], and shaping the immune system [27]. Altogether, a "healthy" GIT microbiota composition, which is composed of a balanced and diverse number of beneficial microbiota, has generally been linked to a healthy host [2]. What is considered a "healthy" GIT microbiota varies between individuals, however, and research on establishing a baseline microbial composition is active.

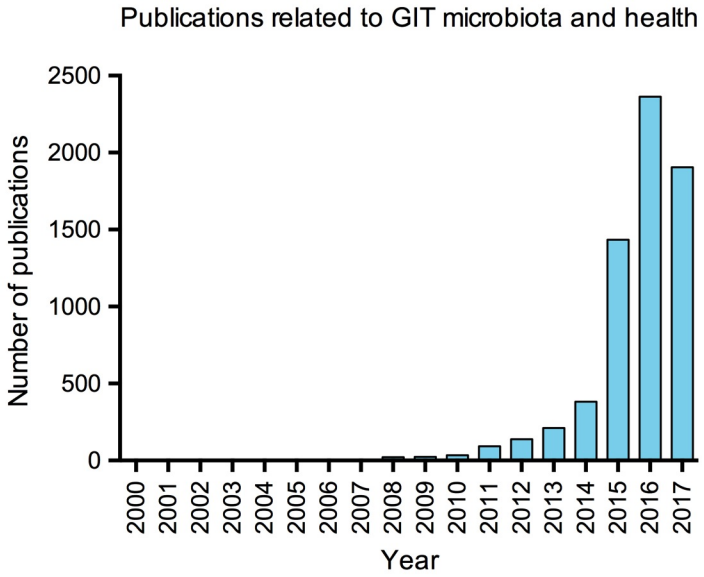

Figure 3 – Number of published articles on GIT microbiota and human health and disease from 2000-2017. Data obtained by searching PubMed (https://ncbi.nlm.nih.gov/pubmed/) with the terms 'gut microbiota', 'gut flora', 'intestinal microbiota', and 'intestinal flora'.

There are many factors that contribute to the altering of the microbial balance, which could hinder the beneficial microbial mechanisms. The factors that helped shape the stable GIT microbiota, such as the mode of delivery and diet, as well as potential perturbations thereafter (*e.g.* antibiotics), can effectively contribute to the development of an imbalanced microbial composition or the shifting of a balanced composition to one that is imbalanced [10, 28, 29]. This imbalanced state, which is known as dysbiosis, usually involves a lower ratio between beneficial and potentially harmful microbiota, as well as a decrease in microbial diversity [30]. These imbalances can reduce the ability of the GIT microbiota to resist perturbations and make it more susceptible to disease-causing species, which could eventually lead to developments of disease [30]. Recent studies have thus aimed at the implications of GIT microbiota dysbiosis on an individual's health. Dysbiosis has been associated with numerous disorders and diseases, such as obesity [31], allergies [32], diabetes [33], inflammatory bowel disease (IBD) [34], celiac disease [7], autoimmune diseases [35], cancer [36], and neurodegenerative diseases [37, 38], while more links are being discovered in preliminary studies, with relationships ranging from an imbalance in individual species to a community of different species (Table 1).

Table 1 – Associations between diseases and dysbiosis, with summary of changes in the GIT microbiota associated with each disease. Adapted from de Vos *et al.* [39].

Disease	Implicated microbiota[a]	References
Crohn's disease	↓ Microbial diversity, ↓ *Faecalibacterium prausnitzii*, variations in *Bacteroides* species	[40, 41]
Ulcerative colitis	↓ Microbial diversity, ↓ *Akkermansia muciniphila*	[42, 43]
Irritable bowel syndrome	Differences in microbial signatures, ↑ *Bacteroidetes/Firmicutes* ratio, ↑ *Dorea*, ↑ *Ruminococcus*	[44]
Clostridium difficile infection	↓ Microbial diversity, presence of *C. difficile*	[45]
Colorectal cancer	Variations in *Bacteroides* species, ↑ *Fusobacteria*	[46, 47]
Celiac disease	Differences in microbial compositions, variations in *Firmicutes* and *Proteobacteria*	[7]
Type 1 and 2 diabetes	Differences in microbial signatures, ↑ *Bacteroidetes/Firmicutes* ratio	[48, 49]
Obesity	Differences in *Bacteroidetes*/*Firmicutes* ratios, ↑ *Lactobacillus*	[31, 50]
Alzheimer's disease	↓ Microbial diversity, ↑ *Bacteroidetes/Firmicutes* ratio, ↓ *Bifidobacterium*	[51]
Atherosclerosis	Differences in microbial signatures, ↑ *Enterobacteriaceae*, ↑ *Streptococcus*	[52]
Autism	Differences in microbial compositions, ↑ *Clostridium*, ↑ *Sutterella*	[53]
Chronic fatigue syndrome	Differences in microbial compositions, ↑ *Alistipes*, ↓ *Faecalibacterium*, ↑ *Bacteroides*	[54]
Depression and anxiety	Shifts in *Bacteroidetes*/*Firmicutes* ratios, ↓ *Bacteroides*, ↑ *Clostridium*	[55]
Multiple sclerosis	Differences in microbial signatures, ↑ *Psuedomonas*, ↑ *Mycoplana*, ↑ *Haeomophilus*, ↑ *Blautia*, ↑ *Dorea*	[56]
Parkinson's disease	Presence of *Helicobacter pylori*, ↓ *Prevotellaceae*, ↑ *Enterobacteriaceae*, ↓ *Blautia*, ↓ *Coprococcus*, ↓ *Roseburia*, ↓ *Faecalibacterium*	[37, 57]

[a]Changes between diseased and healthy models with studies conducted *via* analyzing human stool samples and using animal models.

The recent surge in dysbiosis-associated disease research has opened up numerous avenues to treat diseases and maintain health through the GIT microbiota. A notable treatment is fecal microbiota transplantation (FMT), which is an infusion of a fecal suspension from a healthy individual into the GIT of a diseased individual to re-establish a balanced microbial composition and treat a specific disease [58, 59]. FMT has been used primarily to treat *Clostridium difficile* infections (CDI) in patients [60], while it also has seen successes in treating IBD and irritable bowel syndrome (IBS) [61], as well as having some beneficial effects in the improvement of motor and neurologic functions in autoimmune and neurologic disorders [62]. Hence, the use of microbiota to treat diseases enables the concept of developing "microbial pills", much like prescribed medicine, which could be readily administered to treat specific conditions.

Probiotics, along with prebiotics and synbiotics supplements can be seen as the most representative of these "microbial pills" to date, with potential mechanisms in promoting balance in the microbial composition, maintaining GIT health, and treating various diseases [63, 64]. More widely used, probiotics contain beneficial microbiota, for example *Bifidobacterium* and *Lactobacillus*, which when administered in adequate amounts confer health benefits on the host. Prebiotics, on the other hand, are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and the activity of beneficial microbial species already present in the GIT to improve host health. Synbiotics are a combination of both probiotics and prebiotics, to which the host can benefit from an influx of beneficial microbiota from the probiotics, while prebiotics aid in the growth and maintenance of the administered probiotics in the GIT. Collectively, treatments using these supplements have shown improvements in the conditions of patients with intestinal diseases such as CDI, IBD, IBS, and lactose intolerance, as well as in patients with obesity, diabetes, and colorectal and cervical cancer [64, 65].

Research in the contributions of microbiota to human health and disease, including diseases associated with dysbiosis and development of microbial therapies, has shown promising findings. However, the results are indeed still preliminary, limited, and not conclusive, and many microbial functions and features remain unknown or not fully understood. While many studies to date have focused on the associations and correlations of microbiota with diseases, in which various microbial species or groups relate to a healthy or diseased profile, new efforts have turned to investigate the intricate cause-and-effect relationship between GIT microbiota and disease, and particularly the question of whether dysbiosis is the cause or a consequence of disease [39, 66]. It is crucial to elucidate and establish the mechanisms of host-microbiota interactions, such as the identification of key signaling pathways and potential therapeutic targets, which could lead to the development of strategies for maintaining health and treatment of disease [9, 67]. Further characterizations of the correlations, causality, and mechanisms of the GIT microbiota would enable researchers to grasp a more comprehensive understanding of the GIT microbiota and its relationship to host health.

2.4 Studying the GIT microbiota using *in vivo* and *in vitro* approaches

2.4.1 *In vivo* methods in studying the GIT microbiota

The lack of technology capable of conducting systematic and quantitative experiments on complex microbial communities has been a large bottleneck that has hindered the full understanding of the GIT microbiota and its role in health and disease. Investigations involving human subjects represent the best model to study the GIT microbiota, however, they are limited by the inaccessibility and ethical concerns surrounding sampling the human GIT [9, 68]. Sampling the main internal digestive organs such as the stomach, small intestine, and colon require invasive methods while numbers for volunteer patients are low, which have severely limited systematic experimentation.

The majority of microbial studies have thus been conducted with *in vivo* animal models, most commonly with mice. They offer advantages such as low cost of maintenance and high reproductive rates, along with having comparable GIT physiology and anatomy to humans. The accessibility of the intestinal contents at autopsy provides a significant sampling advantage without heavy ethical concerns. Therefore, germ-free, or gnotobiotic, mice can be administered with external microbes, usually done through transplanting a cultured mixture of microbiota or the fecal microbiota of human donors to simulate the microbial profile of the donor [69, 70]. They are primarily used to investigate the contribution of dysbiosis to a particular pathology by comparing mice colonized with the microbiota of diseased patients to mice colonized with the microbiota of healthy controls. They are also used to investigate the effects of specific diets as well as the metabolic effects of drugs and toxins on microbiota, while immunological and neurological studies have also been shown [70]. While using mice, or *in vivo* animal models in general, have enabled important experiments in understanding and probing the dynamic and

complex relationships between the GIT microbiota and the host, these models do differ substantially from humans in terms of physiology, anatomy, and genetics, which may contribute to colonization of different and mice-specific microbial species, and as such the results of these *in vivo* models are not clearly translatable to humans (Fig. 4a-d) [70, 71].

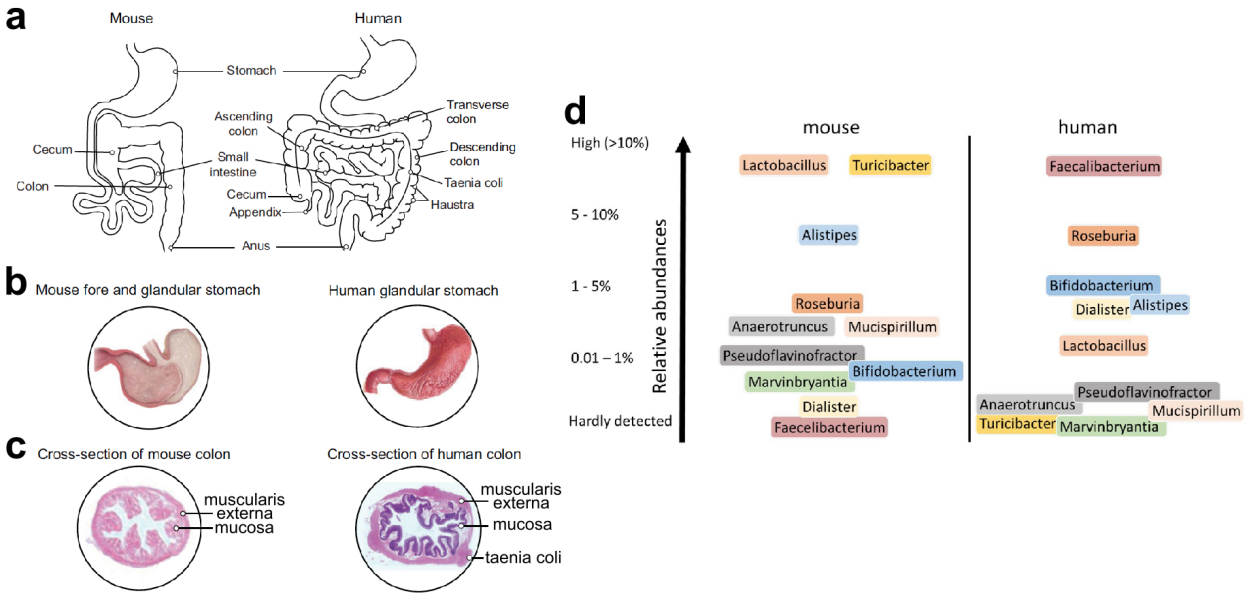

Figure 4 – The physiological and microbial differences between mouse models and humans. (**a**) Comparison of the GIT of the mouse to that of a human. (**b**) The differences in muscle formation of the stomach, whereby the human stomach is lined with a glandular mucosa that secretes gastric acid, while the mouse stomach has two regions, a glandular stomach that secretes gastric acid and a fore-stomach that stores ingested food. (**c**) The different cross-sections of the colon, with the human colon having a thicker muscular wall and mucosa compared to the mouse colon. (d) The relative differences in colonized microbial genera, with distinct profiles for both mouse and human. Images adapted from Nguyen *et al*. and Hugenholtz *et al.* [70, 71].

2.4.2 *In vitro* GIT systems simulating the GIT microbiota

The limitations of *in vivo* models have led researchers to study the GIT microbiota *in vitro*, which enable systematic experimentation and sampling, and provide the means to efficiently control and manipulate specific microbial compositions or responses to uncover their roles and functions [6, 9, 68]. *In vitro* systems range from simple batch cultures without process control to sophisticated large-scale artificial GIT simulators, while recent developments have also included miniaturized organ-on-a-chip systems.

2.4.2.1 Chemostat-based artificial GIT systems

The simplest approaches use batch cultures, which involve the use of a flask or microwell plates, allowing large numbers of microbial samples to be tested in parallel. However, they lack control and complexity in microbial parameters such as pH and continuous transfer of nutrients, and as a result can only be used for short-term studies [9]. The use of chemostat vessels enables a more complex *in vitro* GIT microbiota fermentation system, establishing conditions such as pH control, anaerobiosis, and temperature, and providing continuous nutrients to support the microbial inoculum long-term. By interconnecting several chemostats and operating them simultaneously, a complex and dynamic artificial GIT system can be created, which can simulate particular regions of the GIT, recreate a physiological GIT environment and host digestive functions, and more importantly, simulate the GIT microbiota [6, 9, 68].

Molly *et al.* (1993) developed a dynamic GIT system, referred to as the Simulator of Human Intestinal Microbial Ecosystem (SHIME), which comprises a series of five chemostats with the first two vessels simulating the stomach and small intestine and the last three simulating the colon (ascending, transverse, and descending) (Fig. 5a) [72]. The stomach is seeded with nutrients (food media) and simulates the process of ingestion, while subsequent transfer of the

nutrients downstream to the small intestine along with the addition of pancreatic juice (containing digestive enzymes) mimics the process of digestion. The colon vessels are inoculated with fresh fecal samples, which simulate complex microbiota corresponding to the composition of the donor, and are fed digested nutrients from the small intestine in a continuous manner to effectively support the microbiota. Numerous physiological parameters are mimicked to enable suitable conditions for microbial growth, including an anaerobic environment and controlling pH to *in vivo* ranges within the vessels, while the vessels are also continuously mixed and maintained at 37°C. This system has been widely used in applications such as investigating microbial metabolism and the effects of various probiotics and prebiotics on microbiota [73-76]. McDonald *et al.* (2013) have also developed an *in vitro* GIT system, referred to as the RoboGut, which is comprised of a single chemostat simulating the colon that is multiplexed to run two fecal samples concurrently (termed "twin-vessel") (Fig. 5c) [77]. Similar to the SHIME, nutrients are fed continuously to the colon vessel, while pH control, anaerobic conditions, mixing, and temperature (37°C) are established within the vessels to support the microbiota from the fecal sample. The RoboGut has been used in applications such as investigating bacteriophage communities in human feces and preparing a stool substitute from cultured microbiota derived from a healthy donor for use in FMT to treat recurrent CDI [78, 79].

Another system, developed by the TNO (the Netherlands Organization for applied scientific research) (2000), is the TNO Intestinal Model (TIM) (Fig. 5b) [80, 81]. The TIM is composed of two modules, with TIM-1 simulating the stomach and small intestine, and TIM-2 simulating the colon. TIM-1 is comprised of eight vessels and has the abilities to mimic digestive enzyme and bile secretions, and absorptive capacities similar to *in vivo* conditions, while TIM-2 is comprised of four vessels seeded with fecal matter and is capable of mimicking peristaltic mixing and

metabolite uptake and absorption. The glass-jacketed vessels contain a flexible wall inside, whereby applying pressure on the flexible walls creates peristaltic movements, and they also contain dialysis membranes to enable constant absorption of water and fermentation products. Moreover, pH and temperature are controlled within each vessel. The TIM has been used in applications such as predicting the performance of pharmaceutical drugs and drug products through its absorptive capacity [82], as well as studying the survival of probiotics through the upper GIT (TIM-1) and their effects on the cultured microbiota in the colon (TIM-2) [83-85].

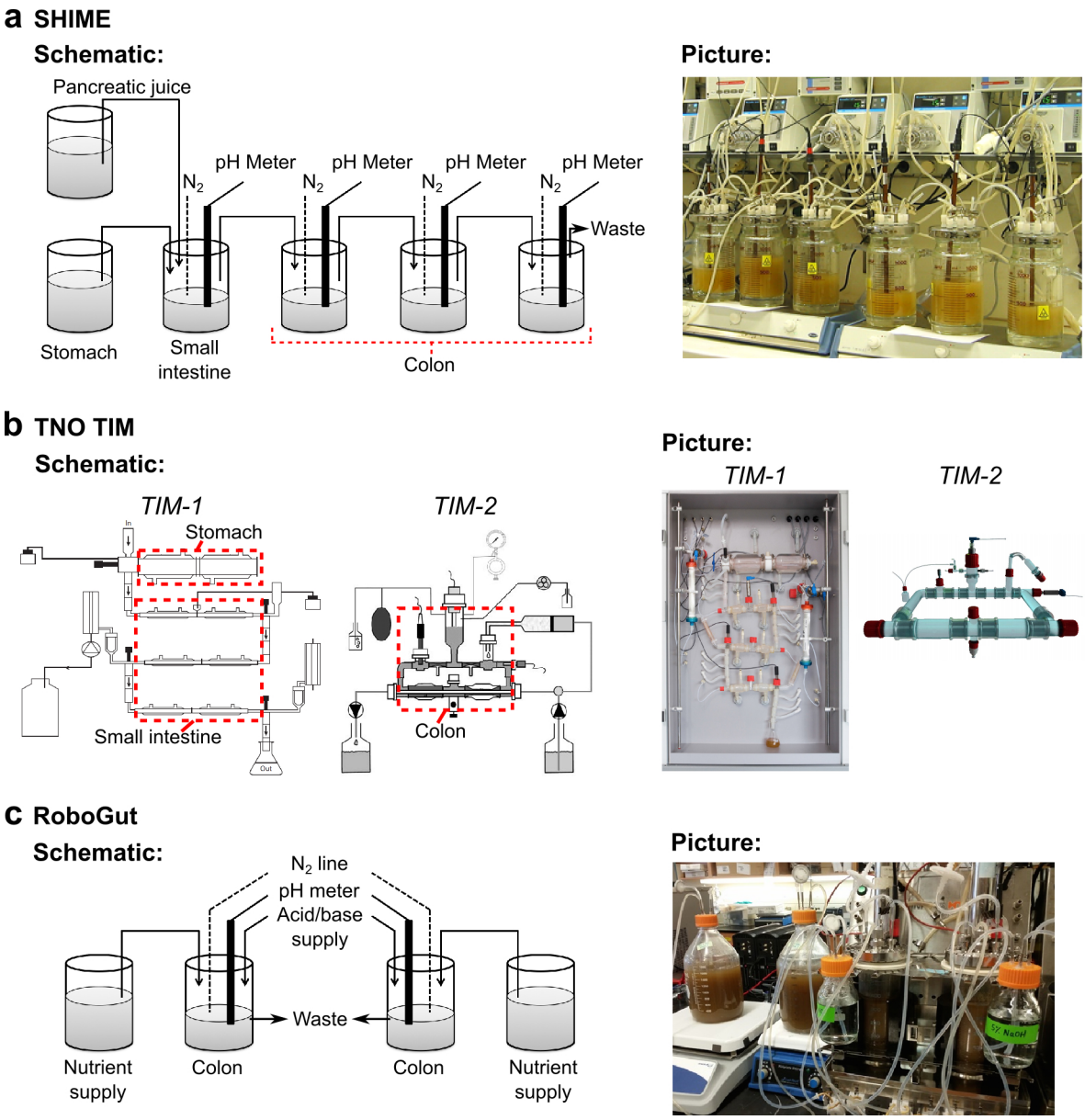

Figure 5 – Chemostat-based artificial GIT systems. (**a**) The SHIME system that is comprised of five vessels simulating the stomach, small intestine, and colon. Parameters such as anaerobiosis, mixing, pH control, and temperature are mimicked within each vessel, while the SHIME is able to support complex microbiota through culturing fecal matter. Adapted from Molly *et al.* [72]. (**b**) The TIM systems comprises of the TIM-1, which simulates the stomach and small intestine, and the TIM-2, which simulates the colon. Peristalsis motions can be mimicked, as well as absorption capacities similar to *in vivo* conditions through dialysis membranes modified in the vessels. Adapted from Verwei *et al.* and Minekus *et al.* [80, 81]. (**c**) The RoboGut system, which is composed of a single chemostat simulating the colon. Similar to the SHIME, the RoboGut can support complex microbiota through culturing fecal matter, while able to perform two experiments with the same sample simultaneously through multiplexing its chemostats ("twin-vessel"). Adapted from McDonald *et al.* [77].

However, these artificial GIT systems face some important limitations such as high cost of acquisition and operation, thus limiting labs to only one instrument. In addition, their large components and construction involving multiple liter-sized vessels, large pumps, magnetic stirrer plates, and pH control modules, require considerable amounts of resources for operation as well as lengthy turnaround time between experiments. In particular, the SHIME and RoboGut are hampered by lengthy microbial stabilization times of 21 and 35 days, respectively, following fecal seeding and are slow to respond to new stimuli [77, 86, 87]. Both systems thus are severely limited in experimental throughput and many trials are conducted once only without replicates, thereby failing to meet the common standard of at least three replicate experiments. In addition, both systems have also shown large shifts between their stable *in vitro* microbial composition and the original *in vivo* fecal composition with over 50% variance, which can result in stabilized states that do not necessarily mimic the microbiota in physiological conditions [72, 77]. While the TIM has shown faster stabilization times following fecal seeding, with less than one week in most cases and a single day in one study, and a more accurate *in vitro* representation of the *in vivo* fecal composition, experiments were performed for only a short period (less than three days), and thus it is unclear whether this system is able to support a stable composition that is representative of *in vivo* conditions, as well as to perform experimentation long-term [81, 88].

2.4.2.2 Miniaturized and chip-based GIT systems

Recently, miniaturized systems and "lab-on-a-chip" devices with reduced sample volumes and accelerated reactions have shown to address certain shortcomings associated with large-scale chemostat-based GIT systems. Developments have concentrated on miniaturized organ-on-a-chip, more specifically gut-on-a-chip devices, which are capable of simulating various physiological functions and components of the GIT in a micro- to milli-liter size range, and offer higher throughput experimentation and versatility in studying microbiota [89, 90]. Mäkivuokko *et al.* (2005) developed a four-stage colon simulator, referred to as the EnteroMix, which is comprised of four milliliter-sized (3-9 mL) vessels that simulate the colon (Fig. 6a) [91]. This system is capable of controlling pH and maintaining anaerobiosis similar to conditions *in vivo*, as well as supporting complex microbiota through culturing fecal matter seeded within the vessels. More importantly, it has the ability to run four experiments simultaneously using the same fecal sample in each vessel. However, experiments could only be performed for short periods and were limited to two days, and it is uncertain whether the microbiota reached steady state, and whether the short experimentation duration was sufficient for the microbiota to respond completely to the simulated environment or testing stimuli [91].

Kim *et al.* (2012) recently introduced a gut-on-a-chip system that simulates the host-microbe interface of the GIT (Fig. 6b) [92]. This one-chamber system is composed of two microfluidic channels separated by a porous membrane that is coated with extracellular matrix, with intestinal epithelial cells on one side and aerobic microbes on the opposite side. This system is capable of mimicking *in vivo*-like peristaltic motions through cyclic stretching of the membrane by applying suction to its vacuum chambers, which are positioned laterally from the main channels. In addition, it was able to support the formations of *in vivo*-like intestinal villi and the co-culture of

intestinal epithelial cells with *Lactobacillus rhamnosus* GG, an aerobic bacterium found in the GIT, while more recently it was used as a model for intestinal bacterial overgrowth and inflammation [92, 93]. Shah *et al.* (2016) developed another gut-on-a-chip system that simulates the host-microbe interface of the GIT, referred to as the human microbial crosstalk (HuMiX), which is comprised of three co-laminar microfluidic channels that host intestinal epithelial cells, microbes, and perfusion media, respectively (Fig. 6c) [94]. A nanoporous membrane separates the epithelial cells and microbes, which allows for interaction and exchange of soluble factors. A distinct feature of this system is that epithelial cells are supplied with oxygenated nutrients across a microporous membrane while bacteria can be cultured in anaerobic conditions, as shown with co-culture experiments involving both *Lactobacillus rhamnosus* GG (facultative anaerobe) and *Bacteroides caccae* (obligate anaerobe). In addition, preliminary data on the co-culture between *Lactobacillus rhamnosus* GG and immune cells (CD4+ T cells), which were inoculated in the perfusion channel, was also shown briefly in this system [94].

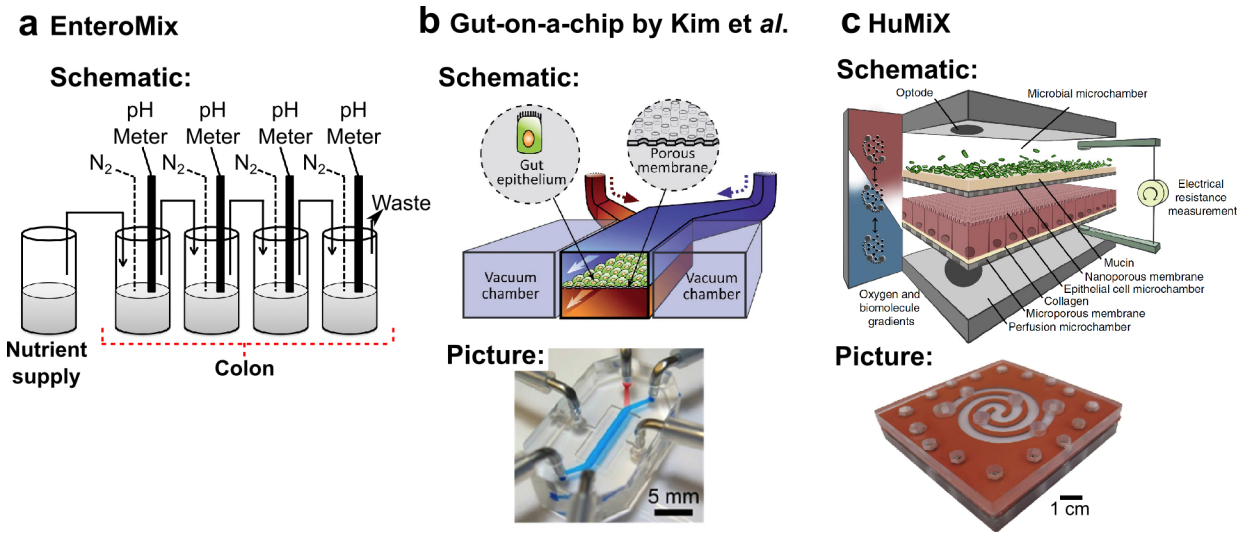

Figure 6 – Miniaturized and chip-based GIT systems. (**a**) A milliliter-sized EnteroMix system that simulates the colon and is able to perform four replicate experiments with the same fecal sample simultaneously [91]. (**b**) A gut-on-a-chip microfluidic system that simulates the host-microbe interface through a two-layer design separated by a membrane with intestinal epithelial cells and microbes on each side. Peristaltic motions can be mimicked through cyclic suction of vacuum chambers located laterally from the culture chambers. Adapted from Kim *et al.* [92]. (**c**) A gut-on-a-chip, HuMiX, microfluidic system simulating the host-microbe interface through a three-layer design. Intestinal epithelial cells and microbes are separated by a nanoporous membrane, while the epithelial cells are separated by a microporous membrane to the perfusion channel. Epithelial cells can be grown aerobically, while microbes can be grown anaerobically. Adapted from Shah *et al.* [94].

However, the limited relevance of physiological and microbial parameters of these gut-on-a-chip systems has been a major drawback. Both microfluidic systems have only shown support for a single bacteria strain in their microbial culture channels, which does not capture the diverse and complex microbiota inhabited in the GIT (> 1000 species) [92, 94]. The simple flow through design, along with the single reaction chamber (< 1 mL) of these chip-based systems do not afford an accurate simulation of the numerous crucial physiological parameters and functions that define the GIT, such as different intestinal regions and their distinct residence times, supply of digestive enzymes for digestion, controlling pH to region-specific ranges, and mixing. In fact, these systems target only one parameter, the host-microbe interface, of the many that are featured

in the GIT, and thus could make it difficult to translate findings to the complex conditions *in vivo*. In addition, it is challenging for these simple chip-based systems to perform more comprehensive and advanced studies, such as investigating specific complex microbial compositions, interplays, and responses as pertained to various dynamic conditions and perturbations of the GIT.

To address the aforementioned drawbacks of current chemostat-based as well as chip-based systems, we proposed and developed an *in vitro* system that is miniaturized, while able to recapitulate numerous complexities of the GIT, such as different regions represented by 40 mL mini-bioreactors, and a physiological environment that includes mixing, pH control, and establishing anaerobiosis. More importantly, the system has the capability to support complex microbiota and enable accelerated microbial stabilization (< 5 days) and responses (< 24 h). The development of such a system allows a simulation of the GIT microbiota that is more representative of *in vivo* conditions and facilitates systematic experimentations that may aid in advancing research on the GIT microbiota.

3 Gastrointestinal Tract-in-a-Box

This chapter is presented as a manuscript, which is currently under internal review by the authors. Once completed, the manuscript will be submitted for publication in a peer-reviewed journal.

Gastrointestinal Tract-in-a-Box

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3.1 Abstract

The microbiota of the human gastrointestinal tract (GIT) has emerged as an important contributor to human health and disease, however, many functions and features of the microbiota in disease remain poorly understood due to the inaccessibility in sampling the GIT. While *in vitro* GIT systems simulating the GIT microbiota enable systematic experimentation, the performance of current systems is limited owing to their size, cost, throughput, or minimal physiological relevance. Here, we present a miniaturized GIT system that is enclosed in a box (GITBox), which comprises of three 40 mL mini-bioreactors that simulate the stomach, small intestine, and colon, and recapitulates critical physiological features of the GIT necessary for microbial growth, including mixing, pH control, and establishing anaerobiosis and temperature. The growth of single strain microbial species was first demonstrated over a period of 180 h. A cyclic 4 h feeding procedure, involving transfers of food media and digestive enzymes mimicking the human digestive process, allowed for the continuous culture of a complex microbiota community derived from a human fecal inoculum. The microbiota, which includes various facultative and obligate anaerobic communities, stabilized within five days, while faithfully retaining the original fecal composition. Next, the GITBox was used to study the response of the stabilized microbiota to antibiotics and then probiotics in 14-day trials, whereby each targeted community exhibited quick (< 24 h) collapse and recovery responses upon each treatment. The GITBox is a powerful tool for studying and simulating GIT microbiota and will

be useful to dissect the interplay between microbiota compositions, diets, and drugs, and may provide a deeper understanding of the GIT microbiota in human health and disease.

3.2 Introduction

The human gastrointestinal tract (GIT) is a digestive organ that consists of several distinct regions including the stomach, small intestine, and colon, and hosts an environment involving functions such as pH, mixing, supply of digestive enzymes, and region-specific transit times [3]. The GIT, being rich in nutrients and spacious (*e.g.* estimated surface area of 300 m^2), represents a major colonization site for microorganisms [2]. These microorganisms, composed mostly of bacteria, along with archaea, viruses, and eukaryotes, are abundant (10^{14}) and form a diverse and complex microbiota, which is overall referred to as the GIT microbiota. In particular, the GIT microbiota has emerged as a major influence in human health and disease [1, 2]. Studies have associated dysbiosis of the GIT microbiota to various disease conditions such as obesity [31], inflammatory bowel disease (IBD) [34], diabetes [33], cancer [36], autoimmune diseases [35], and neurodegenerative diseases [37]. Moreover, microbial therapies, based on modulating the GIT microbiota, to treat certain diseases have also been developed, such as fecal transplants [58] and probiotic supplements [65]. However, the inaccessibility of the human GIT for sampling has significantly limited systematic experimentation, rendering the full understanding of many microbial functions and features underlying the disease or therapy to be largely enigmatic and complex [95, 96].

The development of *in vitro* GIT systems that can recapitulate critical physiological aspects of the human GIT to support the complex GIT microbiota enables systematic experimentation, providing the means to efficiently study, control, and manipulate specific GIT microbiota

compositions or responses to uncover their roles and functions [6, 68, 88]. The most notable systems include the Simulator of Human Intestinal Microbial Ecosystem (SHIME) [72] and the RoboGut [77], which are chemostat-based systems that are seeded with human fecal matter (10% in physiological saline) and support the growth of complex microbiota through culturing. These systems enable *in vitro* testing and studying of the microbial response to different foods, drugs, or supplements. They are comprised of a series of reaction vessels that simulate various regions of the GIT, with the SHIME simulating the stomach, small intestine, and colon, and the RoboGut simulating solely the colon. Moreover, numerous physiological parameters are mimicked to enable suitable conditions for microbial growth, including pH control, mixing, anaerobiosis, and temperature. The transport of nutrients (containing food and digestive enzymes) along the GIT is replicated by serially transferring aliquots from one reactor to the next, and repeating this procedure at regular time intervals, effectively feeding and supporting the growth of fecal microbiota seeded in the colon reactor. These systems however face some important limitations such as high cost of acquisition and operation, and labs typically only have one instrument. Moreover, their large components and construction involving multiple liter-sized vessels (> 0.5 L) along with large pumps, magnetic stirrer plates, and pH control modules, require considerable amounts of resources for operation as well as lengthy turnaround time between experiments. In addition, long microbial stabilization times of 21-35 days following fecal seeding create an initial time lag and slow down the experimental throughput as these systems take a long time to respond to a new stimuli [72, 77, 87]. Many trials are thus conducted only once without replicates, thereby failing to meet the common standard of at least three replicate experiments. Moreover, the *in vitro* microbial composition was considerably shifted compared to the original

in vivo fecal composition (> 50% variance), thus questioning the physiological accuracy of these systems [72, 77, 86, 87].

Miniaturized systems such as lab-on-a-chip devices have helped reduce the resource consumption while increasing the throughput of numerous chemical and biological processes. More recently, organ-on-a-chip systems, in particular gut-on-a-chip systems that recreate miniaturized versions of the GIT have been proposed [89, 90]. Mäkivuokko *et al.* developed a miniaturized GIT system comprised of four milliliter-sized (3-9 mL) vessels that simulate the colon [97]. This system recreates physiological conditions such as pH and anaerobiosis, supports complex microbiota through culturing fecal matter seeded within the vessels, and allows four experiments to be performed simultaneously using the same fecal sample in each vessel. However, the duration was limited to two days and it is unclear whether the microbiota reached a steady state and whether it was representative of *in vivo* conditions. Ingber and colleagues introduced a gut-on-a-chip system that includes mammalian cells and recreates a minimal host-microbe interface [92]. This one-chamber system is composed of two microfluidic channels separated by a porous membrane, with intestinal epithelial cells on one side and aerobic microbes on the opposite side. *In vivo*-like peristaltic motions were mimicked through cyclic stretching of the membrane by applying suction to lateral vacuum chambers connected to the membrane. This system supported the formations of *in vivo*-like intestinal villi [92], and more recently, was used as a model for intestinal inflammation [93]. Shah *et al.* introduced another system that simulates the host-microbe interface, which is comprised of three microfluidic channels that host intestinal epithelial cells, microbes, and perfusion media, respectively [94]. Similarly, a porous membrane separates the epithelial cells and microbes, and allows for interaction and exchange of soluble factors. A distinct feature is that microbes can be cultured in

anaerobic conditions, while epithelial cells are supplied with oxygenated nutrients across a second porous membrane. Both microfluidic GIT systems however have only shown support for a single bacterial strain and use a continuous sample flow through procedure with one microliter-sized chamber (< 1 mL). Thus, they do not capture the parameters that define the GIT such as the microbial complexity, distinct regions and their specific residence times and pH ranges, supply of food and digestive enzymes, and active mixing.

Here, we present a gastrointestinal tract-in-a-box (GITBox) that comprises of three interconnected mini-bioreactors mimicking the stomach, small intestine, and colon, and supports complex microbiota through culturing human fecal inoculum, all enclosed in a small box. The working volume of the reactors is 30-34 mL, representing over an order of magnitude of miniaturization compared to existing chemostat-based systems. The GITBox is programmable and computer controlled, uses N_2-pulsing for both mixing and establishing anaerobiosis, provides pH control through a proportional algorithm, maintains temperature, and is outfitted with sampling ports for retrieving aliquots from the reactors. The support and stabilization times of microbiota in the GITBox was characterized, including individual microbiota species and a complex microbiota community from a fecal inoculum *via* continuous culturing that involves timed transfers of food and digestive enzymes mimicking the digestive process, in multiple replicate experiments. To illustrate the potential of the GITBox for drug studies, the microbiota was treated with antibiotics followed by probiotics, and the microbial populations were monitored at regular intervals before, during, and after administration.

3.3 Results and discussion

3.3.1 GITBox system design

The GITBox design is comprised of three serially connected 40 mL mini-bioreactors, simulating the stomach, small intestine, and colon (Fig. 7a,b). The reactors were connected to one another using flexible Tygon tubing and peristaltic transfer pumps, which transferred defined volumes between the reactors at pre-programmed time intervals to simulate the digestive process (Fig. 7a; Supplementary Information Fig. 12a). Each reactor was connected to a gas line controlled by a solenoid valve for sparging N_2 into the reactor for both mixing and establishing anaerobiosis. The small intestine and colon reactors were each equipped with a pH port integrated with a pH meter and inlets for supplying acid and base solutions. A "pancreatic reservoir" was connected to the small intestine and supplied pancreatic enzymes for digestion. A heating pad was added to establish physiological temperature (37°C). Sampling ports were formed by drilling holes into the reactors and sealing them with a septum cap, in which a syringe needle could be inserted to draw aliquots from the reactors. The reactors were assembled on top of the inverted lid of a polypropylene box lined with insulation foam, and enclosing the system with the box itself helped maintain anaerobiosis and homogeneous temperature. The box was outfitted with three capped ports that aligned with the sampling ports of the reactors. Hence, sampling could be performed without removing the box and without detectable disturbance to the anaerobic conditions and temperature inside the box (Supplementary Information Fig. 12b).

Arduino microcontrollers (UNO and MEGA), connected to a PC, were used for real-time monitoring and control of each electro-mechanical component (Fig. 7c). Two microcontrollers were used to prevent interruption errors, and UNO controlled the pH, which included pH meters and pumps, while MEGA controlled fluid transfers and mixing *via* peristaltic transfer pumps and a solenoid valve, respectively. A circuit connecting the microcontrollers to the various

components reduced voltage fluctuations and prevented component damage [98]. The heating pad was controlled by an external digital thermostat controller.

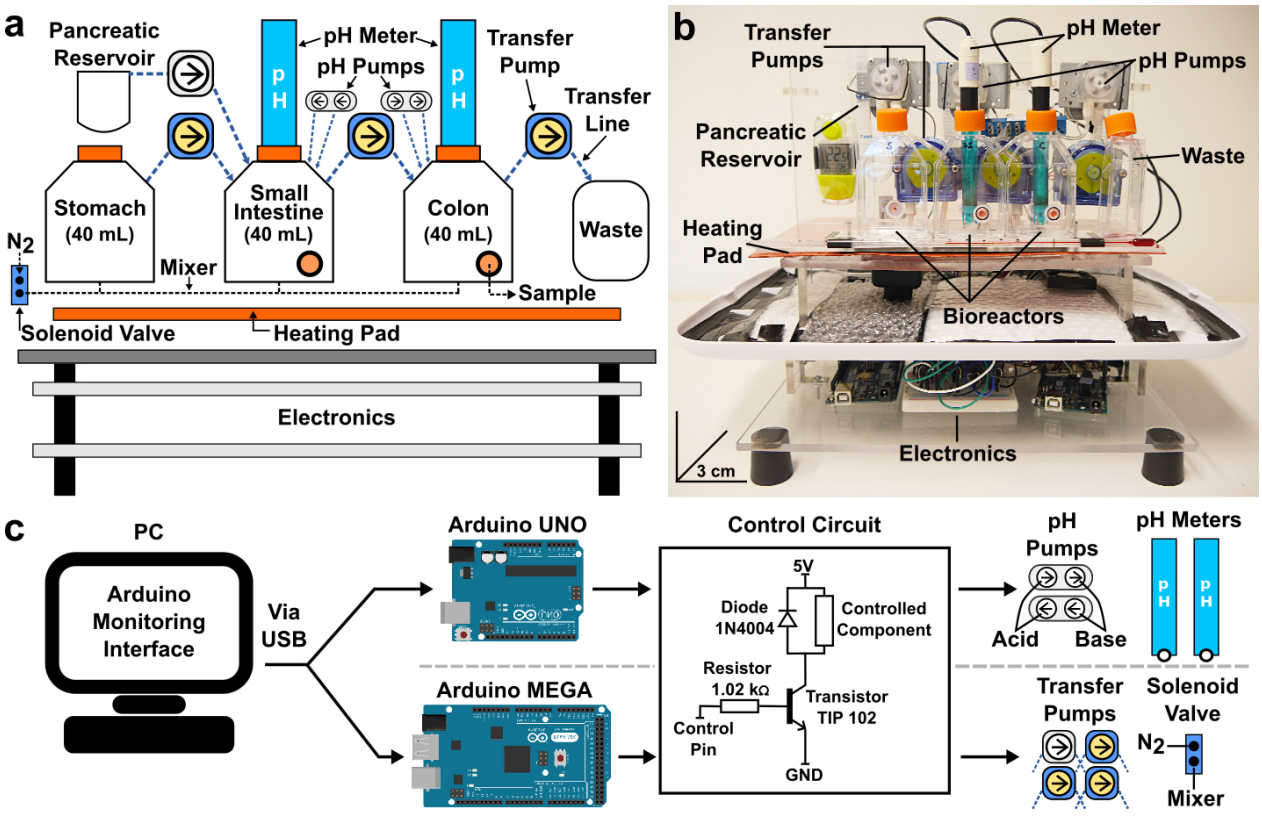

Figure 7 – The Gastrointestinal Tract-in-a-Box. (**a**) Diagram and (**b**) picture of the GITBox, which is comprised of three 40 mL mini-bioreactors that simulate the stomach, small intestine, and colon, and recreates functions for mixing, pH control, and establishing anaerobiosis and temperature. (**c**) Schematic of the control, programming, and monitoring system of the GITBox.

3.3.2 Characterization of the GITBox

Mechanisms for mixing and pH control, along with anaerobiosis, which are critical in establishing physiological conditions suitable for microbial growth, were characterized.

It is essential to homogenize ingested foods and secreted enzymes for digestion in the GIT. Mixing was mimicked, and realized, by automated sparging of N_2 into the reactors, in which a two-way solenoid valve connected to an N_2 gas line and a mixer needle in each reactor delivered pulsed N_2 bubbles at 20 kPa for 0.5 s, followed by an off-time of 2.5 s, leading to effective mixing of the interior contents (Fig. 8a).

The pulsed N_2 further contributed to establishing anaerobiosis in the reactors, which is important for microbial growth since over 99% of GIT microbiota are anaerobes [5, 99]. Anaerobic indicator strips were used and helped establish that an oxygen-free atmosphere was achieved within five minutes and could be rapidly re-established over extended periods (~10 h) (Supplementary Information Fig. 13a,b).

The GIT is characterized by different pH ranges, which modulate the activity of digestive enzymes and microbial growth. The pH was measured using glass-electrode pH meters and controlled by supplying acid and base solutions with peristaltic pumps. An On/Off proportional threshold control algorithm was implemented, which dispensed a set amount of acid or base solution based on the difference between the measured and set-point pH, and allowed for independent pH fluctuations within set-point ranges (thresholds) to mimic pH levels and conditions *in vivo* (Fig. 8b). Characterization of the pH control system was performed by adjusting for higher and lower pH levels in a fecal solution by adding 0.08 mL of either 0.25 M NaOH (base) or HCl (acid), showing rapid (< 1 min.) and precise pH adjustment, with minimal over-shooting error during the adjustment period (Fig. 8c,d).

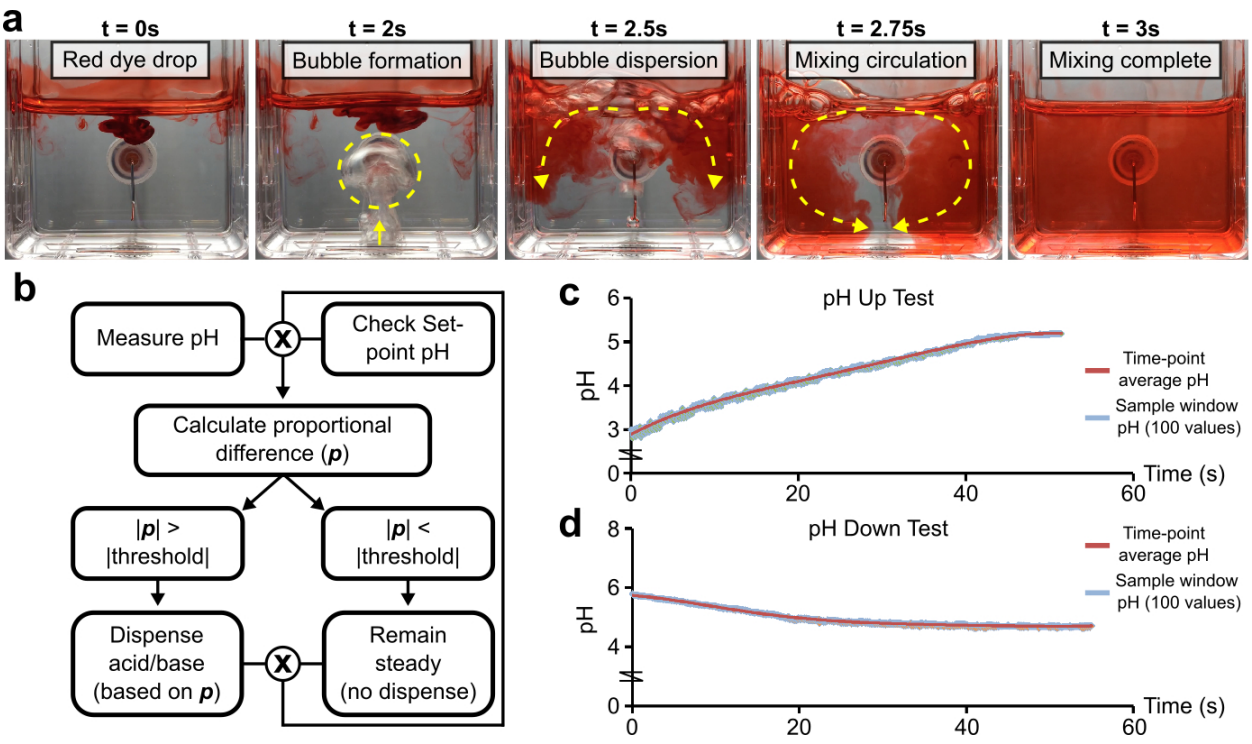

Figure 8 – Characterization of the GITBox system. (**a**) Time lapse pictures of the nitrogen-pulsed bubble mixing mechanism. Yellow arrows depict the bubble pulsation driven mixing. (**b**) Process flow diagram of the On/Off proportional threshold pH control algorithm. (**c**) pH up test from pH 3 to 5 and (**d**) pH down test from pH 6 to 4.5 in a fecal solution, showing rapid and precise adjustment. The measured pH was computed as an average of 100 measurements within the sample window.

3.3.3 Culture of single strain microbial species in the GITBox

To test whether the GITBox can support microbial growth, we initially performed parallel batch cultures of single strain *Bifidobacterium longum* (*B. longum*), *Bifidobacterium infantis* (*B. infantis*), and *Lactobacillus rhamnosus* (*L. rhamnosus*) (Fig. 9a). Each bacterium (30 mL) was seeded in an individual reactor and grown for 180 h with N_2-pulsed mixing and anaerobic conditions, and maintained at 37°C. The GITBox supported rapid and abundant growth of all three bacteria, with *B. longum*, *B. infantis*, and *L. rhamnosus* entering their exponential growth phases within 30 h (Fig. 8b-d). Subsequently, stabilization was quickly achieved with *B. longum* (Fig. 9b), *B. infantis* (Fig. 9c), and *L. rhamnosus* (Fig. 9d) stabilizing at approximately 100 h ($4.67 \pm 0.21 \times 10^6$ CFU/mL), 70 h ($6.09 \pm 0.19 \times 10^6$ CFU/mL), and 75 h ($5.75 \pm 0.82 \times 10^6$ CFU/mL), respectively.

Standard static incubator cultures, in which reactors were sealed and placed in an incubator with manual N_2 flushing at hourly intervals, were compared to the GITBox cultures. The growth and stabilization characteristics of all three bacteria were comparable (Fig. 9b-d). The GITBox cultures, however, exhibited higher population counts than those of their static culture counterparts, suggesting that the GITBox supported microbial growth at higher densities. These results demonstrate that the GITBox was capable of recapitulating a suitable microbial environment to effectively support single strain growth and stabilization.

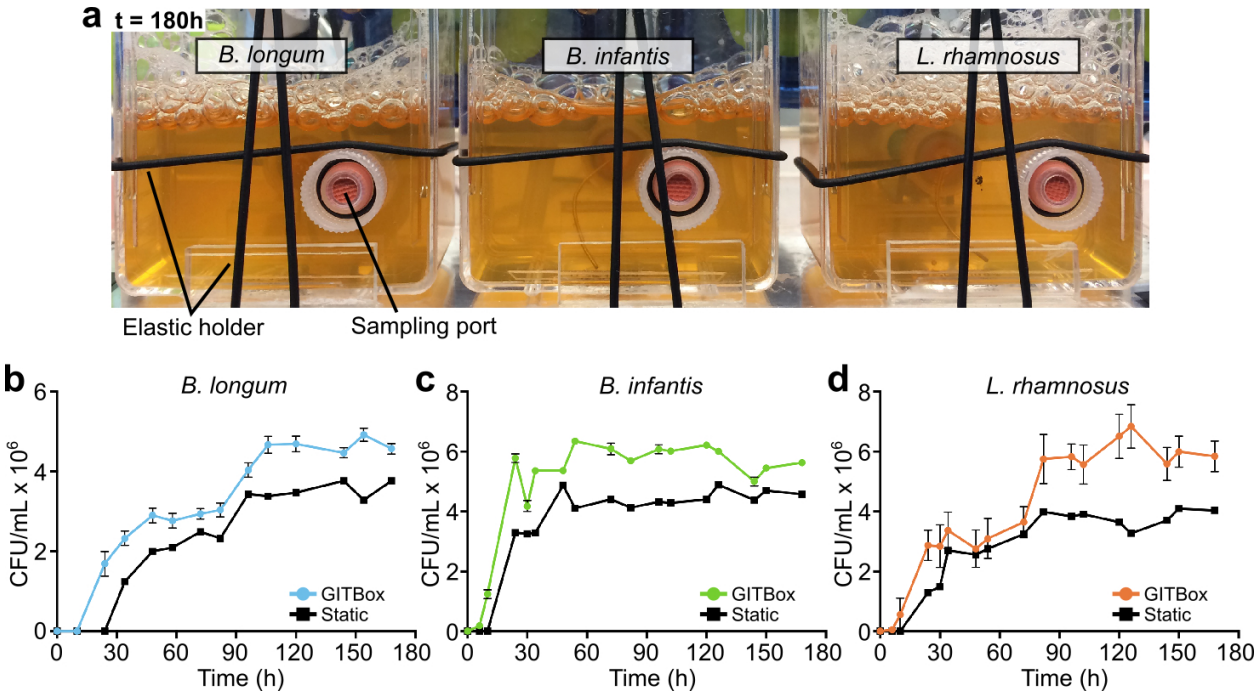

Figure 9 – GITBox culture of single strain microbial species. (**a**) Picture of the GITBox reactors seeded with *B. longum*, *B. infantis*, and *L. rhmanosus* in parallel cultures at time 180 h. (**b**) *B. longum*, (**c**) *B. infantis*, and (**d**) *L. rhamnosus* growth in the GITBox with N_2-pulsed conditions, and in static incubator culture with hourly N_2 flushing. Graphs in **b-d** display average with standard error of the mean. $N = 3$.

3.3.4 Culture procedure for complex microbiota

Following the establishment of the capability to support the growth of individual microbial species in the GITBox, we investigated whether the GITBox could support complex microbiota through culturing fecal matter from a human donor [6]. In existing chemostat-based systems, liter amounts of nutrients are transferred to the fecal inoculum in day-length intervals. Using such large volumes suggests that the fecal sample is constantly diluted, and consequently the initial microbial growth is impeded, resulting in slow stabilization times (21-35 days) [72, 77, 87]. The diluted sample suggests that the initial fecal composition could also be altered over time, as shown with the shifted (> 50% variation) *in vitro* compositions of the chemostat-based systems [72, 77, 87].

We implemented a continuous feeding procedure that mimics the human digestive process (Fig. 10a,b). We initially seeded the stomach, small intestine, and colon reactors with acidified food media (pH 2), a mixture of food and digestive enzyme solution, and a fresh fecal solution (10% in physiological saline), respectively. These solutions simulated the native contents in the specific regions of the GIT. Each cycle began by incubating for 0.5 h in the stomach, simulating ingestion of food. The food (3 mL) from the stomach, along with the addition of digestive enzymes (1 mL), was then transferred to the small intestine and incubated for 0.5 h, while the pH was controlled between 7.0-7.4, simulating digestion. Thereafter, 4 mL of the digested mixture was transferred to the colon and incubated for 3 h, with the pH maintained between 6.5-6.9 that simulated the conditions of the descending colon, effectively feeding the fecal microbiota. After transferring from the small intestine to colon, a 4 mL aliquot was removed from the colon and delivered to the waste, except at times of sample collection, whereby 1 mL of sample was retrieved prior to transferring 3 mL to waste. The feeding routine was repeated automatically on a 4 h cycle for the duration of the experiment, while samples for analysis were collected every 24 h.

The content volumes of the small intestine and colon were maintained between 30-34 mL, a scale-down of more than tenfold of existing chemostat-based systems [72, 77], and in line with the small intestine and colon of adult humans [4, 100]. The stomach functioned as a food reservoir, which was manually loaded with fresh food (40 mL) daily, and thus did not have a constant volume. The transfer volumes were less than 15% of the working volumes, providing a continuous, frequent, and sufficient supply of nutrients to the microbiota to enable steady growth and adequate waste removal. The transfer principle maintained the original contents in each

reactor without significant dilutions, which may potentially aid in faster stabilization and less alterations to the original fecal composition.

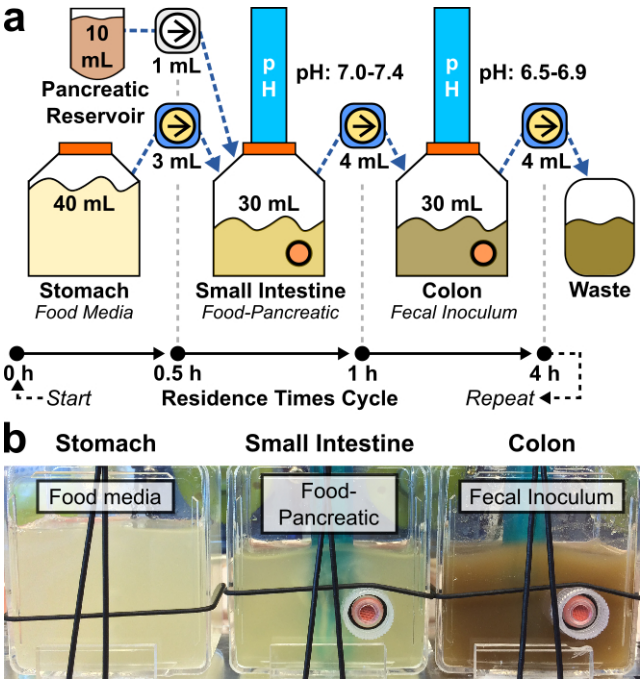

Figure 10 – The fecal culture procedure of the GITBox. (**a**) Schematic of the culture procedure, which mimics the human digestive process, showing interconnected reactors with their specific contents and volumes, transfer volumes, pH conditions, and the residence time cycle. (**b**) Picture of the interconnected reactors seeded with food media, a food and pancreatic mixture, and a fecal inoculum, simulating the native contents in the specific regions of the GIT.

3.3.5 Culture of complex microbiota in the GITBox

The GITBox was used to culture human fecal inoculum and the microbial stabilization time and composition were evaluated by monitoring five facultative anaerobic (aerobic) communities including the *Enterococcus* genus, *Lactobacillus* genus, *Streptococcus* genus, total Coliform group, and total aerobic group, as well as four obligate anaerobic (anaerobic) communities including the *Bifidobacterium* genus, *Clostridium* genus, total Gram-Negative group, and total anaerobic group, within five sequential 7-day trials. Samples (1 mL) were taken daily from the colon and assayed by colony counting on exclusion agar plates.

Population stabilization (CFU/mL) was observed for all communities within 3-5 days of culture (Fig. 11a,b). We then analyzed the daily population variations, which showed between 0-7% change for all communities (Supplementary Information Table 3). The largest variations, between 2-7%, were found between days 2 and 3, which likely reflected initial microbial growth and the transition of *in vivo* fecal microbiota to an *in vitro* culture, and subsequent *in vitro* stabilization (Fig. 11a,b; Supplementary Information Table 3). Comparison between days 5 and 6 showed minimal variations with ranges between 0-2%, while analysis of longer-term culture with 12-day duration showed very little change beyond day 5 (0-4%), indicating that the microbiota had stabilized (Supplementary Information Table 3). Moreover, comparisons to single strain cultures (Fig. 9b-d) showed that the *Lactobacillus* genus and single strain *L. rhamnosus* both stabilized in approximately 3-4 days, while the *Bifidobacterium* genus and single strains *B. longum* and *B. infantis* all stabilized between 4-5 days. These patterns strongly suggest that the complex microbiota stabilized within 5 days.

Next, we evaluated the microbial composition in the GITBox after the *in vivo* to *in vitro* transition. Comparison of the stabilized *in vitro* composition on day 6 in the GITBox to the

composition of the original *in vivo* human fecal matter (day 0) showed a population variation less than 10% for all communities (Fig. 11a,b; Supplementary Information Table 3). These results indicate that the GITBox supported, over extended periods of time, a complex microbiota representative of the original fecal composition.

3.3.6 Perturbation of complex microbiota

Next, we studied the response of the microbiota to antibiotics (ampicillin and gentamicin) as well as probiotics (*L. rhamnosus*, *L. fermentum*, and *B. infantis*), which were administered after stabilization, within three sequential 14-day trials (Fig. 11c,d). The response of the communities was tracked by analyzing daily samples (1 mL) by colony counting on exclusion agar plates.

After a 5-day stabilization period, a mixture containing food media and 0.185 mg/mL doses of ampicillin and gentamicin was fed to the microbiota for 24 h. Most microbial communities were sensitive to the antibiotics and collapsed within 24-48 h, including all "beneficial" genera (*Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*), and total Coliform, Gram-Negative, aerobic, and anaerobic groups (Fig. 11c,d). In contrast, the *Clostridium* genus population remained relatively unaffected (Fig. 11d). To examine whether these communities could be recovered, we used probiotics, consisting of *Lactobacillus* and *Bifidobacterium* (1.0×10^8 CFU/mL for each strain) added to food media, and administered them daily in every feeding cycle, between days 7 and 14. The majority of microbiota responded quickly, showing signs of re-establishment and recovery within 24 h of probiotic treatment on day 8. The *Streptococcus* and *Bifidobacterium* genera exhibited additional delays, taking 48 h to show signs of recovery (Fig. 11c,d), while the *Clostridium* genus, again, showed minimal response (Fig. 11d). Continuous treatment of probiotics aided in steady microbial recovery as analyses on day 14

showed that all microbial communities were representative of their initial stabilized compositions, with population variations of less than 5% when comparing days 5 and 14 for all communities (Supplementary Information Table 4).

Between experiments, we observed a larger range in the shift of the complex microbiota populations after antibiotics treatment, as shown on day 7 (Fig. 11c,d). Regardless, the GITBox could enable further studies to identify the potential microbial interplays or mechanisms underlying the different types of shifts in microbial populations or responses when perturbed by external stimuli.

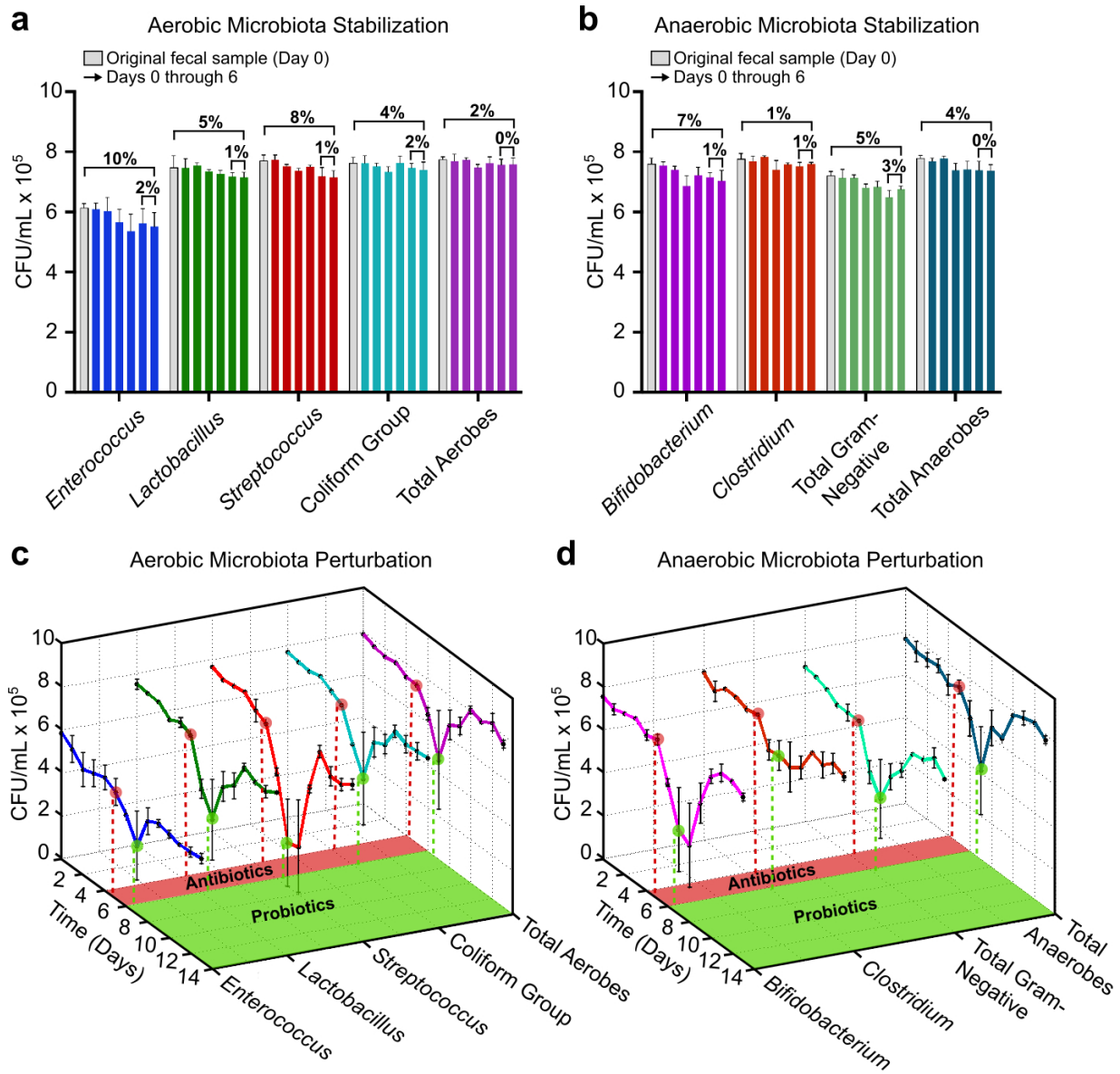

Figure 11 – The culture and perturbation of complex microbiota in the GITBox. (**a**) Aerobic and (**b**) anaerobic microbiota populations from day 0 through day 6, showing population stabilization within five days of culture, while preserving the original fecal composition. Response of (**c**) aerobic and (**d**) anaerobic microbiota following the addition of antibiotics and probiotics in the food media. Graphs in **a-d** display average with standard error of the mean. $N = 5$ and 3 for the stabilization and perturbation trials, respectively.

3.4 Conclusion

We introduced the GITBox, which is comprised of three 40 mL reactors simulating the stomach, small intestine, and colon, and recreates a physiological GIT environment that includes mixing, pH control, and establishing anaerobiosis, and temperature. The growth of single strain microbial species was first demonstrated over a period of 180 h. A feeding procedure, based on a 4 h cycle that mimics the human digestive process, was configured, and allowed the continuous culture of complex microbiota derived from a human fecal inoculum. Stabilization was achieved within five days, while an *in vitro* composition that was representative of the original fecal matter was retained (< 10% variance). The capabilities of the GITBox for studying the response to antibiotics and probiotics were demonstrated, in which the complex microbiota community exhibited fast microbial responses (< 24 h) upon each treatment.

Multiple sequential experimentations (N = 5 replicates for stabilization tests, N = 3 replicates for perturbation trials) were conducted, which to the best of our knowledge is the most complex study in providing an adequate number of replicates in an actively controlled GIT system. In this study, sampling was performed only every 24 h, and the early response of the microbial communities was not captured, which could be addressed by using more frequent sampling rates (*e.g.* every 8 h) and reducing the sampling volume from 1 mL to ~300 µl so as to not deplete the content in the reactor. The modularity, relative low cost, and small footprint of the GITBox opens the way for operating multiple systems in parallel, allowing both replicates and controls to be conducted simultaneously while increasing the experimental throughput. Owing to the automation and programmability of the GITBox, operational parameters such as different feeding cycles or diet schemes, pH conditions, and mechanical mixing conditions could readily be tested.

The faithful maintenance of microbial communities and rapid response time of the GITBox should be useful in modeling healthy and diseased GIT microbiota – for example in obesity – to study the effects of foods, drugs, or supplements. The GITBox could also be used to study and optimize healthy stool substitutes for fecal transplants, whereby microbiota derived from a healthy fecal donor could cultured and optimized with various beneficial microbial strains or additives, which could then be further isolated for transplantation.

Further miniaturization of the GITBox could help further parallelization and improve throughput, but will require replacing the bulky glass-electrode pH meters used currently, and identifying or developing small, affordable, and robust pH meters capable of operating reliably over weeks while immersed in a fecal inoculum. It will also be critical to verify that a complex microbiota community representative of *in vivo* conditions can be supported in smaller reactors. Finally, an important step will be to incorporate mammalian cells and a microbial-epithelial interface, which will open up the study of host-microbe interactions [102].

3.5 Materials and methods

3.5.1 GITBox fabrication and assembly

The GITBox is comprised of three 40 mL mini-bioreactors (Fisher Scientific, Whitby, ON) representing the stomach, small intestine, and colon, interconnected *via* peristaltic pumps (Williamson, UK) and Tygon tubing, and altogether assembled onto a custom laser-cut poly(methyl methacrylate) (PMMA) manifold (McMaster-Carr, USA). The reactors were modified with ports for a mixing needle. The ports were made by drilling holes into the reactors and subsequently sealing them with a septum cap, while the mixing needle was made by modifying a syringe needle (Fisher Scientific, Whitby, ON). The small intestine and colon included an additional port for a pH meter (RobotShop, Mirabel, QC), as well as inlets for pH acid and base peristaltic pumps (Williamson, UK). Sampling ports were fashioned onto the reactors by drilling holes and sealing them with a septum cap. A 10 mL culture tube (Fisher Scientific, Whitby, ON) and a waste reservoir (Fisher Scientific, Whitby, ON) were connected to the small intestine and colon, respectively, *via* peristaltic pumps (Williamson, UK). A heating pad (Cole-Parmer, Montreal, QC), which was connected to a digital thermostat controller (McMaster-Carr, USA), was fashioned onto the PMMA manifold. All the components were enclosed in a polypropylene box (Sterilite, Montreal, QC) that was foam-insulated and contained a viewing window as well as sampling ports that were in line with the sampling ports of the reactors. The electronics platform, which resided below the closed box, consisted of two Arduino microcontrollers (RobotShop, Mirabel, QC) that were connected to each electrical component *via* a control circuit soldered onto a circuit board. Programming (C language) was done on the PC Arduino interface program.

3.5.2 Characterization of the GITBox

Characterization of the mixing mechanism, establishment of anaerobiosis, and pH control system are shown in Fig. 8. The mixing mechanism was characterized using 30 mL deionized water with the addition of 0.5 mL red food dye. The pH control system was characterized using 30 mL of 10% total human fecal matter in physiological saline, and supplying 0.25 M HCl or NaOH. Anaerobic conditions were characterized with anaerobic indicator strips (Sigma, Oakville, ON) inserted in a reactor.

3.5.3 Culture of single strain microbial species

The strains *Bifidobacterium infantis* 702255 and *longum* 71123, and *Lactobacillus rhamnosus* 5221 were obtained from NCIMB (National Collection of Industrial Food and Marine Bacteria, UK) culture collection. Cells were maintained in Man-Rogosa-Sharpe (MRS) media (Sigma, Oakville, ON) with 20% glycerol at -80°C until use. Cultures were started by streaking frozen stocks on MRS-agar plates and grown at 37°C for 18-48 h aerobically (*Lactobacillus rhamnosus*) or in anaerobic culture conditions (*Bifidobacterium* strains) established with anaerobic atmosphere generation bags (Sigma, Oakville, ON). Cultures were expanded in liquid MRS culture for 18 h for *Lactobacillus rhamnosus* and 48 h for *Bifidobacterium* strains and used immediately after incubation.

Cultures were adjusted to 1.0×10^9 CFU/mL and a 1:10 dilution was used in the GITBox, with 30 mL seeded within each reactor in parallel batch cultures, as shown in Fig. 9. Growth was monitored *via* Optical Density (OD) using the Nanodrop ND-1000 Spectrophotometer at 600 nm, sampled twice per day. The OD was then converted to CFU/mL through pre-computed standard curves for the three microbial strains.

3.5.4 Culture of complex microbiota from fecal matter

Human fecal samples were obtained from a male volunteer (25 years old), without a history of antibiotic or probiotic usage in the past 6 months. Fecal slurry, which was prepared fresh, contained 10% total fecal matter in physiological saline. The food media, which is the same as used by the SHIME [72], contained arabinogalactan (1 g/L, Sigma, Oakville, ON), pectin (2 g/L, Alfa Aesar, USA), xylan (1 g/L, Sigma, Oakville, ON), corn starch (3 g/L, Sigma, Oakville, ON), glucose (0.4 g/L, Sigma, Oakville, ON), yeast extracts (3 g/L, Sigma, Oakville, ON), peptone (1 g/L, Bioshop, Burlington, ON), mucin (4 g/L, Sigma, Oakville, ON) and cysteine powder (0.5 g/L, Alfa Aesar, USA). The pancreatic solution consisted of sodium bicarbonate (12 g/L), oxgall (6 g/L, Fisher Scientific, Ottawa, ON) and pancreatin (0.9 g/L, Sigma, Oakville, ON). Feeding occurred according to a 4 h cycle, as shown in Fig. 10.

3.5.5 Antibiotic and probiotic treatments

Antibiotic preparation was done by reconstituting ampicillin (Sigma, Oakville, ON) and gentamycin (Sigma, Oakville, ON) stocks to 0.185 mg/mL in physiological saline, and added to the food media. The probiotic solution included 1.0×10^8 CFU/mL each of *Lactobacillus rhamnosus, Lactobacillus fermentum*, and *Bifidobacterium infantis* strains, which was added to the food media.

3.5.6 Tracking the growth of complex microbiota

The complex microbiota community was assayed by colony counting on exclusion agar plates, prepared according to the manufacturer's directions (Table 2).

Samples (1 mL) from the GITBox colon compartment were taken every 24 h and eight serial dilutions (1:10) were made in sterile physiological saline. Two dilutions (10^5 to 10^7) for each exclusion plate were plated in duplicates. Anaerobic plates were grown for 48 h while aerobic plates were grown for 24 h before counting.

Table 2 – Microbial groups tested along with media and growth conditions.

Microbial Group	Medium	Condition	Source
All Aerobic Group	BBL Brain-Heart Infusion	Aerobic	Becton Dickson, Toronto, Canada
All Anaerobic Group	Brain-Heart Infusion	Anaerobic	Becton Dickson, Toronto, Canada
Lactobacillus genus	LAMVAB *	Aerobic	Sigma Aldrich, Oakville, Canada
Bifidobacterium genus	Raffinose-*Bifidobacterium* media	Anaerobic	HiMedia, Toronto, Canada
Enterococcus genus	*Enterococcus* selective agar	Aerobic	Sigma Aldrich, Oakville, Canada
Staphylococcus genus	Mannitol salt agar	Aerobic	Hardy Diagnostics, USA
Clostridium genus	Tryptose sulphite cycloserin agar	Anaerobic	Oxoid, Nepean, Canada
Coliforms	MacConkey agar	Aerobic	Becton Dickson, Toronto, Canada
Gram-Negative bacteria	Violet-red agar	Aerobic	Quelab, Montreal, Canada

*LAMVAB media: 104.4 g/L MRS, 0.5 g/L cysteine-HCl, 40 g/L agar, 2 mg/mL vancomycin, bromocresol green

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3.6 Supplementary Information

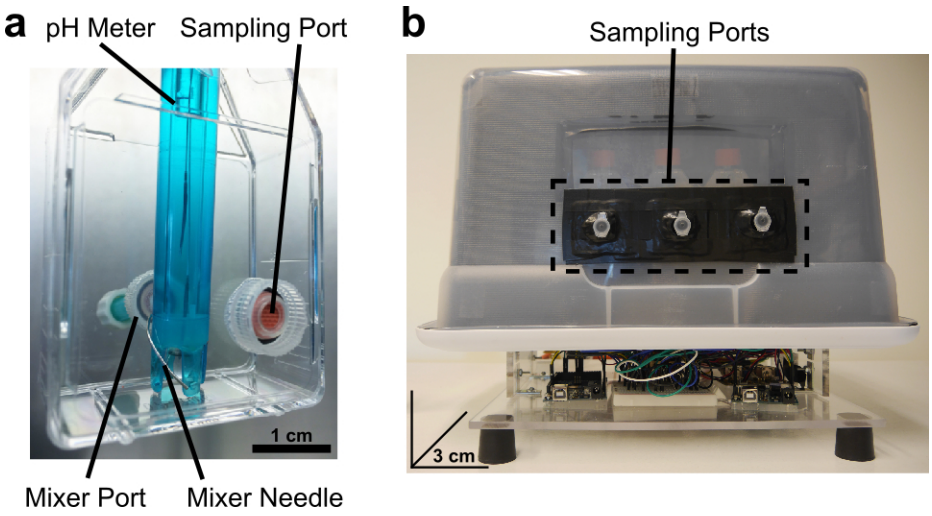

Figure 12 – Bioreactor and closed-box design of the GITBox. (**a**) Picture of the GITBox bioreactor consisted of a pH meter, mixer port and needle, and sampling port. (**b**) Picture of the closed box GITBox with external sampling ports that line up with the interior sampling ports of the bioreactors. The box allows easy sampling without disturbing the interior closed-system conditions.

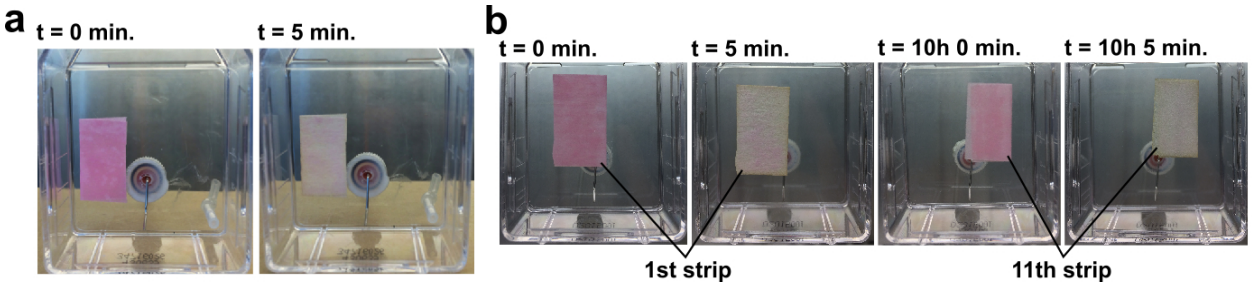

Figure 13 – Time-lapse images of the anaerobic indicator strip in the bioreactor with nitrogen-pulsed mixing conditions. (**a**) The pink strip turning white within 5 min. when exposed to anaerobic conditions. (**b**) Examining anaerobiosis long-term, whereby strips were replaced every hour for 10 h to ensure anaerobiosis was continuously established.

Table 3 – Population variations of complex microbiota communities between days of culture.

	Day 0/1	Day 1/2	Day 2/3	Day 3/4	Day 4/5	Day 5/6	[a]Day 5/12	[b]Day 0/6
Aerobic Microbiota:								
Enterococcus genus	0%	1%	7%	5%	5%	2%	1%	10%
Lactobacillus genus	0%	1%	3%	1%	1%	1%	2%	5%
Streptococcus genus	1%	3%	2%	2%	4%	1%	3%	8%
Coliform Group	0%	1%	2%	4%	2%	2%	2%	4%
Total Aerobic	0%	0%	3%	2%	1%	0%	1%	2%
Anaerobic Microbiota:								
Bifidobacterium genus	1%	2%	7%	5%	1%	1%	2%	7%
Clostridium genus	1%	2%	5%	2%	1%	1%	0%	1%
Gram Negative Group	1%	0%	5%	0%	5%	4%	4%	5%
Total Anaerobic	1%	1%	5%	0%	3%	3%	1%	4%

[a]Population variation between microbiota on day 5 and long-term microbiota on day 12.

[b]Population variation between original, pre-cultured, fecal sample on day 0 and stabilized microbiota on day 6.

Table 4 – Population variation between stabilized microbiota before antibiotics perturbation (day 5) and recovered microbiota after probiotics perturbation (day 14).

	Day 5/14
Aerobic Microbiota:	
Enterococcus genus	3%
Lactobacillus genus	4%
Streptococcus genus	2%
Coliform Group	5%
Total Aerobic	3%
Anaerobic Microbiota:	
Bifidobacterium genus	4%
Clostridium genus	1%
Gram Negative Group	4%
Total Anaerobic	5%

4 Conclusion

4.1 Project summary

In this project, we introduced a miniaturized gastrointestinal tract-in-a-box, which is comprised of three 40 mL mini-bioreactors simulating the stomach, small intestine, and colon, and recreates a physiological GIT environment that includes mixing, controlling pH to *in vivo* ranges, and establishing anaerobiosis and temperature. The culture of single strain microbial species *via* batch culturing was first demonstrated over a period of 180 h, establishing the environment of the GITBox in supporting microbial growth. A continuous culture procedure mimicking the human digestive process was established to support complex microbiota from a fecal inoculum, with reactor working volumes (30-34 mL) and residence times (0.5-3 h) representative of *in vivo* parameters. The complex microbiota community displayed rapid stabilization (< 5 days), and retained an *in vitro* microbial composition that was representative of the original *in vivo* fecal composition (< 10% variance). The capabilities of the GITBox for studying the response to antibiotics and probiotics were demonstrated, in which the complex microbiota community exhibited fast microbial responses (< 24 h) upon each treatment. The GITBox is a versatile and robust system, and capable of effectively recapitulating various critical parameters of the GIT and supporting a complex GIT microbiota, and could be used to systematically study a wide-range of microbiota population properties, which may prove important in advancing research and therapeutic developments on the GIT microbiota pertaining to human health and disease.

4.2 General discussion and outlook

We demonstrated that the GITBox was able to facilitate reproducible sequential experimentation (N = 5 replicates for stabilization tests, N = 3 replicates for perturbation trials), as well as enable

advanced experimentation involving both antibiotics and probiotics in the same trial, which to the best of our knowledge have not been performed before in any GIT system. While the microbial responses observed were within 24 h when perturbed by the external stimuli, the tracking of the responses was overall limited by the sampling rate (every 24 h), and thus the early response of the microbial communities was not captured, which could be addressed by using more frequent sampling rates (*e.g.* every 8 h) and reducing the volume from 1 mL to ~300 µl so as to not deplete the reactor contents.

The GITBox enables a stabilization time that is shortened by 10-30 days when compared to existing chemostat-based systems [77, 87], which significantly reduces the pre-experimental preparation time required before microbial testing could be performed, thereby resulting in considerable cost savings and personnel time. The ability of the GITBox to maintain an *in vitro* composition that is representative of the original *in vivo* fecal composition provides a more accurate simulation of the microbial profile of the human donor, contrasting the compositions of large-scale chemostat-based systems that effectively establish an *in vitro* signature that is not representative of the original fecal microbiota [77, 87], and may render studies difficult to translate to *in vivo* conditions.

The development of the GITBox can facilitate a wide-range of advanced experiments on the GIT microbiota. The GITBox could be used to simulate various diseased GIT microbiota models derived from the fecal matter of patients, opening up avenues in investigating the microbial species and communities underlying the specific disease, and studying the effects of certain foods, drugs, or supplements on the microbiota for treatment of the disease. For instance, the microbiota of obese individuals could be simulated and various diets or foods could be tested. Moreover, the effects of different drugs and supplements (*e.g.* probiotics) on the microbiota of

individuals with diseases such as IBD could also be investigated. In addition, the GITBox could be used to study and optimize healthy stool substitutes for fecal transplants, whereby microbiota derived from a healthy fecal donor could cultured and optimized with various beneficial microbial strains or additives, which could then be further isolated for transplantation. Since the GITBox provides a representative microbial composition to that of the donor's fecal sample, it may also be useful for personalized medicine and testing. The modularity and low cost of the GITBox allows it to be readily multiplexed, which can enable high throughput, parallel, experimentation of many microbial parameters, and proper control experiments. In addition, more reactors could also be readily connected to simulate and study various regions of the GIT, such as simulating a more complete colon including the ascending, transverse, and descending regions, which would allow the monitoring of the microbiota in each specific region. The configurability of the software and electro-mechanical components of the GITBox could also permit a wide-range of studies on factors that may influence microbial functions such as different feeding cycles or diets, digestive enzymes, pH conditions, and mechanical mixing conditions.

4.3 Future directions

Future directions will involve further miniaturization of the GITBox. We encountered a miniaturization bottleneck in the current GITBox due to the commercial-scale sizes of many electro-mechanical components, such as the pH meters and transfer pumps, which were inexpensive, however, took up space. We will explore developing miniaturized electro-mechanical components, such as screen-printed microelectrode pH meters and electromagnetically actuated micropumps, while maintaining the ease of use and integration, and low-cost. Consequently, the miniaturization of the GITBox components would enable a further

miniaturized system, as well as efficient development and multiplexing of the system. The incorporation of a complex microbial-epithelial interface within the GITBox could also be explored, which would enable a more accurate simulation of the GIT and allow studies pertaining to the interactions between complex microbiota and the host. To date, Marzorati *et al.* developed an add-on module to the SHIME that can support complex microbiota flowed in from the SHIME, as well as intestinal epithelial cells, which are separated by a semi-permeable membrane, to investigate complex host-microbial interactions [103]. However, the module is still limited due to the aforementioned limitations of the SHIME, with throughput a major bottleneck, and has only shown capabilities in performing short-term experiments (< 2 days). Therefore, it would be beneficial to explore a complex microbial-epithelial interface that is integrated within the reactors themselves, such as using a two-layer bioreactor separated by a membrane where it can continuously support both complex microbiota and epithelial cells, and thus can be sustained long-term for systematic host-microbe studies.

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