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REVIEW

Designs and methodologies to recreate in vitro human gut microbiota models

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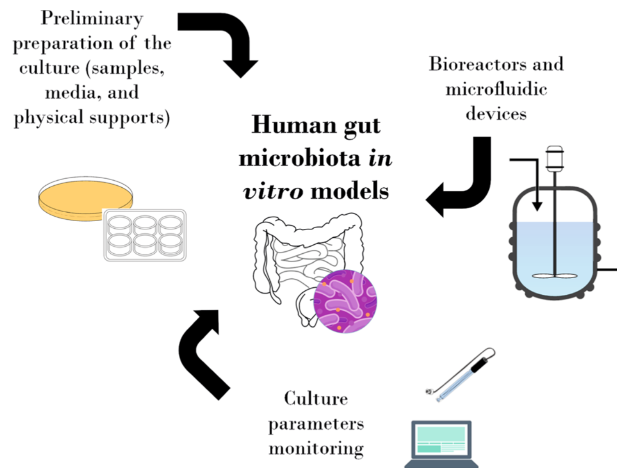
Abstract

The human gut microbiota is widely considered to be a metabolic organ hidden within our bodies, playing a crucial role in the host's physiology. Several factors affect its composition, so a wide variety of microbes residing in the gut are present in the world population. Individual excessive imbalances in microbial composition are often associated with human disorders and pathologies, and new investigative strategies to gain insight into these pathologies and define pharmaceutical therapies for their treatment are needed. In vitro models of the human gut microbiota are commonly used to study microbial fermentation patterns, community composition, and host-microbe interactions. Bioreactors and microfluidic devices have been designed to culture microorganisms from the human gut microbiota in a dynamic environment in the presence or absence of eukaryotic cells to interact with. In this review, we will describe the overall elements required to create a functioning, reproducible, and accurate in vitro culture of the human gut microbiota. In addition, we will analyze some of the devices currently used to study fermentation processes and relationships between the human gut microbiota and host eukaryotic cells.

Graphic abstract



Keywords Gut microbiota · In vitro models · Bioreactors · Host-microbiome · Fermentation

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Introduction

The human gut microbiota is a trending research topic. Besides supporting the digestive process, protecting the host from endogenous and exogenous infections, and shaping the anatomy and functionality of the entire gastrointestinal tract [1–5], gut microorganisms are also implicated in physiological processes involving other distal organs (e.g., the microbiota-gut-brain axis) [6, 7].

In vivo experimental models (e.g., mice, rats, pigs) have been widely used to study the effects of the gut microbiota on various organs [8–10]. Although in vivo models are quite common, they are recognized to have low reproducibility, ethical and cost restrictions, and to represent other physiologies than human ones. In addition, due to intrinsic difficulties in taking samples frequently from animals, in vivo models do not typically allow continuous monitoring of intestinal conditions and interactions between gut microorganisms and the host. Contextually, the focus on in vitro models that mimic the gut environment has grown in recent years thanks to the advent of innovative culture techniques, materials, technologies, and screening systems that have allowed researchers to recreate, study, and understand the effects of the human gut microbiota on the host (Fig. 1). For example, artificial co-cultures made up of the human gut microbiota, intestinal components (i.e., mucus), and mono- or multi-layered tissues have become an incredibly powerful and novel tool to study this intricate tangle of cellular and ecological interactions. This is also thanks to the development of more physiologically relevant tissue models, such as organoids [11].

In this review, we aim to analyze and discuss some of the "ingredients" needed to make a reliable in vitro model of the human gut microbiota (Fig. 2) and how they have already been used to study each facet of the microbiota universe. The reliability of an in vitro model is principally related to its reproducibility over different experiments and its accuracy in recreating a physiologically relevant model (e.g., microbial composition and metabolic functions) similar to the in vivo state. The latter can be difficult to achieve for in vitro models because not all of the systems described in the next sections can promote the stability and functions of the human gut microbiota. These are clearly critical points to be evaluated when an in vitro model of the human microbiota has been designed and set up.

In particular, this review will first focus on the several choices that must be made prior to culturing the gut microbiota (e.g., selection of the appropriate sample and culture medium, choosing the environmental parameters to consider). Then, we will describe the technological advances in gut microbiota in vitro culture, especially highlighting the features of the main systems presented in the literature before now.

Fabricating an in vitro model of the human gut microbiota: setting the operation parameters

Recreating human gut microbiota composition: not only a problem of numbers

The most ambitious goal of recreating the human gut microbiota in vitro is to replicate the complex network of bacterial metabolisms ex vivo [11]. This is not necessarily associated with the precise composition of the microbial community. However, careful selection of the microbial sample to be inoculated in the in vitro model has to be considered as the first step in designing the perfect model (Fig. 2). Although the majority of studies in the literature use human stool or samples obtained through endoscopic procedures, aspiration of intestinal fluids, ileostomies, or endoscopic capsules [12–14] as sources of colonic and small intestine microbiota, others prefer using a few target bacteria able to synthetize metabolites and chemical compounds which reproduce the principal metabolic pathways found in the intestine [15, 16]. Recreating a complex community such as the human gut microbiota can require two approaches. The "top-down" approach is used when, starting from a large microbial community and varying environmental parameters, bacteria are selected to reproduce a certain metabolic process. Conversely, the "bottom-up" approach is used when the information derived from multi-omics technologies is employed to recreate a certain metabolic process from the microbial selection [17]. For example, Petrof et al. recreated a narrow microbial population, metabolically comparable to the gut microbiota, using 33 species of bacteria, and demonstrated that a fecal transplant with this suspension could resolve *Clostridioides difficile* infections [15]. From an in vitro perspective, Krause et al. described a simplified gut microbiota model (SIHUMIx) that included the eight most abundant bacterial species of the human gut microbiota [16, 18]. The aim of these approaches is not only to reduce the interconnection variables between the various species but also to perform more replicable experiments by maintaining a bacterial core able to guarantee certain metabolic pathways of the human gut microbiota. Nevertheless, the dramatic reduction of microbial richness, biodiversity, and interactions can be problematic when settings like these are designed.

Finding the best culture medium

One of the crucial points in the in vitro culture of the human gut microbiota is to identify a suitable culture medium that guarantees the survival and replication of most of the microorganisms which constitute the microbial community. Although gut microbiota medium (GMM) is known to encourage reproduction of a diverse microbial community

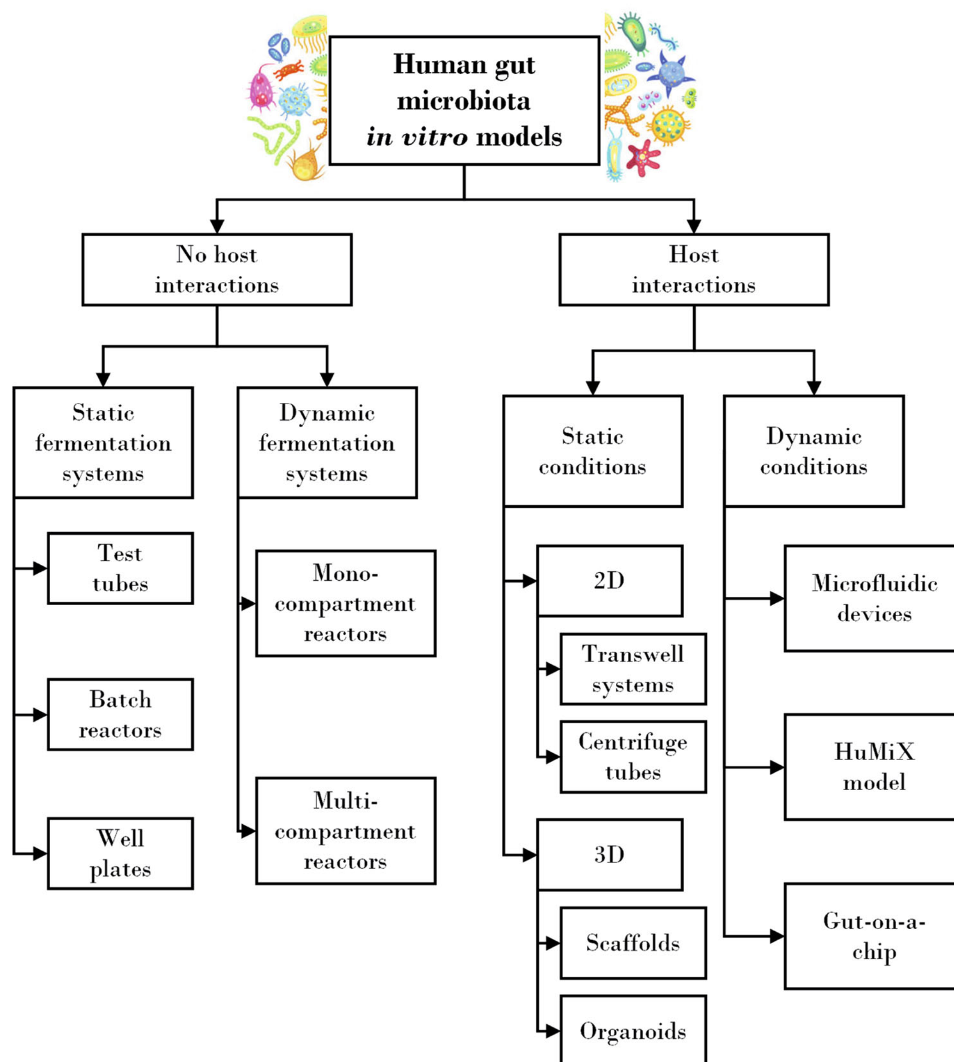

Fig. 1 Classification of the various in vitro models of the human gut microbiota

[19], a "universal" medium to culture all the microorganisms of the human gut microbiota still does not exist. The composition of a culture medium for microbiological studies is typically water, a carbon source, a nitrogen source, and some mineral salts [20]. In addition, some demanding microorganisms need other elements to grow, such as amino acids, vitamins, purines, and pyrimidines [20]. Different media have been tested to study the final composition of in vitro-cultured microbiota [21]. Kim et al. used three different culture media, i.e., brain heart infusion broth (BHIB), high concentration carbohydrate medium (HCM), and low concentration carbohydrate medium (LCM), to culture the human gut microbiota extracted from fecal samples, demonstrating that LCM inoculated with a fecal suspension at a final concentration of 3% granted the highest microbial abundances of the principal *phyla* within the human gut microbiota (e.g., *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*) after a short period culture time (i.e., 18 h of incubation) [22]. Li et al. tested the effects of inorganic salts, bile salts, short-chain fatty acids (SCFAs), and mucins on the functionality of the human gut microbiota by metaproteomics [23]. Yousi et al. tested four bacterial culture media (BHIB, GMM, fastidious anaerobe broth (FAB), and BGM) and demonstrated the differences in terms of microbial composition and SCFA production of the cultured microbiota [24].

Selecting the appropriate environmental conditions

The environmental parameters constantly change throughout the entire length of the gastrointestinal tract, with different regions of the intestine harboring microbial populations that are directly shaped by these different environmental conditions. For instance, pH, partial oxygen pressure, quality of nutrients, gut peristalsis, and concentration of hormones and bile salts are only a few of the shaping factors that characterize the ecological niche in which each microorganism is allowed to live and multiply [25–27]. Even in the diametral

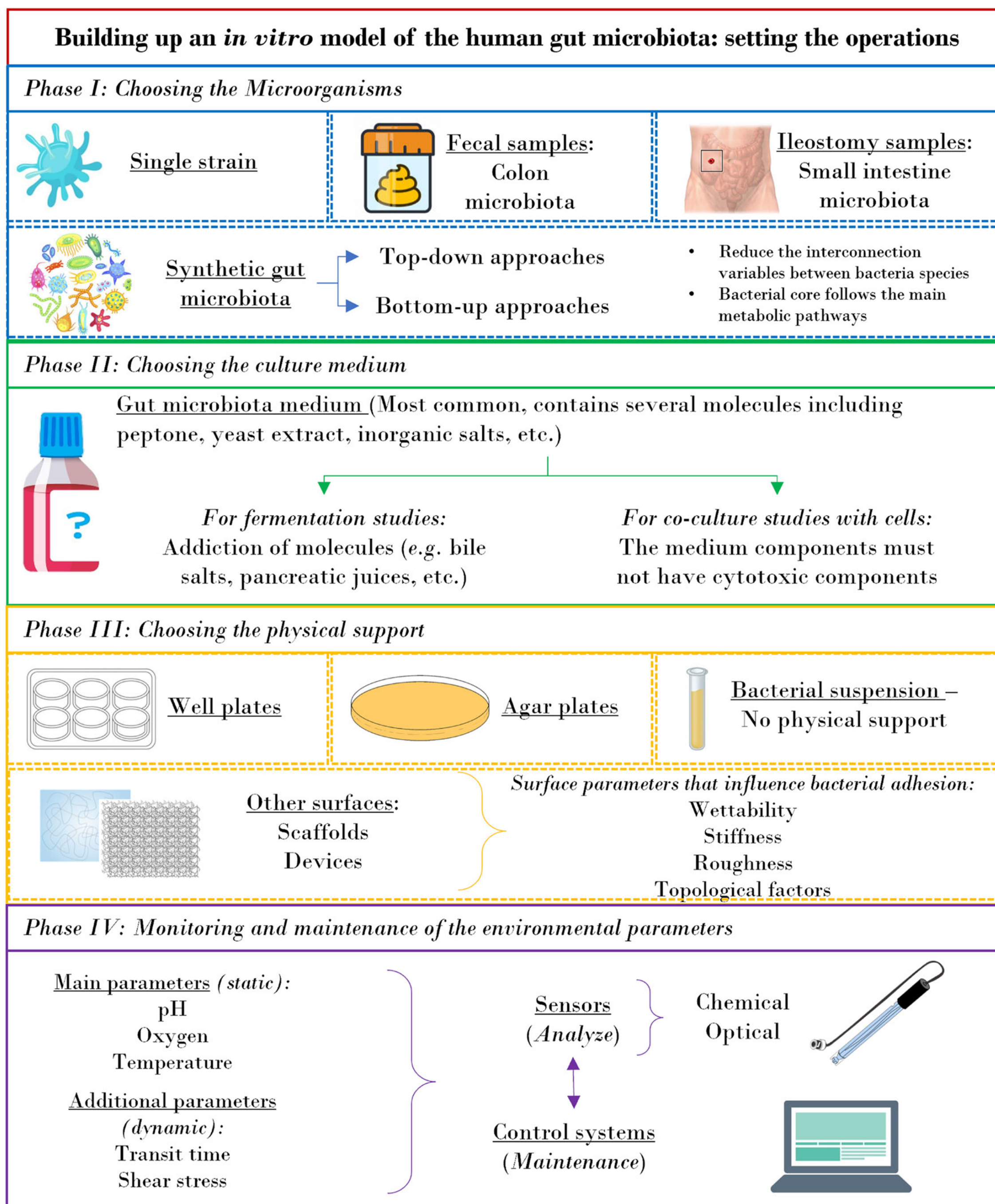

Fig. 2 Workflow for starting a human gut microbiota culture

section of the intestine, environmental factors such as oxygen profoundly differ [28]. Specifically, the small intestine is mainly populated by *Lactobacillaceae* and *Enterobacteriaceae*, with a low overall bacterial load due to low pH levels and compounds secreted by intestinal, hepatic, and pancreatic cells, such as bile salts and antimicrobial peptides [27]. In the large intestine, on the other hand, one finds the highest number of microbial species, with a prevalence of *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* [27]. Especially in this part of the intestine, the pH is widely variable from person to person or even in the same individual, depending on diet-driven fermentation processes, thus leading to changes in the bacterial clusters that inhabit this area [26, 29, 30].

From an in vitro culture perspective, several works have shown how changing one or more of these environmental

parameters leads to growth inhibition or stimulation of certain bacterial *taxa*. Duncan et al. noted a strong decrease in *Bacteroides* species (spp.) when moving from a controlled condition of pH 6.5 to 5.5 [26]. In addition, a pH shift from 6 to 4 resulted in a less biodiverse microbial population with lower abundances of acetate and propionate producers but more lactate-secreting bacteria [26]. Haindl et al. showed an increase in acetate and propionate levels and in the abundance of *Bacteroidetes* and *Verrucomicrobia*, as well as a decrease in the concentration of butyrate and in the abundance of *Actinobacteria* and *Firmicutes* in parallel with the rising pH of the culture [29].

Transit time (i.e., transit of luminal content along the gastrointestinal tract) is one of the most common parameters used to describe gut motility [31]. Since gut motility is related to nutrient availability in the different intestinal regions, it is reasonable to deduce that the composition of the human gut microbiota is linked to transit time [32, 33]. Using a blue-dye method, Asnicar et al. showed that some bacterial species (i.e., *Akkermansia muciniphila*, *Bacteroides* spp., and *Alistipes* spp.) are linked to longer transit time [34]. Furthermore, Tottey et al. followed an in vitro approach using the 3-Stage Environmental Control System for Intestinal Microbiota (3S-ECSIM), which is a multi-compartmental reactor that simulates the physiochemical parameters of the proximal, transverse, and distal portions of the colon, to describe how human gut microbiota composition and metabolism change with transit time [35]. In particular, lower biomass was found in the distal colon compartment, where there is an increased transit time and increased fermentation activity of microorganisms.

A surface issue

Similar to eukaryotic cells in vitro, cultured microorganisms grow better on surfaces than they multiply suspended in culture medium [36]. Microbial adhesion to a surface is a complex biophysical process which can be divided into two main phases. In the first phase, a reversible adhesion occurs in which bacteria adhere to the surface due to thermodynamic phenomena (all these are well reviewed in [37]). The Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloidal stability is typically applied to describe the different chemical/physical phenomena occurring in this phase of the adhesion process [38]. In particular, the extended DLVO theory describes the Gibbs free energy, which is the sum of the Lifshitz–van der Waals interactions, electrostatic double-layer interactions, and acid–base bonds. This model is commonly applied to microbial adhesion process to a surface because bacterial mean size fits the colloidal particle dimensions (0.5–2 μm) [39]. Following this formulation, a resulting negative Gibbs free energy promotes bacterial adhesion, while positive free energy may inhibit it. Some results have shown that this general model is accurate because bacteria, which are characterized by a hydrophobic external surface, are attracted to hydrophobic materials, and vice versa [40]. Furthermore, the presence of external appendages on the bacterial surface of certain microorganisms (e.g., flagella, pili, fimbriae) creates a connection with the surface of the material that promotes this reversible adhesion by acting as a spring [41–44].

The transition from reversible to irreversible adhesion, the second phase of the adhesion process, derives from a series of physical and chemical mechanisms involving, for example, production of extracellular polymeric substance (EPS) and consequent formation of the so-called “biofilm.” A biofilm is a structured community of microorganisms encapsulated in their EPS and attached to a surface [45]. Bacterial biofilms are well known in the field of medicine for being the leading cause of artificial-implant failure, oral-cavity disease, and bacterial resiliency after drug and disinfectant treatment [46].

Changing the properties of a surface (e.g., surface charge, wettability, roughness, topography, or stiffness) can lead to conflicting results in bacterial adhesion [47]. One example is the surface charge density of the material used as the physical support for the culture. Typically, bacterial surfaces are characterized by the presence of a net negative charge due to carboxyl, amine, and phosphate groups, which influence the initial adhesion, making them attracted to positively charged materials [42, 48, 49]. However, in some cases, some bacteria also adhere to negatively charged surfaces thanks to their appendages and surface polymers typically found in Gram-negative bacteria (e.g., lipopolysaccharides [50]). In extreme cases, both negatively and positively charged materials reduced the adhesion of *Pseudomonas aeruginosa* on polystyrene plates [51]. The same contradictory results can be observed for the wettability, topography, and stiffness of a certain surface (Table 1). These opposite behaviors clearly demonstrate the complexity in selecting a universally valid surface for bacterial culture. In fact, ideal physical supports for culturing the human gut microbiota are difficult to achieve due to the complex microbial composition and the remarkable differences in adhesive surface properties of various bacterial species.

Adherence of bacteria to a physical substrate is not only dependent on its mechanical and physical properties but also on its chemical characteristics. Different molecules have been studied for their effects on bacterial adhesion to material surfaces [36]. Fibronectin, for example, promotes adhesion of *Staphylococcus aureus* [68, 69], but inhibits that of *Staphylococcus epidermidis* [70]. Mucins are molecules that can be added in an in vitro model to reproduce more faithfully the gut environment and its resident microbiota. They are glycoproteins produced by the epithelial tissue of the gastrointestinal tract, and several works have already suggested their importance in the attachment process of certain microbial species

Table 1 Relation between surface properties (i.e., material stiffness, topological features, chemical surface) and different in vitro microbial cultures

Material stiffness

Material	*Elastic modulus (kPa)*	*Cultured microorganisms*	*Comment*	*Ref*
Agar (0.75%–3% w/v)	6.6–110	*Bacillus* spp., *Pseudoalteromonas* spp.	*Pseudoalteromonas* spp. adhesion increased with higher stiffness, while *Bacillus* spp. adhesion increased with lower stiffness	[52]
Polyelectrolyte multilayer non-porous film	1000, 10 000	*S. epidermidis*, *E. coli*	Adhesion was positively correlated with increasing stiffness	[53]
Polydimethylsiloxane (PDMS)	100–2600	*E. coli*, *P. aeruginosa*	Adhesion was higher in physical substrates with lower stiffness	[54]
Polyacrylamide	$G' = 0.017–0.654$	*S. aureus*	Adhesion decreased with increasing stiffness	[55]

Topological features

Material	*Topological features*	*Cultured microorganisms*	*Comment*	*Ref*
PDMS	Squares (2–100 µm) with distance between features ranging from 5 to 20 µm	*E. coli*	The presence of squares increased biofilm formation regardless of the feature's dimension and the space between them	[56]
	Squares, ridges, and grids	*E. coli*, *S. aureus*, *P. aeruginosa*	Features smaller than bacteria size decreased their attachment	[57]
	Cones	*E. coli*, *S. epidermidis*	Adhesion depended on the cones dimension but not on the distance between these features	[58]
Polyurethane (PU)	Pillars (diameter 410–430 and 500–560 nm; pillar row separation 350–400 nm and 450–500 nm, height 690–700 nm and 640–650 nm)	*Staphylococcus* spp.	Adhesion to the textured PU was reduced	[59]
Silicon	Micropillars	*S. aureus*, *E. coli*	Retention, growth, and proliferation decreased with micropillar size	[60]
	Honeycomb structures	*E. coli*, *S. aureus*	Micropatterns with dimensions less than 1 µm reduced bacterial adhesion and proliferation	[61]
Polystyrene + Fibers	Surface texturization	*P. aeruginosa*	Adhesion was lower in the presence of fibers	[62]
Gelatine	Random structures (electrospinning process)	*E. coli*, *Entero. faecalis*, *C. albicans*, *C. innocuum*, *B. fragilis*, human gut microbiota from fecal samples	*E. coli*, *C. albicans*, *C. innocuum*, *B. fragilis*, human gut microbiota from fecal samples showed major adhesion on the electrospun structures with respect to positive controls (well plates)	[63]

Table 1 continued

Topological features				
Material	*Topological features*	*Cultured microorganisms*	*Comment*	*Ref*
Gelatine, polycaprolactone (PCL)	Random structures (electrospinning process) with and without a mucin coating	Human gut microbiota from fecal samples	Human gut microbiota showed better adhesion on the PCL structures at 24 and 48 h but on the gelatine structures at 72 h and 7 days. Mucin coating reduced adhesion and proliferation of microorganisms	[64]
Chemical surface				
Material	*Hydrophobic/Hydrophilic*	*Cultured microorganisms*	*Comment*	*Ref*
Poly-4-hydroxybutyrate and polypropylene	Different wettability	*S. aureus*, *E. coli*	Hydrophilic materials promoted lower adhesion than hydrophobic materials	[65]
Polystyrene	Different wettability	*E. coli*	Superhydrophilic and superhydrophobic materials exhibited low bacterial adhesion. Higher levels of bacterial adhesion were obtained with moderate hydrophobicity	[66]
Teflon, polycarbonates, polyurethanes, titanium, silicone, borosilicate glass	Different wettability	*E. coli*, *P. aeruginosa*, *S. epidermidis*, *C. albicans*	Hydrophobic materials promoted biofilm formation	[67]

List of microorganisms: *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans*, *Clostridium innocuum*, *Bacteroides fragilis*

and in the resulting composition of the human gut microbiota [71, 72]. In fact, the mucus layer in the gut is the home of different microbes such as *Akkermansia muciniphila* and *Lactobacillus* spp., which are able to colonize the mucosal layer under healthy conditions [72]. *Lactobacillus reuteri*, for example, binds the mucins thanks to the presence of mucus binding domain on its outer membrane [73]. Culturing a complex population such as the human gut microbiota may lead to different results due to the presence of microorganisms not able to adhere to mucins. For example, in our previous work, we demonstrated that biofilm production of a fecal microbiota on electrospun gelatine structures was lower in the presence of mucins [64]. Also, using the M-SHIME bioreactor, Van den Abbeele et al. demonstrated diametrically opposite differences in the composition of the human gut microbiota in the luminal and the mucosal environment, with a prevalence of *Firmicutes* in the mucus layer [74]. For this reason, introducing the mucus layer to an in vitro model of the human gut microbiota could be useful if coupled with non-mucins layer to enhance the adhesion and replication of both mucus- and non-mucus-adhesive microorganisms.

Shear stress studies

The effects of moving fluid on microorganisms attached to a physical substrate can be of critical importance for creation of an in vitro model of the gut microbiota. Studying the mechanical behavior of bacteria departs from the microbiological tradition, in which bacterial adhesion and proliferation is guided mainly by the chemical environment [75]. It is known, for example, that circulation of a culture medium tends to boost microbial proliferation by carrying anabolites and washing away waste products. However, the flow could also inhibit microbial proliferation, affecting the density of the formed biofilm and reducing its size, depending on the intensity of flow [76, 77]. Also in this case, there are remarkable differences in the behavior of different microbes, with adhesion forces dependent on both environment and surface properties [78]. For example, Lecuyer et al. showed with a microfluidic device that the rate of attachment of *P. aeruginosa* increases up to a shear stress of 3.5 Pa on different surfaces (i.e., hydrophilic glass and hydrophobic polydimethylsiloxane (PDMS)) [79]. Detachment of *P. aeruginosa* occurs when the shear stress suddenly decreases, thus showing that bacteria respond dynamically to shear velocity by modifying the adhesive state accordingly. The

relation between the nature of surfaces and shear stress is also described by Moreira et al. [80]. In their study, *Escherichia coli* was exposed to different fluid flows, showing that up to a threshold level of 8–10 mL/s, the species bound better to the hydrophobic physical substrate (wall shear stress was evaluated with computational fluid dynamics and ranges from 0.05 to 0.07 Pa). Then, beyond this threshold, a diminished number of attached bacteria were observed, thus demonstrating a correlation between shear stress and adhesion of *E. coli*.

Other examples are reported in Table 2. Some of these highlight a correlation between increased shear stress and a higher rate of bacterial attachment on the surface, which is a diametrically opposite behavior than that of most eukaryotic cells cultured in vitro [81].

Biofilm topology is also affected by flow. Rusconi et al. found formation of long filamentous structures in *E.coli* culture following the direction of fluid flow [82]. The formation of these streamers is also enhanced by the presence of surface irregularities. Uncontrolled development of these streamers could be problematic in an in vitro culture, clogging the channel and consequently stopping the flow [83, 84]. For these reasons, the absence of sharp edges, which enhances these effects, could be added as a design specification for an in vitro culture device.

Culture medium for co-culture with human cells

Optimization of the culture medium is crucial for success of the co-culture between the gut microbiota and human eukaryotic cells, as it is responsible for the nourishment and viability of the different cell types involved. Still, it is laborious and time-consuming due to the huge number of possible combinations, as highlighted in the review by Vis et al. [88].

Co-culture strategy and medium composition are the first two variables to consider when designing an in vitro co-culture (Table 3). Then medium volume, waste product accumulation, and reuse of the medium itself must be taken into account.

Medium volume affects primarily the concentration and dilution of waste products and produced metabolites [98–100]. A higher medium volume leads to lower concentrations of cell secretomes (i.e., soluble factors and extracellular vesicles), which is particularly important for cell–cell communication, cell proliferation, and differentiation [98]; however, a lower medium volume is more cost-effective and favorable for culturing some cell types such as neuron-like cells and adipose tissue-derived mesenchymal stem cells [92].

Concerning the problem of waste accumulation, the medium must be replaced periodically to maintain proper concentrations of nutrients and growth factors and allow the removal of waste products generated by cellular metabolism. Typically, this goal is achieved with continuous reactor systems. However, changing the medium implies the removal of secretomes, whose production determines further stress for cells and has a negative impact on their viability [101]. To overcome this issue, dialysis systems have drawn the attention of experts in in vitro cultures [102–105]. Their integration in in vitro culture systems enables the selective removal of waste products and reintroduction of nutrients and vitamins into the medium, while ensuring retention of cell secretomes. This allows reuse of the culture medium and helps in creating a more physiological environment for cells [88]. In addition, dialysis membranes show promise as they are already used in indirect co-culture systems to perform physical separation of different cellular types and/or microorganisms [106–108].

For co-culture of microorganisms and mammalian cells, an additional issue must be considered. The majority of the microbes constituting the human gut microbiota are obligate anaerobic bacteria, which constantly crosstalk with the colonic epithelium in a mucosal anoxic–oxic interface [109]. This constraint establishes the need for two different culture media, one anoxic and the other oxygenated, to guarantee cellular survival and suitable environmental conditions. Therefore, indirect co-culture and partitioned culture environments are the most frequently implemented solutions for in vitro co-culture of the human gut microbiota in the presence of host cells, as described in the next section.

Studying an in vitro model of the human gut microbiota: what are we looking for?

Now that various preliminary steps are understood, we will move on to discuss how the human gut microbiota could be studied in an in vitro model, taking into consideration its complex physiology and richness. Fermentation studies (whose purpose is mainly to replicate as closely as possible the intestinal conditions to investigate the response of microbial fermentative pathways to the presence of specific dietary compounds, toxic molecules, pathogens, etc.) can be distinguished from interaction studies, which involve co-culture with human cells.

Fermentation models

The fermentation processes carried out by the human gut microbiota play a key role in physiological digestion of food [110]. Metabolites produced by these pathways are mostly absorbed by the intestinal mucosa and, while some have health benefits, others have harmful effects on the host [111]. The fermentation patterns associated with the intake of specific nutritional components have been widely studied through different systems described in the literature.

Table 2 Effects of shear stress on different microbial cultures

Bacterial adhesion with fluid-flow condition			
Cultured microorganisms	Device/physical support	Comment	Ref
P. aeruginosa	Soft lithography microchannel	No variations were observed up to 3.5 Pa independent of surface type	[79]
E. coli	Parallel-plate flow chamber	Flux of 8–10 mL/s decreased adhesion (wall shear stress ranged from 0.05 to 0.07 Pa)	[80]
	Microchannels with different heights	Proliferation was inhibited at a shear stress level of 10 mPa after inoculation	[85]
	Silicone microfluidic devices with different stiffness	Adhesion was similar to soft and stiff silicon with low shear. For high shear, adhesion was greater on soft silicone	[86]
S. aureus	Collagen-coated coverslips inserted into a parallel-plate flow chamber	The maximum adhesion was at a shear stress of 0.3–0.5 Pa on type I collagen, 0.5 Pa on type II collagen, and 0.1–0.3 Pa on type IV collagen	[87]

List of microorganisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

Table 3 Different co-culture strategies and approaches used for selecting the best culture medium (general information taken from [88, 89]; more specific references to culture examples are reported directly in the table)

Co-culture strategy	Direct co-culture	Physical contact between different cell types or shared intermediate, promoting direct communication through surface receptors [90, 91]
	Indirect co-culture	Physical separation between different cell types in the co-culture. This can be achieved through different methods, including semi-permeable membranes, hydrogels, gels, agar beads, or different liquid phases [92, 93]
	Indirect co-culture with conditioned medium	The culture medium of the first cell type is used to culture the second cell type [94]
Co-culture medium optimization strategies	Mixed medium	The specific media of all the different cells or microorganisms are combined in specific ratios [95]
	Supplemented medium	A general base medium is supplemented with growth factors and other additives that stimulate proliferation of different cultured cell types or microorganisms without adversely affecting them [96]
	Physically partitioned culture medium flows	Cells and microorganisms receive their specific medium while direct contact between them is still allowed [97]

An initial distinction must be made between static and dynamic fermentation systems. Static systems are typically batch fermentation models built with a closed and controlled environment (i.e., flask, beaker, closed vessel) that simulates one stage at a time (e.g., mouth, stomach, small intestine, colon) [112, 113]. Different methodologies and protocols have been published in an attempt to standardize the culture conditions, such as environmental parameters and digestive fluid composition, digestion time, and operation steps, as well as the post-process (e.g., determination of enzyme activity, collection of samples during the digestive process) [114–116]. These models are simple and have good reproducibility but lack the absorption process by the mucosal component, and transit time between the different compartments of the gastrointestinal tract is not considered. Also, in these static systems, the culture conditions are difficult to standardize due to cell activity and resource concentration. Conversely, dynamic systems are characterized by single or multiple reactors that, thanks to the setting of compartment-specific environmental parameters such as pH, oxygen, temperature, and transit time, can more accurately recreate the intestinal environment. Most of the fermentation models, especially in dynamic conditions, are well reviewed in [117, 118]. In particular, we must consider that the stability of the microbial profile is not always guaranteed in these systems, especially in the mono-compartment models,

as described by Liu and colleagues [119]. Here we aim to highlight some of the technological enhancements of these fermenters over the last 40 years.

Dynamic fermentation systems can be classified into mono- and multi-compartment models, and which of these are selected depends only on the experimental specifications. As explained by Firrman et al. [120], both models are able to develop microbial communities with different species compositions from the same initial sample. In mono-compartment fermentation models, only a single region of the gastrointestinal tract is reproduced. For example, the artificial colon (ARCOL) reproduces the colon environment of humans and animals in vitro (Table 4) [121–124]. The ARCOL bioreactor is equipped with various probes and ports and can be inoculated with fresh stool from healthy animals or human volunteers. The temperature and pH are kept constant by adding NaOH. This model is the first to allow a continuous anaerobic condition inside a fermenter solely through the metabolic activity of bacteria. The Reading Model developed in 1988 by Gibson and co-workers [125] is one of the first examples of a multistage bioreactor. The three vessels used to mimic the proximal, transverse, and distal colon are aligned in series. The pH, the chemical components inside the three vessels, and the fermentation substrate are predefined to simulate food fermentation in the gut. While the first vessel has a mildly acidic environment and high substrate concentration to induce microbial growth as in the proximal colon, the others have neutral pH and a few substrates to resemble the conditions in the transverse and distal colon. Also, the microaerophilic environment is maintained with the insertion of N_2 and O_2 and controlled by a dissolved oxygen sensor. Thanks to its simplicity and easy customization, this model is still used to examine the effects of prebiotic and dietary components on the human gut microbiota [126, 127]. Another example of customizable technology which has evolved during the last 30 years is the in vitro dynamic model of the gastrointestinal tract (TIM) described for the first time in 1995 [128]. The first configuration (TIM-1) comprises four compartments (i.e., stomach, duodenum, jejunum, and ileum) connected to each other by peristaltic pumps that allow chyme transport between the vessels. The TIM system has been heavily customized and improved over the years. For example, the tiny-TIM system is a smaller version of the TIM that comprises two compartments resembling the stomach and small intestine only [129], while the TIMagc simulates the specific conditions in the corpus and antrum part of the stomach [130]. In addition, the TIM-2 system operates with high-density gut microbiota samples to mimic the dynamic and metabolic conditions in the colon (Table 5) [131].

To simulate the gastrointestinal tract, most fermentation systems use working volumes similar to the physiological ones. This choice leads to an increase in costs, mainly due to the culture medium used, and in the physical space required for the overall system. The MiniBioReactor arrays (MBRAs) and the smallest intestine (TSI) (Tables 4 and 5) are two examples of dynamic mono- and multi-compartment systems, respectively, where the working volume inside the system is drastically reduced. The TSI, being constituted by five reactor units enclosed in a box where the environmental parameters are constantly controlled, replicates transit through the small intestine [132]. A dialysis chamber is used to simulate the absorption of nutrients. The results obtained from a study involving the TSI reveal that several strains of *Lactobacillus* have been successfully cultured inside the model [132].

A common problem among the different in vitro fermentation systems is the inoculation of the fecal sample. In fact, most systems use a liquid fecal suspension as inoculum without a physical substrate, resulting in several limitations like the absence of biofilm-associated microorganisms [133]. To overcome this issue, the PolyFermS system uses immobilized microorganisms (Table 5). The system is composed of an inoculum reactor made of micro-encapsulated microorganisms from the human gut microbiota. This reactor is used to supply other reactors disposed in parallel, which have varying environmental conditions. This model has been found to maintain a stable microbial community for 38 days [134]. Another approach in guaranteeing a stable microbial profile, even where cells are suspended, is described by Li and colleagues [135]. In fact, they demonstrated that unlike a continuous reactor system, a looped mass transfer can stabilize microbial communities over a long period of time.

Co-culture systems

One of the bioengineering challenges over the last 10 years has been to create an in vitro model explaining how the human gut microbiota interacts with eukaryotic cells from the host. Several strategies derived from tissue engineering principles have been applied to microbiology to reproduce a co-culture between microorganisms and mammalian cells. These models are well reviewed in [148–154]. Some examples of these co-culture approaches, with a focus on the devices, cultured cells, and bacteria, are reported in Table 6 and Fig. 3.

The purpose of this section is to describe some of these technologies to evaluate if and how they can be adapted to study the effects of the human gut microbiota on the host.

Transwell culture models are particularly useful for studying the interaction between bacteria and intestinal epithelial cells in aerobic conditions [159]. Typically, these wells consist of a lower compartment on which the first cell line can be placed, and a removable upper insert consisting of a microporous membrane, on which a second cell line can be seeded. The pores are large enough for the passage of growth factors and other molecules released by the cell, but too small to

Table 4 Principal fermentation mono-compartment models of the human gut microbiota (all systems were temperature-controlled and cultured at 37 °C)

Mono-compartment fermentation models		
Used to simulate in vitro environment of a single gastrointestinal tract		
Dynamic gastric model (DGM)	*Tract*: Stomach *Environment control*: pH *Working volume*: 800 mL *Mixing*: contraction by water pressure with piston and barrel *Section used*: saliva, gastric juice, HCl	[136–138]
Artificial colon (ARCOL)	*Tract*: large intestine *Environment control*: pH *Anaerobiosis*: available (generated by the microbial metabolism) *Mixing*: rotary stirring *Nutrient absorption*: hollow fiber membrane *Microorganisms*: human gut microbiota from fecal samples	[121–124]
micro MatrixTM	*Tract*: distal colon *Environment control*: pH and dissolved oxygen *Working volume*: 1–10 mL *Microorganisms*: human gut microbiota from fecal samples	[139]
MiniBioReactor arrays (MBRAs)	*Tract*: distal colon *Environment control*: pH (6.8) *Mixing*: magnetic stirrer *Working volume*: 15 mL *Microorganisms*: human gut microbiota from fecal samples	[140]
Proximal environmental control system for intestinal microbiota (P-ECSIM)	*Tract*: proximal colon *Environmental control*: pH (5.75) *Anaerobiosis*: available (generated by the microbial metabolism) *Mixing*: magnetic stirrer plates *Working volume*: 2 L *Microorganisms*: human gut microbiota from fecal samples	[141]

allow passage of the cells themselves. Recently, a variant of this Transwell culture system has been developed, allowing analysis of host-microbe interactions between Caco-2 cells and anaerobic *Fecalibacterium prausnitzii*, as reported in the study by Ulluwishewa et al. [160]. Caco-2 cells were grown on microporous membrane inserts. Due to the polarization of the Caco-2 monolayer (i.e., cells are arranged in an organized manner with the basal part on the bottom of the membrane and the apical part on the top), two culture media were used. In the basal compartment, an aerobic medium was used to prevent cellular death due to hypoxia. In the apical compartment, an anaerobic medium was used to culture *F. prausnitzii*, instead. The overall system was isolated from the external environment and the co-culture chamber was placed in an anaerobic workstation. The well was also equipped with a pair of electrodes to assess the integrity of the cell monolayer junction. It is interesting to note how Maier et al. adopted the "mixed medium" approach for their Transwell system [155]. Thus, *F. prausnitzii* was cultured in an anaerobic medium composed of 50% M199 (cell-culture medium) and 50% BHI (bacterial-culture medium). This combination improved not only the viability of *F. prausnitzii* but also attachment of HEK293-TLR2-Luc cells on the collagen-coated inserts. Conversely, the basal compartment was filled with aerobic Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), to allow generation of the anoxic–oxic interface and guarantee HEK293 cell survival. Although these models are most commonly used by the pharmaceutical industry, they cannot reproduce the physiological morphology of three-dimensional (3D) intestinal cells and tissues as well as some of the key intestinal functions (i.e., mucus production and villi formation). Furthermore, being static models, they present the problem of periodic manual change of the culture media to avoid toxic waste product accumulation and cannot support culture of the human gut microbiota together with human intestinal cells for more than one day [153].

Bioreactors are another example of devices used in tissue engineering to promote cell proliferation and differentiation. These systems perform "incubator" functions, ensuring dynamic environmental conditions with a greater physiological relevance. Among these millifluidic devices, the host-microbiota interaction (HMI) [157] is designed to be connected to a SHIME system [142] (Fig. 3b). This indi-

Table 5 Principal fermentation multi-compartment model of the human gut microbiota (all systems were temperature-controlled and cultured at 37 °C)

Multi-compartment fermentation models		
Used to simulate in vitro environment of a single gastrointestinal tracts		
Simulator of human intestinal microbial ecosystem (SHIME)	*Tract*: stomach, small and large intestine *Environment control*: different pH for the different tracts, dissolved oxygen *Mixing*: magnetic stirrer *Working volume*: different for the different tracts *Secretion tested*: gastric juice (pepsin, lipase, HCl), bile, pancreatin, $NaHCO_3$ *Absorption of nutrients*: porous membrane *Microorganisms*: human gut microbiota from fecal samples **ADD-ON** • **M-SHIME**: addiction of a compartment for the mucus adhering microbes	[74, 142–145]
The TNO gastro intestinal model (TIM-2)	*Tract*: large intestine *Environment control*: pH (5.8), dissolved oxygen *Mixing*: changes in water pressure (to recreate a peristaltic movement) *Working volume*: 1.6 L *Secretion tested*: gastric juice (pepsin, lipase, HCl), bile, pancreatin, trypsin, NaCl, $NaHCO_3$ *Microorganisms*: human gut microbiota from fecal samples **ADD-ON** •**TinyTIM**: addiction of a compartment for the small intestine •**TIMacg**: addiction of a compartment for the stomach	[128–131]
Computer-controlled dynamic simulator of the gastrointestinal tract (SIMGI)	*Tract*: stomach, small intestine, and colon *Environment control*: different pH for the different tracts, dissolved oxygen *Mixing*: magnetic stirrer + peristaltic movement *Secretion tested*: gastric juice, bile, pancreatin, $NaHCO_3$, HCl, NaOH *Microorganisms*: human gut microbiota from fecal samples	[146]
PolyFermS	*Tract*: different (5 reactors) *Environment control*: pH, dissolved oxygen *Mixing*: magnetic stirrer *Microorganisms*: immobilized bacteria through micro encapsulation	[147]
The smallest intestine in vitro model (TSI)	*Tract*: different (5 reactors that simulate transit through the small intestine) *Environmental control*: pH, dissolved oxygen *Mixing*: magnetic stirrer *Working volume*: 12 mL (minimum) *Absorption of nutrients*: dialyzing system *Microorganisms*: three *Lactobacillus* strains	[132]

rect co-culture bioreactor has two compartments separated by a semi-permeable membrane and an artificial mucus layer made of mucin and agar. The lower compartment is dedicated to cell culture, while the upper compartment carries the products from the SHIME. The system was tested with fermentation products from the yeast *Saccharomyces cerevisiae*, and the group found that Caco-2 cells remained viable after 48 h of co-culture [157].

Although dynamic conditions promote bacterial proliferation as well as recirculation of the culture medium and elimination of waste products, these systems present some problems related to their size and feasibility. To overcome some of the limitations described, microfluidics devices have been properly implemented to study the human gut microbiota. A microfluidic device can be defined as a perfusion device that hosts one or more cell types and aims at reproducing key structures, functions, and aspects of human metabolism of a given tissue or organ in normal and pathological physiology [153]. Miniaturization of these devices still allows integration of control, sensors, imaging systems, and other analytical components [161]. There are several examples of microfluidic devices in the literature with dif-

Table 6 Principal studies of the interaction between the human gut microbiota and eukaryotic cells using co-culture approaches

Culture system	Bacteria and cell culture	Comment	Ref
Apical anaerobic co-culture systems (a custom-made 12-well co-culture chamber equipped with a Transwell insert)	Microorganisms: *F. prausnitzii* DSM17677 cultured in Medium 199, without FBS or antibiotics Cell lines: Caco-2 cells HTB37 cultured in Medium 199 containing FBS	*F. prausnitzii* altered the expression of a larger number of immunomodulatory genes in Caco-2	[155]
The human oxygen-bacteria anaerobic (HoxBan) (co-culture of glass-adherent human cells in liquid medium and anaerobic bacteria in solid agar medium, Fig. 3a)	Microorganisms: *F. prausnitzii* cultured on freshly autoclaved YCFAG-agar medium Cell lines: Caco-2 cells, DLD-1 cells or human HepG2 cells cultured on DMEM medium (without antibiotics)	Caco-2 cells with *F. prausnitzii* led to a unique profile of excreted and consumed metabolites, indicating that these cells modify each other's metabolism	[156]
The host microbiota interaction (HMI™) module (Two compartments that simulate the luminal part and the epithelium, separated by a functional double-layer. The functional double-layer was composed of an upper mucus layer and a lower semi-permeable polyamide membrane, Fig. 3b)	Microorganisms: Human gut microbiota (fecal samples) and *S. cerevisiae* cultured on the medium from a fermentative bioreactor (carbohydrate-based nutritional medium and pancreatic and bile liquid) Cell lines: Caco-2 cells maintained in DMEM without antibiotics and antimycotics	After 24 h and 48 h of co-culture, the morphology of Caco-2 cells during and at the end of the treatment period was comparable with that of the cells at the beginning of the experiment	[157]
HuMiX (consisting of three co-laminar microchannels: a medium perfusion microchamber, a human epithelial cell culture microchamber, and a microbial culture microchamber, Fig. 3c)	Microorganisms: *L. rhamnosus* and *B. caccae* (cultured DMEM medium) Cell lines: Caco-2, CCD-18Co, primary CD4 + T (cultured in anoxic and oxygenated DMEM)	Individual transcriptional responses from human epithelial cells in co-culture agreed with in vivo data	[158]
Gut-on-a-chip (This microdevice contained two compartments separated by a porous, flexible, extracellular matrix-coated PDMS membrane)	Microorganisms: GFP-labeled *E. coli* cells and VSL#3 (contains different microbial strains, cultured in antibiotic-free DMEM medium) Cell lines: villi forming Caco-2BBE; peripheral blood mononuclear cells (PBMCs) (cultured in DMEM)	Results suggested that a person with hyperpermeability of the intestinal epithelium may be more vulnerable to continuous microbial attacks and aggressive immune infiltration	[109]
Anoxic–oxic interface-on-a-chip (a modified gut-on-a-chip with an increased height of the lumen microchannel creating an anoxic–oxic interface, Fig. 3d)	Microorganisms: *B. adolescentis*, *E. hallii* (cultured in anoxic antibiotic-free cell culture medium (DMEM)) Cell lines: villi forming Caco-2BBE (culture in antibiotic-free cell culture medium (DMEM))	Successfully co-cultured obligate anaerobic bacteria with human cells	[97]

List of microorganisms: *Faecalibacterium prausnitzii*, *Saccharomyces cerevisiae*, *Lactobacillus rhamnosus*, *Bacteroides caccae*, *Escherichia coli*, *Bifidobacterium adolescentis*, *Eubacterium hallii*

ferent and peculiar features. The "gut-on-chip" described by Kim et al. was fabricated by soft lithography and composed of two parallel micro-channels [162]. These two compartments were separated by a porous PDMS membrane coated with an extracellular matrix (ECM) solution composed of rat type I collagen and Matrigel in serum-free DMEM. The culture medium was perfused through the microchannels, representing the fluid flow and shear stresses present in the human intestine, while cyclic deformations were applied to mimic peristalsis movement. Inside this device, Caco-2 cells were arranged to produce cellular monolayer and villi structures, and *Lactobacillus rhamnosus* was co-cultured. Results showed that after 96 h of co-culture in dynamic conditions, *L. rhamnosus* continued to adhere to the Caco-2 surfaces, ensuring a 95% viability rate of the monolayer, while in the control (i.e., a Transwell system in static condition) the death of the epithelial monolayer was observed after only 48 h. Jalili-Firoozinezhad et al. proposed a similar device that allowed the human intestinal epithelium to be cultured together with the human gut microbiota [163]. Using this device, it is possible to culture in vitro microorganisms in direct contact with host cells and their naturally produced mucus layer for at least 5 days. Culturing a complex microbial community together with host cells over a long period is difficult due to the high growth rate of bacteria compared to that of mammalian cells. Bacteria can also invade and kill epithelial cells, so separating microorganisms from host cells may overcome this problem. Pajoumshariati et al. described another system where different enteric bacterial species (i.e., *E. coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *P. aeruginosa*) and other

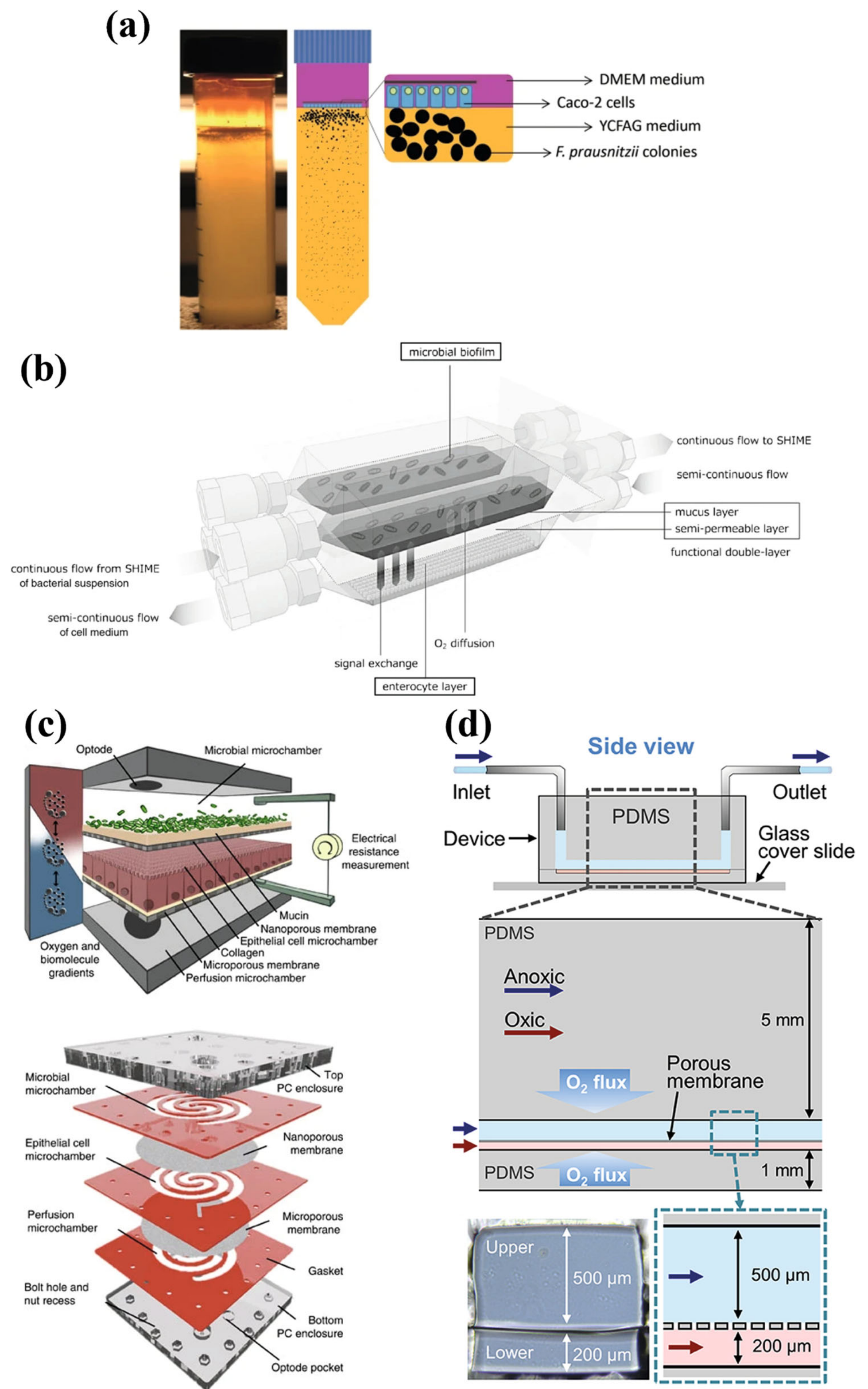

Fig. 3 Scheme of other devices used to study interactions between the human gut microbiota and eukaryotic cells. **a** The HoxBan system used for static co-culture between microorganisms, *F. prausnitzii*, and Caco-2 cells (reproduced from [156], Copyright 2015, with permission from the authors). **b** The HMI module consists of two chambers (the upper one contains bacteria, while the lower one contains enterocytes) separated by a polyamide semipermeable membrane and a mucus layer that form a double functional layer (reproduced from [157], Copyright 2014, with permission from the authors). **c** The HuMiX module is composed of three silicone rubber gaskets, each of which defines a distinct spiral-shaped channel (200 mm in length, 4 mm in width, and 0.5 mm in height), separated by two semipermeable membranes (a microporous membrane between the perfusion chamber and the cell chamber and a nanoporous membrane between the cell chamber and the microbial chamber). The whole structure is enclosed between two polycarbonate sheets (reproduced from [158], Copyright 2016, with permission from the authors). **d** The anoxic–oxic interface-on-a-chip module is fabricated through soft lithography and is composed of two PDMS parts. Anoxic (blue) and oxic (red) culture media are supplied through two different microchannels, separated by a porous PDMS membrane, to recreate an oxygen gradient (reproduced from [97], Copyright 2019, with permission from the authors)

bacterial species isolated from the ileum of patients with Crohn's disease (CD) were incorporated within chitosan-coated alginate-based microfibers (60 µm in diameter) that spatially separated microorganisms from co-cultured cells [164]. This encapsulation in a physical substrate successfully recreated the biofilm-associated microorganisms in the intestine. To reproduce the gut environment, mucins were also incorporated into the alginate. The results showed that

this model is able to retain bacteria within the microfibers for an acceptable period, thus providing a 3D microenvironment for bacterial growth and proliferation. The model also allows cells to more closely mimic their natural growth than planktonic and 2D in vitro methods.

Culturing cells and bacteria in a microfluidic system, particularly in the long term, can also lead to other problems, including fluid leakage [165], clogging [166], and unwanted accumulation of bubbles in the channels. Although some of these problems are manageable through a careful selection of materials or protocols, bubble accumulation is a frequent obstacle that is extremely difficult to avoid in most PDMS microfluidic systems [167]. Bubbles have a high probability of forming in the connection between the channel and the tube from which the fluid arrives. In addition, bubbles can gradually grow in the channel due to temperature and pressure variation. The presence of air bubbles can damage cells, rupture the cell membrane, or even wash away the cells. For this reason, some devices may involve the use of "traps" to eliminate these bubbles [168].

Ultimately, one of the new frontiers of research is to co-culture the human gut microbiota and intestinal organoids. The latter are self-assembling 3D cellular constructs made from stem cells that represent and reproduce the main physiological properties of an organ. From a purely physical and geometric point of view, an intestinal organoid can be considered as a closed 3D geometry with an internal cavity where a low level of oxygen is present. Furthermore, intestinal cells exhibit polarity and are typically arranged with the apical part toward the inside of the cavity. One of the first attempts to co-culture the gut microbiota and an organoid was made by inserting intestinal microorganisms directly into the cavity by micro-injection [169–173]. Although this technique is very easy and straightforward, it presents many risks related to co-culture over long periods such as disruption of the organoid membrane, a lack of medium recirculation, and a high probability of infection. Another possibility is to make organoids with the apical cellular part in contact with the external environment [174]. In this way, it becomes possible to insert the microbial suspension directly into the culture medium, preventing leakage problems during the micro-injection phase. Another method that can be applied is to linearize the structure of an organoid by switching from a 3D to a 2D construct. Indeed, standardized organoids can be fragmented and cultured to make monolayers [175, 176]. Using this method, several systems including anaerobic microorganisms have been realized through the formation of oxygen gradients or anaerobiosis chambers [177, 178]. This method can also be applied to microfluidic systems by inserting monolayers derived from organoids [97]. In this way, it is possible to use these systems for long-term cultures (i.e., 24 h for the Transwell systems above described [177, 178]) and, thanks to dynamic conditions, accumulation of a mucus layer, with a thickness similar to that found in vivo, can be observed [179].

Concluding remarks

Revealing the connections between the composition of the human gut microbiota and the consequent alteration of the normal physiological state is currently one of the most challenging research topics. Different studies have attempted to bridge this gap by exploiting in vitro models, drugs, pathogens, or highly predictive tools to show the effects of dietary components, on the activity and composition of a complex microbial community such as the gut microbiota, as well as to unravel the dense network of interactions between microorganisms and eukaryotic cells in different physiological states. Recreating a complete in vitro model of the human gut microbiota requires several initial steps, and a priority at this stage is comprehension of the ultimate purpose of the study itself. When fermentative processes and metabolic pathways carried out by the gut microbiota are investigated, more attention must be paid to the selected culture medium and maintenance of the culture parameters. On the other hand, when studying the effects of the human gut microbiota on eukaryotic cells, creation of an oxygen gradient between bacteria and cells, as well as the presence of cytotoxic molecules produced by microorganisms, becomes important priorities. Although extensive experience has been gained with in vitro systems over the last 20 years, most of the results are inconclusive and comprise single bacterial strains as opposed to a complex microbial profile like the human gut microbiota. In the future, researchers should primarily focus on designing human gut microbiota models whose activity and composition remain constant over time, or at least have only small fluctuations, and which are comparable with those of in vivo communities. Furthermore, it will be necessary for these technologies to be supported by in silico tools to create more adequate predictive models.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human or animal subjects performed by any of the authors.

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