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REVIEW

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Technological tools and strategies for culturing human gut microbiota in engineered in vitro models

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Abstract

The gut microbiota directly impacts the pathophysiology of different human body districts. Consequently, microbiota investigation is an hot topic of research and its in vitro culture has gained extreme interest in different fields. However, the high sensitivity of microbiota to external stimuli, such as sampling procedure, and the physicochemical complexity of the gut environment make its in vitro culture a challenging task. New engineered microfluidic gut-on-a-chip devices have the potential to model some important features of the intestinal structure, but they are usually unable to sustain culture of microbiota over an extended period of time. The integration of gut-on-a-chip devices with bioreactors for continuous bacterial culture would lead to fast advances in the study of microbiota-host crosstalk. In this review, we summarize the main technologies for the continuous culture of microbiota as upstream systems to be coupled with microfluidic devices to study bacteria-host cells communication. The engineering of integrated microfluidic platforms, capable of sustaining both anaerobic and aerobic cultures, would be the starting point to unveil complex biological phenomena proper of the microbiota-host crosstalks, paving to way to multiple research and technological applications.

KEYWORDS
anaerobiosis, bioreactors, gut-brain axis, microfluidic systems, organ-on-a-chip

1 | INTRODUCTION

1.1 | Gut environment and microbiota-host interaction

The gut microbiota refers to the whole of symbiotic microorganisms colonizing the human intestine and growing in physiological synergy with cells (a condition defined as eubiosis) (Bäckhed, 2011; Dieterich et al., 2018; Quigley, 2013).

The gastrointestinal (GI) tract (Figure 1), where gut microbiota resides, has a complex structure which is divided into three regions: the stomach, the small intestine (duodenum, jejunum, and ileum) and the colon (ascending, transverse and descending). Each region has physicochemical peculiarities influencing bacterial composition and strain abundance (Arrieta & Finlay, 2012; Boeri et al., 2019; Chambers et al., 2015; Conlon & Bird, 2015; Keshavarzian et al., 2012; Kim et al., 2014, 2016; Manach et al., 2004; Morrison & Preston, 2016; Stokholm et al., 2016). Indeed, the concentration of

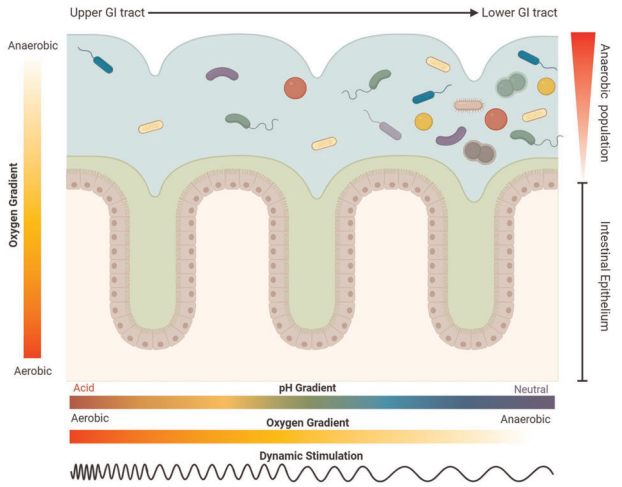

FIGURE 1 The gastrointestinal tract is a complex system both from a physicochemical and biological point of view. Moving from the upper to the lower GI tract, there are different parameters that vary accordingly to the site, such as oxygen partial pressure, pH and intensity of the dynamic stimuli, as well as an increased number and diversity of bacteria shaping the microbiota. Regardless from the tract considered, the distribution of bacterial strains is influenced by the oxygen gradient. Figure made with biorender (https://biorender.com) [Color figure can be viewed at wileyonlinelibrary.com]

bacteria per gram content increases along the GI tract: 10^1 bacteria/g in the stomach; 10^3 bacteria/g in the duodenum; 10^4 bacteria/g in the jejunum; 10^7 bacteria/g in the ileum; 10^{12} bacteria/g in the colon (Dieterich et al., 2018; Drissi et al., 2017; Shah et al., 2020). However, the exact composition of the bacterial species of the GI is still discussed and its detection depends on the experimental analytic techniques used (e.g., culturomics, metagenomics). Recently, targeted studies have investigated these aspects, even if the results are not yet generalizable (Rinninella et al., 2019). One example is a work by Maihle and collegues where they found 110 species in the stomach and 235 in the left colon by using culturomics as detection technique (Mailhe et al., 2018). Microbiota community also varies along the transversal section of the intestine, from the gut wall to the internal lumen. This depends on chemical and nutrient gradients, antimicrobial immune response, epithelial activity, and morphological features of the gut walls (Donaldson et al., 2015).

Moreover, the composition and abundance of the gut microbiota is related to sex, age, health, mental conditions and diet of the host, among others (Sekirov et al., 2010). Other parameters like pH, flow rate, transit time of digesta, thickness of the mucus layer, activity of immune cells, enzyme secretion, bile acid concentration, redox potential and oxygen concentration are also crucial to determine microbiota features along the different GI tracts (Espey, 2013; Tuohy & Scott, 2015). The stomach has the lowest bacterial concentration due to its unfavorable physicochemical properties, (pH 2), high concentrations of pancreatic enzymes and bile acids. The gastric extreme environment actively filters the ingested bacteria, allowing for the survival and/or successful passage into the small intestine of only few microbial strains (e.g., *Helicobacter pylori*, *E. coli*, *Salmonella*, and *Shigella*) (Tuohy & Scott, 2015). Along the three small intestine regions the pH raises from 6 to 7.7 (Hillman et al., 2017; Koziolek et al., 2015). In these tracts, the high flow rate of digesta, high micronutrient digestion and adsorbtion, and the intense reactivity of immune cells demonstrated to mainly influence the residing micriobiota (Judkins et al., 2020). The continuous excretion of digestive enzymes and bile acids, and a short transit time cause continuous bacterial renewal and, hence, a dynamic microbiota pattern (Zoetendal et al., 2012). Reactive immune cells residing in the Peyer's patches along the walls of the small intestine, contribute to gut homeostasis and microbiota colonization (Tuohy & Scott, 2015). Differently, the colon is characterized by the most stable microbiota community, accounting for more than 400 species according to culturomics analysis (Mailhe et al., 2018). This is mainly due to less critical pH values, low transit time of digesta and the availability of food-derived energy. Since the ascending colon is a site of intense bacterial fermentation, the lumen becomes slightly acidic in the proximal portion, while turning neutral in the distal one (Tuddenham & Sears, 2015; Tuohy & Scott, 2015).

Oxygen concentration is one of the key factors influencing bacterial composition in the whole GI tract, since it determines the survival and growth of facultative or obligate anaerobes. An oxygen gradient exists along both the radial and longitudinal axes of the intestine (Zheng et al., 2015). Within the radial section, the oxygen partial pressure (P_{O2}) decreases precipitously from the submucosa (80–100 mmHg) to the lumen (<10 mmHg) (Espey, 2013). Cell heterogeneity and distribution along the gut villi mirror oxygen requirements: the cells consuming oxygen at higher rates (i.e., Paneth cells and intestinal stem cells) are located at the basis of the intestinal crypt, while the cells consuming oxygen at lower rates (i.e., bacteria and enterocytes) are located at the lumen interface (Clevers, 2013). Along the longitudinal axis, P_{O2} decreases gradually while descending the GI tract. It reduces from ~77 mmHg in the stomach to <1 mmHg in the rectum (Espey, 2013). These oxygen gradients are mainly due to the structural anatomy and blood vessel distribution. For instance, at the basis of the gut villus the blood flow and nutrient exchange is maximum and it decreases towards the lumen (Zheng et al., 2015).

Such a complex environment influences the gut microbiota, and the communication between bacteria and host-cells. This interplay has recently attracted the attention of researchers, due to the increasing awareness of its direct or indirect effect on the state of health of different body districts, even if anatomically distant from the GI tract. Increasing resources were devoted to model the gut envirоinment and to investigate microbiota-human cell communication to elucidate different biological questions. In this view, one of the most exciting examples of microbiota-host interaction is the microbiota-gut-brain axis as a new paradigm for neuroscience research. Several hypotheses have been proposed to describe the microbiota-gut-brain axis (such as neuroanatomical and neuroendocrine pathways) and were reviewed in detail in other works (Cryan et al., 2019; Serra et al., 2019; Wang & Wang, 2016).

The gut microbiota influences brain development and functionality through neural networks (i.e., enteric nervous system [ENS] or vagus nerve), and the immunomodulatory effects of microbial factors, which can be released both at peripheral and central level (Braniste et al., 2014; Dinan & Cryan, 2017a, 2017b; Fung et al., 2017; Heijtz et al., 2011; Michel & Prat, 2016; Neufeld et al., 2011; Raimondi et al., 2020; Rea et al., 2016; Rook et al., 2014; Silva et al., 2020; Williams et al., 2014; Yunes et al., 2016). The shift of microbiota population towards a pathologic profile is defined as dysbiosis and its role in neurological and neurodegenerative diseases has been deeply investigated (Brial et al., 2018; Keightley et al., 2015; Leclercq et al., 2014; Moser et al., 2018; Patterson et al., 2014; Tilg et al., 2020). Emerging data suggest that dysbiosis could trigger inflammation by acting on the permeability of both gut and blood–brain barrier (BBB), thus affecting disease onset and progression (Pellegrini et al., 2018; Quigley, 2017; Roy Sarkar & Banerjee, 2019; Sampson et al., 2016; Spielman et al., 2018; Vogt et al., 2017).

In the last decades, based on transgenic animal models research, the hypothesis of a connection between microbiota alterations and Alzheimer's disease (AD), Parkinson's disease (PD) or other neurodegenerative disorders was introduced (Cattaneo et al., 2017; Forsyth et al., 2011; Harach et al., 2017; Nair et al., 2018; Tufekci et al., 2011).

Despite in vivo studies were fundamental in highlighting the possible presence of the microbiota-gut-brain axis, they were unable to fully describe the series of biological events underlying this relationship. For this reason, growing resources were dedicated to study in vitro the axis, and in particular the first step of the microbial-host cells molecular dialogue. For instance, it is worthy to notice that the European Research Council funded more than 60 microbiota-related projects in <10 years. Among them, the ERC-CoG-MINERVA project aims at developing an innovative technological platform to investigate the relationship between intestinal microflora and brain functionality, in healthy and pathological conditions (Raimondi et al., 2019, 2020).

Modeling bacteria-host communication in vitro is not a trivial task. Researchers have used two different approaches: to produce and purify microbiota-derived molecules, they have applied bioreactors able to sustain the culture of human microbiota over an extended period (i.e. fermenters), while to study the effect of bacteria-derived molecules on human cells they have exploited advanced in vitro gut-on-a-chip devices (Holmes et al., 2020; Kim et al., 2012; Mailhe et al., 2018; Sontheimer-Phelps et al., 2020; Villa et al., 2020). Apart from the specific focus of the study, these systems face similar issues, such as the optimization of the sample preparation and modeling the complexity of intestinal environment. Indeed, human microbiota is a very variable bio-ecosystem requiring stringent technical specifications to be efficiently cultured. In addition, the faithful reproduction of its chemical-physical and structural features is a difficult task, particularly because dynamic stimulation should be included to model physiological phenomena like intestinal peristalsis and biological fluids flows. In addition, microbiota cultivation is higly dependent on the sampling procedure (Table 1). At the same time, from an engineering point of view, the tools allowing for a prolonged microbiota culture with in vivo-like conditions are based on complex technologies (Marzorati et al., 2014; Venema, 2015). In the following paragraphs, we will focus on the main technologies available for long-lasting human microbiota culturing and the most advanced systems to model in vitro the human gut. In particular, the advantages and disadvantages of recent in vitro approaches to achieve a reliable representation of bacteria-host communication will be discussed.

1.2 | Long-term culture of human microbiota

To study microbiota biodiversity, culture-independent and -dependent methods can be exploited. Culture-independent approaches, whose advantages and disadvantage were already described (Su et al., 2012; Weinstock, 2012) directly characterize native microbiota by molecuar techniques, without the need of bacteria culturing (Gupta et al., 2019; Su et al., 2012; Weinstock, 2012; Woo et al., 2008). Culture-dependent approaches rely on growing microbiota samples in different media formulations Before characterization. In this case, the first challenge is the selection of the inoculation method. An intrinsic issue arising when culturing microbiota is the difference among in vitro and in vivo conditions (i.e., pH, transit time, carbohydrate content in the medium, variations in oxygen levels, absence of mucosal binding sites or selective pressure), which may result in compositional discrepancies between native and cultured microbiota (McDonald et al., 2013; O'Donnell et al., 2016; Payne, et al., 2012). Hence, the isolated microbes may not be representative of the most abundant taxa in situ, leading to ineffective inoculation, regardless of the experimental set-up used for the culture (Stefani et al., 2015). Moreover, from a technical point of view, bacteria can be inoculated in liquid form or immobilized in polymer beads (de Vos, 2015). Even though Silverman et al. (2018) showed that gut microbiota composition was stable over long periods of time, the immobilization reproduces both motile and immobilized microbes, and it helps to ensure bacterial diversity at high cell densities for long periods in continuous intestinal reactors (Cinquin et al., 2004, 2006b).

Regardless of the application, numerous complications should be taken into account when investigating microbiota effect on human health in vitro (Payne, et al., 2012; Sommer, 2015). First, the presence of cells, modeling the human body, inhibits the use of specific bacterial culture broths imposing the use of cell culture medium, which may affect bacterial growth due to a suboptimal culture condition (Ito et al., 2019; Jalili-Firoozinezhad et al., 2019; Yousi et al., 2019). Second, when chronic phenomena such as gut inflammation and its possible role in neurodegeneration are considered, bacterial culture should be maintained for long time frames. This is important not only to accurately model the in vivo situation, but also to reach the microbiota metabolic equilibrium in vitro and, hence, the cellular reaction in time to the steady state condition

TABLE 1 Possible methods for gut microbiota sampling for in vitro culture

	Methods	Pros	Cons	Main application
FECES	Patient collect their stool, usually in bulk and at home. Commercial containers (e.g., Fisherbrand™ Commode Specimen Collection System, EasySampler®, Fe-Col® or BioCollector™) limit their discomfort, prevent sample contamination from urine, toilet water and sanitizers, and provide closed containers for transportation	- Simplicity, repeatability on daily basis, affordability and noninvasiveness - Guidelines and protocols for donor selection, stool collection, transportation and DNA extraction (Wu et al., 2019)	- Difficult collection for diarrhea or baby stool (Videnska et al., 2019) - Use of protectant media (Bellali et al., 2019, 2020; Lagier et al., 2015; Martínez et al., 2019; Million et al., 2020) to maintain anaerobic bacteria - Daily interindividual variations lead to greater differences in microbiota analysis than stool handling (e.g., homogenization or freezing; Cheng & Ning, 2019; Hsieh et al., 2016; Lozupone et al., 2012; Rinninella et al., 2019; Scepanovic et al., 2019; Vandeputte et al., 2016; Zarrinpar et al., 2014) - Possible incomplete removal of fecal bacteria from intestinal flora (Tang et al., 2020) - Samples treated with stabilization buffers (e.g., RNALater®, OMNIgene®·GUT, and FTA cards) to prevent DNA/RNA degradation are unsuitable for culturing and continuous colonic fermentation models (Chan et al., 2016; Morjaria et al., 2019)	- Study of the more transient luminal bacteria of the large intestine. Unsuitable to recapitulate the spatial organization of gut microbiota communities (Jones et al., 2018)
ENDOSCOPIC PROCEDURES	A) Mucosal biopsy: A flexible endoscopy reaches the sampling site from the mouth or the anus. With respect to standard forceps, the Brisbane Aseptic Biopsy Device reduces sample contamination and diversity (Shanahan et al., 2016). In this device, a membrane covers the tip of the endoscope. The biopsy forceps penetrate the membrane, that retracts when the forceps is advanced	- Suitable for the diagnosis of disease type in IBDs (Salvatori et al., 2012)	- Unfriendly, possible bleeding - Bacteria in not-sampling sites are dragged to sampling sites - Small sampling area, leading to sample deviation - Not enough DNA, RNA or proteins for multi-omics technologies - Large amounts of contaminated host DNA	- Assess the composition of mucosal microbiota in different GI (Tang et al., 2020; Zhang et al., 2017)
	B) Luminal brushing: Initially developed to sample lung microbiota (Wimberley et al., 1979) it	- Reduced bleeding and infection, more representative mucosal samples with respect to biopsy	- The general ones of endoscopic procedures	- Study of luminal-associated microbiota (e.g., spatial variations

TABLE 1 (Continued)

	Methods	Pros	Cons	Main application
	couples mucosal biopsy to protected specimen brushing. A plug and sheath protect the sample in the colonoscope working channel (Lavelle et al., 2013)	- Large ratio of bacterial to host DNA		between luminal and mucosal microbiota)
	C) Laser capture microdissection: An infrared laser beam allows for the adhesion of the tissue section on the surface of a biopsy to a thin, transparent film. Then, the film is removed and the sample (e.g., DNA, RNA, proteins) is treated Emmert-Buck et al.,1996)	- Isolation of pure sections - Punctual analysis (a small focal region is transferred to the film) - Easy, specific and accurate sampling (resolution: 5 μm, (Nava et al., 2011)	- Biopsy samples are required - Biopsy preparation before laser treatment (Baarlen et al., 2008) - Nuclei acid degradation and low sample amount - Not for large-scale analyses, but suitable for precision medicine	- Study of mucosal- and crypt-associated microbial communities (Nava et al., 2011; Pédron et al., 2012; Richard et al., 2018; Wang et al.,) - Study of mucus layers and mucus-associated bacteria (Chassaing & Gewirtz, 2019; Lavelle et al., 2015)
ASPIRATION OF INTESTINAL FLUID	A) Capsules: For example, stainless steel capsules connected to a negative-pressure source by a tube: (Shiner, 1963)	- Prevention of contamination (after collection, the sample is isolated from the external environment)	- Technically challenging	- Culture of small bowel microflora for the diagnosis of SIBO (Choung et al., 2011) - In vitro evaluation of orally administered drug products (Litou et al., 2020)
	B) Tubes: For example, tubes for enteral feeding and ingestible tubes (Lavelle et al., 2010; Minekus et al., 1999)	- Prevention of contamination	- Difficult and time-consuming - Possible occlusions due to the viscosity of intestinal fluid	
	C) Endoscopic aspiration: (Rao & Bhagatwala, 2019)	- Alternative to endoscopic biopsy	- Patient discomfort - Contamination of the endoscopic channel by oropharyngeal and GI content - Time-consuming - Possible unsuccessful suction due to the sparseness of fluid aspirates	- Most popular method for intestinal fluid collection

(Continues)

TABLE 1 (Continued)

	Methods	Pros	Cons	Main application
CAPSULE ENDOSCOPY	Swallowable pills move actively or passively through the GI tract by peristalsis. They offer recognition, anchoring and bio-sensing capabilities (Amoako-Tuffour et al., 2014; Hale et al., 2014). Some of them can also collect tissue samples (at least 400 µm in size or aspirate intestinal fluid (> 200 µl) (Cui et al., 2008; Park et al., 2008)	- Low invasiveness - Accurate location of sampling points - Reduction of patient comfort - High technological content (e.g., locomotion mechanisms; wireless connection; temperature, pH, pressure, oxygenation, oxidation/reduction, conductivity sensors; multi-axial accelerometers and gyroscopes for inertial navigation and positioning)	- Risk of capsule aspiration and retention - Possible sample contamination by intestinal fluid from noncollected sites (Cui et al., 2008b) - High costs of the medical procedures - High costs for fabrication, but a reduction is possible with 3D printing (Rezaei Nejad et al., 2019) - Commercial products, but they do not perform biopsy (Pan et al., 2019)	- Study of small bowel diseases (e.g., celiac disease, CD, cancers), assess mucosal activity - Intestinal microbiome sampling and preservation (Koziolek et al., 2015) - Imaging the distal duodenum, jejunum and ileum (Moglia et al., 2009) - Drug release (Cui et al., 2008b)

Abbreviations: CD, Crohn's disease; DBE, double-balloon enteroscopy; IBD, inflammatory bowel diseases; GI, gastrointestinal; MEMS, microelectromechanical; SBE, single-balloon enteroscopy; SIBO, small intestinal bacterial overgrowth.

(Peebo & Neubauer, 2018; Webb, 2017). Short-term in vitro co-cultures of bacteria and cells are excellent tools to study acute phenomena, such as bacterial adhesion and infection of the intestinal walls (Jalili-Firoozinezhad et al., 2019). However, they are not able to model chronic pathological conditions yet, which are typical features of neurodegenerative diseases (Kelly et al., 2017). For this reason, bioreactors for long-term bacterial culture have become particularly attractive even outside their classic fields of use, in particular as suitable upstream systems for the coculture of bacteria with cells in microfluidic devices (Marzorati et al., 2014).

Long-term culture of the microbiota was firstly assessed in fed-batch conditions, where the medium is poured into the culture vessel hosting the biomass, thus avoiding nutrients depletion (Rumney & Rowland, 1992; Venema & Van Den Abbeele, 2013). The biomass can be composed of a single cell type as well as complex microbial communities, usually cultured with a specific medium in a closed glass vessel (Coimbra et al., 2020; Khalil et al., 2014; Mou & Cooney, 1983). The two main factors influencing the biomass growth-profile in a fed-batch bioreactor are the inoculum density and the substrate depletion rate (Modak et al., 1986; Rumney & Rowland, 1992). When properly combined, these parameters induce a shift of the growth curve from the typical quadriphasic trend of the batch culture (i.e., lag, logarithmic, stationary and death phases) to enhanced logarithmic and stationary phases, as demonstrated both experimentally and theoretically (Figure 2a) (Goudar, 2012; Landa et al., 2001; Modak et al., 1986). The possibility to culture the same bacteria population for prolonged times in a single procedure brings some advantages, such as the minimal incidence of mutations and multi-compound fermentation (Kubitschek & Bendigkeit, 1964; Mora-Villalobos et al., 2020). Moreover, the batch and fed-batch conditions can be coupled to operate in nearly stationary regimen, maximizing the control of fermentation conditions before nutritive supplies. Even though these advantages are unquestionable, the fed-batch is a closed system, which drags at least two limitations. First, the logarithmic growth is sustained until fresh medium is supplied (Pompei et al., 2008). This may result in unfavorable temporal constraint, especially for weeks-long experiments like monitoring of the microbiota metabolism over time (Kaarel Adamberg et al., 2020; Cleusix et al., 2008). Second, it is not possible to couple the bioreactor with other downstream systems, which is a *sine qua non* priority when microbiota-host or bacterial-cellular crosstalk is investigated.

To overcome these limitations, closed fed-batch bioreactors evolved to include medium outflow (Rumney & Rowland, 1992). The balance between nutrient availability and waste removal allows for the prolonged maintenance of a univocal correspondence between biomass and environmental conditions (i.e., steady state condition) (Shachrai et al., 2010; Wang et al.,). Only in steady state the changes of molecules' concentration, and hence the physiological state of cells, are negligible and an unaltered biological process can be assumed in time (Korem Kohanim et al., 2018). The control over experimental parameters (e.g., temperature, pH, oxygen concentration and flow rates) and the possibility of their modification with

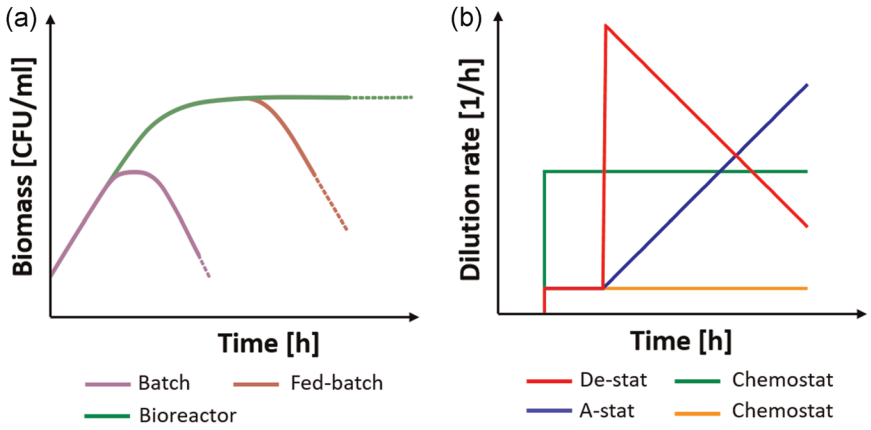

FIGURE 2 (a) The typical growth curves of batch and fed-batch cultures are compared to the continuous culture performed by a bioreactor, such as a chemostat, where the presence of a medium inflow and outflow allow for a potentially infinite stationary phase. (b) The different approaches for the continuous culture in a bioreactor with reference to the chosen method to control D. In particular, the increasing or decreasing of the dilution rate corresponds to the accelerostat or decelerostat condition (red and blu line respectively). Differently, a constant D defines the chemostat approach (in the figure, two different D are represented by the green and orange line). Figure made with biorender [Color figure can be viewed at wileyonlinelibrary.com]

reasonable resources are mandatory prerequisites for continuous culture.

The control of medium flow is a standard strategy to reach the steady state condition. Culture systems are classified accordingly to the method used to dilute the culture by considering the dilution rate,*D*, which is a parameter defined as the value of the medium flow rate divided by the total culture volume (Figure 2b) (Adamberg et al., 2015). The chemostat approach consists in maintaining a constant *D* over time. In this case, *D* should be set not to exceed the maximum specific growth rate of the biomass, thus not diluting the bacterial population faster than its growth capability and leading to a constant depopulation (Bhunia, 2014). Importantly, when the steady state is reached, *D* can be assumed as numerically equivalent to the specific growth rate of the biomass (Doble & Kumar, 2005).

Hence, the control over the process parameters (in/out flows) can be used to engineer important fermentation conditions, such as the biomass in the culture. However, this is not a trivial process. For instance, increasing D to maximize the growth rate is extremely time-consuming, as it requires culture stabilization after each *D* variation step (Adamberg et al., 2015; Gresham & Hong, 2015). Control over the culture can be obtained by the changestat approach, where the environmental parameters are constantly tuned during the culture while preserving the steady state condition (Lahtvee et al., 2011). If D is increased or decreased over time, then the changestat is defined as accelerostat or decelerostat (A-stat or B-stat, respectively). In both cases, *D* variations should be not too high to disrupt the bioprocess equilibrium, but not too low to lead specific phenotypical mutations. Differently, if *D* is maintained constant and other environmental parameters are changed (e.g., pH, oxygen concentration, temperature, medium additives), then the system is defined as dilution rate-stat (*D*-stat) (Adamberg et al., 2015). The A-/De-stat bioreactors are particularly suitable for culture condition and bioprocess optimization. For example, changestats were used in microbiota continuous cultures, revealing that *D* has a substantial effect on microbial prevalence (Feria-Gervasio et al., 2011), molecule production (Child et al., 2006; Macfarlane et al., 1998) and biodiversity maintenance (Tottey et al., 2017). Moreover, the De-stat approach has enormous benefits in terms of time required for reching the targed culture condition (reduction up to 94% compared to chemostat) with only 5% of accuracy loss (Hoekema et al., 2006). Even though these strategies are different, the available bioreactors can switch from one approach to another; for example, passing from a fed-batch pre-stabilization phase to a chemostat/chagestat culture (Fehlbaum et al., 2015; Tanner et al., 2014; Zihler Berner et al., 2013). Hence, the aforementioned classification is useful to define the experimental *ratio*, but it is limited in the description of the experimental set-up. Indeed, the bioreactors for long-term culture of microbiota were designed in different ways, though maintaining the common aspect of medium flowing in the system (Figure 3). The number of serial vessels (reservoirs and waste excluded) composing the bioreactor is the main classification parameter. To date, single-, double-, triple- and multi-stage bioreactors have been described (Payne et al., 2012) (Table 2). Single-stage bioreactors consist in a single glass vessel directly connected to the medium reservoir and the waste container (Cinquin et al., 2004). They can be used to optimize experimental procedures, such as the reproducibility of a new inoculum type or the definition of the best dilution conditions (Adamberg & Adamberg, 2018; McDonald et al., 2013). Also, the effect of certain target compounds, such as ions, fibers, and other nondietary molecules, can be assessed with single-stage chemostats or changestats after a suitable stabilization time. The complexity of the vessel containing the microbiota increases accordingly to the aim of the study and can be designed to include hydraulic accessories to recapitulate relevant biological processes as peristalsis, water absorption and salt dialysis (Minekus et al., 1999). In two-stage bioreactors, a second vessel receives the outflow from a first vessel. The two vessels sustain the culture in different conditions of pH, retention time and working volume to model

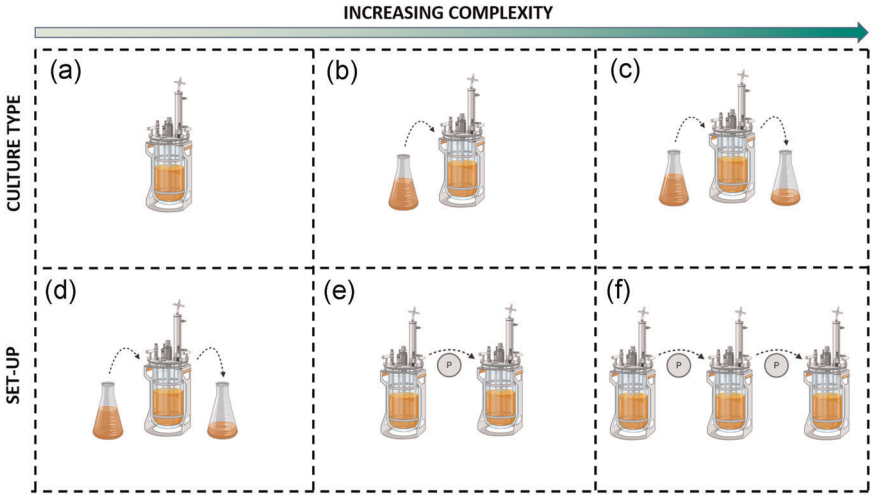

FIGURE 3 Schematic representation of the microbiota culture methods with increasing level of complexity, including batch (a), fed-batch (b), and continuous culture (d). The bioreactors for continuous culture of microbiota were classified accordingly to the number of vessels: single-, double- and triple-stage bioreactors (d, e and f, respectively). Figure made with biorender (https://biorender.com) [Color figure can be viewed at wileyonlinelibrary.com]

two different GI tracts, usually the proximal colon and the transverse or descending colon (Fehlbaum et al., 2015; Zihler Berner et al., 2013). This set-up allows investigating the effect of an in vivo-like environment on microbiota metabolism, composition and other age-dependent bioprocesses (Dostal et al., 2013; Tanner et al., 2014; Zihler Berner et al., 2013). Two-stage bioreactors are more flexible than single-stage ones and open to the possibility of multi-treatment analysis, as well as the presence of a control-culture by substituting the second vessel with multiple vessels in parallel (Fehlbaum et al., 2015). The addition of a third vessel, modeling the remaining colonic tract, represents the three-stage approach (Poeker et al., 2018). Similarly to two-stage bioreactors, the medium is pumped by peristaltic pumps through three vessels bearing different environmental conditions each (Feria-Gervasio et al., 2014). Three-stage systems are the main type of bioreactors for long-time culture of microbiota, as they more accurately model the in vivo modularity (Gibson & Fuller, 2000; Payne et al., 2012). In this way, it is possible not only to study the effect of a certain treatment on microbiota composition, biodiversity and metabolism, but also (at the same time) to distinguish the combined effects of each colonic sub-environment on biomass features (Venema & Van Den Abbeele, 2013b). Three-stage bioreactors for long-term microbiota culture were applied in different fields, including –without being exhaustive— the effects of dietary fibers, age-dependent metabolism, probiotics, prebiotics and mechanisms of bacterial infection (Cinquin et al., 2006a; Macfarlane & Macfarlane, 2007; Payne et al., 2012; Zihler et al., 2010). However, they are more complex than single- or two-stage bioreactors, as they require advanced control systems and empowered resource accessibility (Gibson & Fuller, 2000).

Microbiota culture conditions were further optimized by adding other districts of the GI tract (Venema, 2015). In this way, bioreactors were not only able to model the environment where microbiota is prevalently found in vivo (colon), but also to include other host-related biological processes, such as digestion and bile secretion (Venema, 2015). For example, the TIM1 and TIM2 bioreactors modeled the upper and lower intestine, respectively, and were coupled to investigate the effect of inulin, galacto-oligosaccharides and other dietary fermentation products (Maathuis et al., 2012; Macfarlane et al., 1992; Van Nuenen et al., 2003). Similarly, the SHIME aims at modeling all the compartments of the GI tract (Venema, 2015). These bioreactors were designed with several improvements, such as the possibility to run multiple experiments in parallel or include simple mucus models (García-Villalba et al., 2017; Van Den Abbeele et al., 2013). Despite the relevance of gut multi-stage bioreactors, they will not be described in this review, since already presented in other works (Ceppa et al., 2020).

Regardless of the design, scope and objectives, the culture of microbiota is performed in anaerobic conditions. A modified three-stage chemostat exploited microbiota metabolism to produce different ratios of CO_2 and H_2, thus self-generating anaerobiosis (Feria-Gervasio et al., 2014). However, fluxes of N_2, CO_2, H_2 or their combination (e.g., 90% N_2, 5% CO_2, 5% H_2) remain the preferred strategy to reduce oxygen concentration. Before the experiment, the gases are flushed to remove oxygen from the tubes and vessels; then, the same flux is maintained during the whole culture to guarantee anaerobic conditions (Gaci et al., 2017; McDonald et al., 2013).

The presence of strict anaerobic bacteria in the microbiota is one of the main limitations impairing cocultures with cells. However, the combination of new technologies with increasing interest on microbiota effect on human health has boosted the design of bioengineered platforms able to finely control oxygen concentration.

1.3 | Advanced culture systems to study microbiota-host interaction

In vitro systems that sustain the coculture of obligate anaerobic bacteria with cells are as important as challenging. In the last decade, considerable efforts were employed to engineer complex in vitro models with an anoxic-oxic interface (AOI) inside microphysiological environments (Figure 4 and Table 3). As the intestinal oxygen gradient contributes to the definition and the maintenance of microbiota heterogeneity and distribution, the AOI should be considered a necessary component to be implemented in advanced gut-on-a-chip devices aiming at supporting microbial biodiversity (Zheng et al., 2015).

TABLE 2 Main studies exploiting long terms culture of bacteria and their definition in terms of the experimental ratio and set up

Type of control	Experimental set-up	inoculum	Preculture stability (*d*)	Experiment duration (*d*)	Main application	Ref.
Chemostat	Single stage	Immobilized/slurry	16	54	Effect of the inoculum type on the microbiota biodiversity	Cinquin et al. (2004)
		Immobilized	13	71	Changes in the production of SCFAs with four different prebiotic fructans	Le Blay et al. (2010)
			12	62	Combined effect of glycerol and *Limosilactobacillus reuteri* on microbiota composition	Cleusix et al. (2008)
		Slurry	4	48	Stability of microbiota culture with different inoculum techniques and replicates	McDonald et al. (2013)
			Variable	Variable	Differentiation of the microbiota accordingly to different dilution rate	Adamberg and Adamberg (2018)
			Variable	Variable	Microbiota stability with different dilution rates and dietary fibers	Adamberg et al. (2020)
			2	> 62.5	Monitoring of the mictrobiota steady-state metabolism at different dilution rates	Feria-Gervasio et al. (2011)
	Two stage	Immobilized	13	38	Changes induced by pH variation to the microbiota ecology	Zihler Berner et al. (2013)
			11	54	Swine microbiota composition and diversity in new poly-fermenter system	Tanner et al. (2014)
			14	80	Differences in elderly microbiota composition cultured in multiple environmental condition	Fehlbaum et al. (2015)
			3	70	Impact of Fe availability on the child microbiota composition and metabolism	Dostal et al. (2013)
	Three stage	Slurry	48	120	Effect of mucin on the dissimilatory sulfate reduction and CH_4 production	Gibson et al. (1988)
		Immobilized	12	29	FISH counting efficacy compared to CFUs. Stability of the microbiota culture	Cinquin et al. (2006b)
			> 3	33	EPS and FOS influence on the human infant microbiota	Cinquin et al. (2006a)
			2	42	Metabolic adaptation of child microbiota to obese, normal-weighed and anorectic dietary	Payne, Chassard, et al. (2012)
			10	65	Probiotics effect on salmonella infection and *E. coli* colonization	Zihler et al. (2010)
Changestat	Three stage	Slurry	o.n.	> 16	Monitoring of the mictrobiota metabolism and composition at different retention time	Macfarlane et al. (1998)
			> 10	Variable	Effect of the transient time on the microbiota metabolism and composition	Tottey et al. (2017)

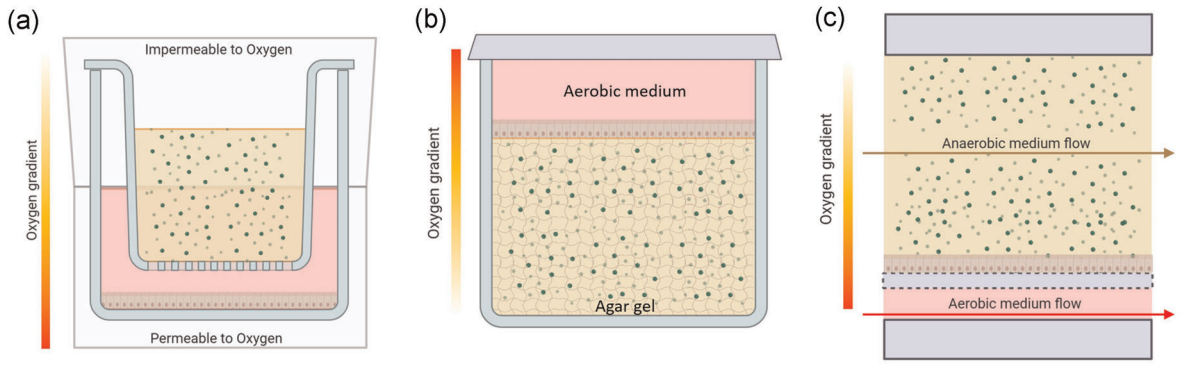

FIGURE 4 The different approaches used to develop in vitro AOI in a crescendo of complexity. The gradient of oxygen was statically engineered by the presence of O_2 permeable/impermeable environments (a) or agar-based gels (b). Differently, the aerobic/anaerobic medium flows allowed modeling AOI in dynamic gut-on-a-chip systems (c). Figure made with biorender (https://biorender.com) [Color figure can be viewed at wileyonlinelibrary.com]

To engineer the AOI, the simplest approach relies on a membrane separating a basal chamber, hosting a model of the intestinal epithelium (usually based on Caco-2 cells), from an apical anaerobic chamber (Figure 4). In this way, the two chambers are ideally decoupled, allowing bacteria to survive in anaerobic conditions, while the oxygen flux coming from the aerobic chamber ensures cell survival. These models are static and indicated as apical anaerobic coculture systems and can be obtained with different levels of complexity (Maier et al., 2018; Ulluwishewa et al., 2015). From a technical point of view, the simplest configuration adds a oxygen-impermeable shell to the standard Transwell® chambers, which is conventionally used to model biological barriers, thus creating anaerobic environment in the apical chamber (Figure 4a). This approach was able to sustain epithelial cell function, but also demonstrated the beneficial effects of bacteria (e.g., *Faecalibacterium prausnitzii*) on certain epithelial features and activity, as increased transepithelial electrical resistance (TEER), and modulation of anti-inflammatory activity. However, the porous membrane fails to finely control oxygen gradient, because the gas may diffuse across the cell monolayer from the basal chamber (permeable to oxygen), eventually saturating the microbial apical side. For this reason, those system demonstrated to sustain anaerobic bacterial survival no longer than 8 h (Ulluwishewa et al., 2015). Even though the coculture of anaerobic bacteria and cells was significantly improved if compared to previous pure oxygen-containing systems, these engineered Transwell® systems are not suitable for prolonged microbiota-host commumunication investigation (von Martels et al., 2017).

The Human oxygen-Bacteria anaerobic (HoxBan) coculture system is an example of a compact and user-friendly system for modeling the AOI in vitro (Sadabad et al., 2015). The aerobic and anaerobic compartments are recreated inside a 50 ml centrifuge tube. Caco-2 cells are seeded on the top of a glass coverslip, which is exploited as a separatory element between the two chambers. On the basal level, anaerobic conditions were obtained by a 1% agar gel, with or without mucin, where the bacteria were (~ 80% of the total) guarantees the generation of a steep oxygen gradient inside the gel, resembling the in vivo situation. Indeed, the oxygen was demonstrated to gradually diffuse into the agar bulk, without reaching the lowest part of the tube, where the obligate anaerobic bacteria grow.

The HoxBan successfully sustained the coculture of Caco-2 cells and *F. prausnitzii* for one day, showing a mutual communication between intestinal epithelial cells and obligate anaerobic bacteria. However, the simplicity of the system is unable to recapitulate the complex gut environment, also because of the absence of important physiological stimuli like mucus flow and mechanical deformations. Moreover, culturing bacteria in the confined volume of a centrifuge tube leads to either bacteria overgrowth or saturation of gases derived from bacterial and cellular metabolism.

All these limitations are mitigated by ventilated and dynamic systems, where the presence of a fluid flow enables material renewal and dynamic stimulation with physiological levels of shear forces (few units of mPa) acting on bacteria (Kim et al., 2016; Marzorati et al., 2014).

Fluidic systems like the HMI module (Marzorati et al., 2014) and the HuMix device (Shah et al., 2016) showed to successfully sustain the AOI interface for suitable strict anaerobic coculture with cells. These systems represent suitable models of dynamic host-microbiota interaction. The concept at the basis of fluidic systems consists of parallel chambers forming the luminal and basolateral compartments for bacteria and cell culture, respectively, intermediated by semi-permeable membranes. In this configuration, the presence of an inlet and outlet in each chamber allows for a controlled seeding of cells and bacteria inoculation, while not impairing sample collection. In this way, the cocultures can be monitored in real-time by biological analysis, including cytokine assays or metabolomics profiling (Shah et al., 2016). Another advantage of the decoupled perfusion is the possibility to inflow different media and gasses in the chambers (e.g., anoxic and/or oxic gasses), establishing a controlled gradient of oxygen from the aerobic environment of the cellular compartment (21% pO_2) to the anaerobic condition for anaerobic bacteria culture (0.8% pO_2) (P. Shah et al., 2016; Shin et al., 2019). In this way, the AOI interface is generated between the two chambers, recreating

TABLE 3 Different fluidic tools used for the modeling of the gut environment and the AOI interface

System type	Pros	Cons	Main application	Ref.
Apical anaerobic coculture systems	- Consolidate intestinal epithelium model - Automated measurements of epithelial barrier integrity	- Impossibility to control the oxygen tension gradient in time - Coculture duration limited by Caco-2 viability for prolonged periods	- *Faecalibacterium prausnitzii* altered the immunomodulatory action of Caco-2 cells	Ulluwishewa et al. (2015); Maier et al. ()2017
HoxBan	- Use of compact and user-friendly culture system made of classic centrifuge tubes - Use of solid agar medium for the coculture of anaerobic gut bacteria - Generation of a physiological steep oxygen tension gradient	- Need of anaerobic culture environment - Absence of dynamic perfusion for controlled metabolites exchange and mechanical stimulation - Bacteria overgrowth and gases saturation	- Demonstration of the metabolic mutualism between Caco2 cells and *F. prausnitzii*	Sadabad et al. (2015)
HMI	- Dynamic fluidic system - Control on cell seeding and bacteria inoculation through separate culture chambers - Complex microbiota culture up to 48 h - Possibility of interfacing with human gastrointestinal tract simulators (i.e. SHIME)	- Limited control on fluid velocities, shear forces and molecules concentration profiles due to the macro scale dimensions.	- Enterocytes exposed to complex microbial community for prolonged time showed to stay vital and sustain the coculture for more than 24 h	Marzorati et al. (2014)
Humix	- Dynamic fluidic system - Intermediate perfusion chamber separates human cells from the microbiota with in vivo-like distance and smooth oxygen tension profile	- Difficult measurements of epithelial barrier integrity - Impossibility of interfacing with a SHIME-like bioreactor	- Individual transcriptional responses from human epithelial cells cocultured with LGG are comparable with in vivo findings	Shah et al. (2016)
Gut-on-a-chip	- Microfluidics allows for fine tuning of flow velocity profiles and shear stress distribution as well as transport of molecules - Mechanical actuation mimicking the intestinal peristalsis	- Caco-2 cells are put in direct contact with anaerobic bacteria through a permeable membrane - Bacteria overgrowth in a micrometric space - Need geometrical adaptation to host anaerobic bacteria.	- Intestinal barrier dysbiosis triggers the onset of intestinal inflammation - The barrier integrity is needed and sufficient to suppress the pro-inflammatory cascades mediated by the host-microbiome cross-talk	Shin and Kim (2018)
Anoxic-oxic interface-on-a-chip	- no need of complex apparatus to maintain the anoxic condition (e.g. incubators, glove box) - Geometry adapted for efficient physiological transepithelial anoxic gradient	- Bacteria overgrowth in a micrometric space - Impossibility of interfacing with a SHIME-like bioreactor	The transepithelial anoxic gradient is necessary and sufficient to coculture obligate anaerobic *B. adolescentis* and *E. hallii* with aerobic host epithelial cells	Shin et al. (2019)

microaerophilic conditions within the mucosal layer. Additionally, dynamic fluid perfusion guarantees continuous metabolite intake and catabolite removal, while avoiding bacteria overgrowth. The achievement of a correct balance between the flow rate, molecule and metabolite diffusion, and the dynamic steady state of the culture requires a careful optimization of the process parameters. Therefore, the maintenance of the AOI interfaces increases the complexity of the experimental set-up. For instance, the absence of oxygen in the anoxic medium needs to be finely controlled by constantly and continuously bubbling nitrogen, which must not interfere in the maintainace of the equilibrium in the diffusion of oxygen coming from the aerobic compartments across the permeable membrane (Shah et al., 2016). To this purpose, the platform is usually provided of sensors for the continuous monitoring of oxygen concentration and, ideally, of an automated control line to restore this equilibrium (Shah et al., 2016).

The dimensions of the dynamic systems can be at the macro- or microscale. The fabrication technique and the final application are determinant for the selection of the dimensions of the fluidic channels. Macro-bioreactors show flow chambers with dimensions in the order of centimetres (e.g., the HMI module has a chamber of 10 × 6 cm). Such dimensions allow for interfacing with bioreactors for the long-term culture of bacteria, which require the displacement of consistent volumes of media at relatively high flow rates (in the order of ml/min) (Marzorati et al., 2014). Reducing the dimensions of the fluidic channels brings several advantages, such as a more accurate control over the velocity profiles in the medium, the shear forces exerted on cells and bacteria, and the concentration profiles of the target molecules. Indeed, at the microscale, the characteristic length (L) of the system is micrometric and its contribution to inertial forces is reduced of three orders of magnitude if compared to macroscale bioreactors. This implies the fall in Reynolds number while increasing the laminarity of the flow (Brennan et al., 2014).

In microfluidic systems, the flow experiences minimal mixing due to the lack of turbulence and the prevailing of viscous forces. The modulation of the advection/diffusion ratio is necessary to control and enhance oxygen diffusion for stable and tailored oxygen tension profiles, which can be controlled by acting on the flow velocity along the principal channel direction (Rivera et al., 2019):

Microfabrication techniques (e.g., soft lithography) allow to fabricate fluidic devices with more than two microchambers. The HuMix device shows a central perfusion chamber between the upper microbial chamber and the lower human cells chamber. The presence of the central space separating human enterocytes and the microbial population recreates the typical distance (about 0.5–1 mm) between epithelial cells and microbiota found in healthy, intact epithelial barriers (Shah et al., 2016). This optimized structure is further coupled with the simultaneous perfusion of -oxic and -anoxic media through the micro-chambers, which leads to the establishment and maintenance of an oxygen gradient close to the in vivo situation.

An advanced gut-on-a-chip device supporting the growth of microbes and cells was presented by Kim and collaborators (Kim et al., 2012). They cultured the intestinal *Lactobacillus rhamnosus* GG (LGG) on the apical surface of a Caco-2 cell monolayer in a microfluidic device. They demonstrated that the intestinal epithelium could coexist in direct contact with bacteria in a dynamic device with peristaltic-like motion, showing enhanced and constant functional TEER with values around 4 kΩcm^2, and the presence of morphological features (villi structure). Peristaltic-*like* deformation is applied by with a controlled vacuum source, which induces the uniaxial stretching of a membrane, reaching cyclic 10% cell strain at the frequency of 0.15 Hz like in the human physiology (Deloose et al., 2012; Kim et al., 2012). In this example, the maintenance of anaerobic conditions inside the microchannel was not an issue, as LGG is a facultative anaerobic bacterium.

Most recently, the so called AOI-on-a-chip was the first example of a microfluidic device where obligate anaerobic bacteria were successfully integrated in a chip for the study of host-microbiota interaction (Shin et al., 2019). In this case, the AOI interface was optimized by tuning the height ratio between the upper and the lower channel. The increased height of the luminal microchamber was exploited to achieve the equilibrium between shear stress values, cell/bacterial viability and flow rates to recreate the physiological transepithelial AOI. Indeed, the authors demonstrated that the presence of a flow of preconditioned anaerobic medium at specific flow rates was sufficient to establish a proper oxygen gradient, without any other equipment or procedure. This not only established a steady-state AOI in vitro, but also supported the growth of anaerobic human gut microbiomes, such as *B. adolescentis* and *E. hallii*, improving the total culture period up to 72 h (Shin et al., 2019). Gut-on-a-chip devices with bacteria in direct contact with cells were suitable to investigate microbiota-host crosstalk, exposing either synergy or antagonism. Still, further improvements are needed to achieve long-term cultures and avoid overgrowth in the range of weeks for more robust recpaitulation of microbiota-gut interactions. The main limitation of these systems when used alone is their impossibility to maintain bacterial cultures for more than few hours, because bacteria overgrowth occurs independently from the dynamic condition of the set-up. This causes bacteria suffering and damage and results in metabolic alterations.

The use of bioreactors for microbiota fermentation—upstream to the gut-on-a-chip device—is a valid tool to overcome this limitation, as demonstrated by the HMI module. Indeed, this gut-on-a-chip device was successfully connected to a SHIME-*like* reactor, where the fecal samples of patients were processed and then inoculated into the module (Marzorati et al., 2014). The combination of bacteria bioreactors and gut-on-a-chip devices allowed to study the effects of microbiota-related molecules on the modulation of the host response.

Even though the gut-on-a-chip devices described above have the advantage of introducing a dynamic stimulation, they separate anaerobic bacteria from the epithelial cell layer with a synthetic membrane. This strategy protects the host cells from direct exposure to microbiota infections, but fails in recapitulating the real microbiota-gut interface, for example including mucus. Indeed, the mucus is not only a biological structure with complex architecture,

but it is also strongly dependent on the microbiota-host crosstalk itself (Sardelli et al., 2019). For this reason, gut-on-a-chip systems evolved to coculture bacteria into the luminal channel, in direct contact with the mucus of intestine epithelial layer, while keeping the microenvironment perfused (Kim et al., 2012; Shin et al., 2019; Sontheimer-Phelps et al., 2020).

Up to date, the AOI has been developed in single organ in vitro models, i.e., gut-on-a-chip devices. However, even though these systems demonstrated to sustain dynamic models of the intestinal barrier, they show some limitations when applied to the study of the intestinal microenvironment (Ceppa et al., 2020). To investigate microbiota relationship with distant organs (e.g., gut-brain axis), AOI in vitro models need to be integrated in multiorgan platforms, defined as body-on-chips (BOC). Generally, they are made up of multichamber devices connected in series and kept in communication by fluidic circuits (Kimura et al., 2018). They were efficiently used to determine the absorption process of drugs, by combining in vitro models of intestine and liver, or to investigate the distribution and excretion of molecules by modeling the blood circulation and glomerular filtration (Imura et al., 2010, 2013; Kimura et al., 2015). However, engineering AOI compartments in BOC represents a challenging technological issue. Indeed, it requires not only the high control over flows to guarantee the correct mass transport among the different body districts, but also to provide the oxygen gradients for the coculture of bacteria and cells. This is particularly difficult when long biological processes are considered, because the presence of continuous bacterial cultures must be integrated into the system without leading to undesired infections. The development of BOC that can cope with AIO, long term bacterial growth and multi-organs models may represens the opportunity for a significative step forward towards more reliable models of complex biological processes, such as molecular axis involving microbiota metabolism, immune response and neurodegeneration (Raimondi et al., 2019).

2 | CONCLUSIONS

Continuous bioreactors allow for the culture of bacteria for months whent the proper control of the experimental parameters is guaranteed. However, their application has mainly regarded the analysis of the fermentation products, and not the investigation of microbiota-host cell crosstalk. This is due to several limitations. First, culturing in vitro the human microbiota is intrinsically difficult: microbiota isolated from patients' samples differs in terms biodiversity and concentration from the in vivo situation, being heavily manipulated by the inoculation process. Moreover, the experimental set-up is extremely elaborate and expensive, reaching the highest complexity in the case of three-stage bioreactors, where a deep technical knowledge is needed for the correct control of the culture parameters (e.g., chemostats or changestat configuration). Second, the available gut-on-a-chip devices are not designed to sustain long-term cocultures of bacteria and cells. Although relevant progresses has been achieved in integrating bacteria into cellular microdevices, the small sizes, low flow rates and the complexity required to maintain an AOI made these systems suitable only for limited applications. For instance, they are valid and efficient tools for the analysis of infection mechanisms and the modulation of gut permeability. However, they do not allow investigating the mechanisms behind complex biological phenomena, such as molecular axis between microbiota products and distant organs homeostatis. A considerable step forward in this field could be achieved by coupling bioreactors for bacterial culture with multicomponent microfluidic systems. Indeed, this new engineered approach may strengthen the advantages of bacterial bioreactors (i.e. long-term cultures, indispensable to recapitulate chronic diseases), with those of microfluidic devices, like the deep control over experimental parameters. In this way, complex phenomena such as the gut-brain axis would be potentially unveiled, opening new medical paradigms and therapeutic scenarios.

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AUTHOR CONTRIBUTIONS

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