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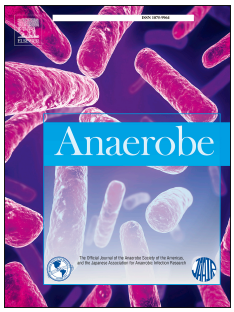

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ACCEPTED MANUSCRIPT

The role of gut microbiota in health and disease: *In vitro* modeling of host-microbe interactions at the aerobe-anaerobe interphase of the human gut

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Key words:
- Anaerobic gut bacteria
- Human intestinal epithelium
- Co-culture system
- Host-microbe interactions
- Transwell co-culture
- Host-Microbiota Interaction (HMI) module
- Human oxygen-Bacteria anaerobic (HoxBan) system
- The human gut-on-a-chip
- HuMiX model

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Highlights:
- Gut microbiota play an essential role in human health.
- Anaerobic bacteria form the major part of the gut microbiota.
- The oxygen-sensitivity of anaerobes hinders interaction studies with oxygen-requiring epithelial cells.
- Host-anaerobe interaction models enable detailed study of this interplay.
- The gut microbiome is an attractive target to modify to improve human health.

Conflict of interest:
HJMH received research funds for MSS from DSM and received a research grant from Chr. Hansen. GD, KNF and JZHVM have no conflict of interest to declare.

Contents

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Abstract

The microbiota of the gut has many crucial functions in human health. Dysbiosis of the microbiota has been correlated to a large and still increasing number of diseases. Recent studies have mostly focused on analyzing the associations between disease and an aberrant microbiota composition. Functional studies using (*in vitro*) gut models are required to investigate the precise interactions that occur between specific bacteria (or bacterial mixtures) and gut epithelial cells. As most gut bacteria are obligate or facultative anaerobes, studying their effect on oxygen-requiring human gut epithelial cells is technically challenging. Still, several (anaerobic) bacterial-epithelial co-culture systems have recently been developed that mimic host-microbe interactions occurring in the human gut, including 1) the Transwell "apical anaerobic model of the intestinal epithelial barrier", 2) the Host-Microbiota Interaction (HMI) module, 3) the "Human oxygen-Bacteria anaerobic" (HoxBan) system, 4) the human gut-on-a-chip and 5) the HuMiX model. This review discusses the role of gut microbiota in health and disease and gives an overview of the characteristics and applications of these novel host-microbe co-culture systems.

Introduction

Anaerobic gut bacteria play a pivotal role in human health and disease, most of which are strict/obligate anaerobes. Due to the oxygen-sensitivity of these bacteria, it is technically challenging to study their interaction with oxygen-requiring gut epithelial cells *in vitro*. Although many of the bacteria can survive oxygen by mechanisms such as sporulation; oxygen-free conditions are required for the anaerobic bacteria to grow. [1] Recently, a

number of different anaerobe-epithelial co-culture systems have been developed. These co-culture systems allow research of both aerobic (i.e. epithelial) cells and specific strains of anaerobic bacteria within one system. Development of representative co-culture systems that can mimic the gastrointestinal ecosystem are valuable tools to study host-microbiota interactions in detail at the mechanistic level. This review will first discuss the role of the human gut microbiota in health and (gut-related) diseases. Secondly, the relevance and the applications of the currently-available anaerobe-epithelial co-culture systems will be discussed.

1. The role of the gut microbiota

The human gut contains a wide variety of different microorganisms. Bacteria, viruses, archaea, yeast and fungi colonize the bowel. [2] The bacterial part of the microbiota is the most studied and best described of these different microorganisms. [3] The trillions of bacteria that inhabit the gut of each individual belong to hundreds of different species. [4,5] The composition of the gut microbiota is highly dynamic and different for each human individual and changes during the course of life. [6] The bacterial phyla Bacteroidetes and Firmicutes are the most prevalent in adults and together they form the majority of the gut bacteria. [4,5] The microbiota in the gut has many crucial functions in human health and affects the host via different host-microbiota interaction pathways. [7-9] For example, intestinal microbiota enable fermentation of complex non-digestible carbohydrates and produce short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate. [10,11] Several anaerobic bacteria that dominate a healthy gut, such as *Faecalibacterium*

prausnitzii and *Roseburia* species, are major butyrate producers. [12,13] Butyrate is known to be an important energy source for colonocytes, and is suggested to enhance intestinal barrier function. [14] Moreover, butyrate is known to possess anti-inflammatory properties and even possible anti-cancer effects. [10-12,15] In addition, the 'healthy gut microbiome' plays an important role in the development of a balanced immune-system. A certain level of immunological tolerance exists for the intraluminal bacteria in a healthy gut. Extensive profiling of the human gut microbiome has shown that several common diseases are associated with "dysbiosis" of the gut microbiota. The term dysbiosis is often used to describe a disturbed balance between 'beneficial' bacteria with anti-inflammatory properties and pathobionts with pro-inflammatory properties. Moreover, many diseases are associated with a decreased diversity of the gut microbiota. [16-18]

For the majority of diseases it remains unclear to which extent the dysbiosis is the cause or the consequence of the disease and/or treatment. [19] This issue is further complicated by the fact that many studies investigate the bacterial composition of the fecal material, which may significantly differ from the bacterial composition attached to the mucosa (mucosa-associated microbiota, MAM) that may be more directly related to the actual disease development. [20] Moreover, the bacterial composition and abundance vary between different parts of the gastrointestinal tract.

It is well established that the two major forms of inflammatory bowel disease (IBD) – Crohn's disease (CD) and ulcerative colitis (UC) – are associated with alterations of the microbiota. [21,22] In both diseases, there is an inappropriate mucosal immune response

133 triggered by the commensal microbiota in a genetically predisposed host. [23-27] Changes in
134 the gut microbiome seem more apparent in CD than in UC. [28,29] Also, CD patients show a
135 less diverse microbiota profile than healthy individuals. [30,31] Typically, a decrease in
136 abundance of *Bacteroides* and Firmicutes is detected, together with an increase in
137 proteobacteria and fusobacteria. [22] A consistent observation is a decreased abundance of
138 butyrate-producing *F. prausnitzii* and an increased number of Adherent-invasive
139 *Escherichia coli* (AIEC) in CD patients. [22,32-36] In addition, an increase of the mucin-
140 degrading bacterium *Ruminococcus gnavus* has been described. [21] CD patients with
141 higher numbers of pathobionts, such as *E. coli*, and lower proportions of *F. prausnitzii*
142 have an increased risk of endoscopic recurrent disease after ileal/ileocecal resection. [37,38]
143 Furthermore, CD patients with the lowest abundance of *F. prausnitzii* often have a less
144 favorable disease course, with worse disease scores and elevated inflammatory markers.
145 [39] In line with these observations, the abundance of *F. prausnitzii* may even function as a
146 biomarker for predicting disease course in CD patients. [40,41]
147
148 Another example of a disease in which an aberrant microbiota composition is observed is
149 celiac disease. In the duodenum of these patients typically an increase in Bacteroidetes is
150 detected. [42-45] Also, an association between the gut microbiome and the development and
151 the progression of intestinal cancer has been described. [46,47] Recent evidence suggests a
152 relationship between aberrant intestinal microbiota and non-gastrointestinal disorders. It
153 is increasingly recognized that common metabolic diseases, such as obesity and type 2
154 diabetes mellitus, are associated with an altered microbiota composition. [48-51] For
155 instance, a recent study shows that a relatively high abundance of *Akkermansia*

156 *muciniphila* is associated with a healthier metabolic status. [51] Finally, associations
157 between an altered microbiota composition and neurologic or psychiatric diseases, such
158 as anxiety, depression and autism are described. [52,53]
159
160 The composition of the gut microbiota is dynamic, complex, and is influenced by both
161 non-adjustable factors, such as age and geographical location, and adjustable factors, like
162 diet and medication. [54-56] The strong link between aberrant microbiota with several
163 common diseases, and the possibility to reshape its composition, makes the microbiota an
164 attractive target for health improvement. [56,57] As a result of a dysbiotic state of the
165 intestinal bacteria, host functions, such as the epithelial barrier and an adequate immune
166 response may be compromised.
167
168 It is apparent that dietary interventions have a strong effect on microbiota composition.
169 [58,59] The western diet, characterized by high sugar and fat content and low amounts of
170 dietary fiber, has adverse effects on the microbiota composition, especially in the context
171 of IBD. [60,61] Certain probiotic (living microorganisms) and prebiotic (non-digestible
172 polysaccharides) supplements can be used to alter the microbiota composition. [62-65]
173 Moreover, different types of medication have adverse effects on the microbiota
174 composition. For example, treatment of bacterial infections with antibiotic drugs is
175 common in modern medicine. However, these drugs should be prescribed in a
176 conservative way, because of the profound effect of these drugs on the microbiota
177 composition. [66-68] Similarly, chemotherapeutic agents may have an even more detrimental
178 effect on the microbiota, with dramatic reductions in the number of anaerobic bacteria.

[69,70] Also, a recent study, combining the data of three large Dutch cohorts, shows that proton pump inhibitors (PPI's) negatively modify the microbiota and predispose to *Clostridium difficile* infection. [71] 'Improving' the composition of the gut microbiota is therefore a promising target for the treatment of many diseases. For *C. difficile* infection, fecal microbiota transplantation (FMT) has already been shown to be an effective and highly successful treatment. [72,73] However, FMT has shown to be less promising for IBD patients. [74] Moreover, FMT has several risks, such as potential transmission of viruses. Also the long-term effects of this treatment are not fully determined yet. Multiple studies have evaluated the effect of prebiotic and probiotic interventions in IBD. In this review we will only discuss a selection of important studies performed in this area. [75]

In UC the role of the probiotic supplement VSL#3 was evaluated. This supplement is a probiotic mixture, consisting of four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus salivarius* subsp. *thermophilus*. VSL#3 intake results in an increase of 'protective' bacteria and may help to prevent a flare-up of intestinal inflammation. [76] Indeed, a recent meta-analysis revealed that VSL#3, when added to conventional therapy, improves remission rates in mild to moderate active UC. In a similar way, this probiotic mixture enhanced remission in chronic pouchitis patients. [77,78] Also in CD, the other major form of IBD, different dietary interventions (i.e. pre- and probiotics) aiming to modify the microbiota composition have been performed. The clinical trials with pre- and probiotics can be considered as rather opportunistic as they test the "known suspects" for their therapeutic potential. However, in many cases the results of such clinical trials are inconsistent. [79] Numerous factors, such as interindividual

genetic variation and differences in environmental circumstances, are frequently encountered in prospective human studies. Of course, these factors influence the outcome of these intervention studies, and may compromise the reliability of the findings. Considering the ethical issues and high costs associated with such clinical trials, it would be of immense value when the potential therapeutic effects of pre- and probiotics could be analyzed in a controlled and reproducible manner. Gnotobiotic animals, such as germ-free mice, seem to be an attractive model between human clinical studies and *in vitro* models. [80,81] Advantages of these germ-free mice consist of a controllable host environment and the opportunity to investigate specific bacterial contributions. However, in recent years, many *in vitro* gut systems have undergone great technological improvements and increasingly become more representative of the *in vivo* situation. These improvements in *in vitro* gut models will likely result in increased usage of these systems, for instance as a screening tool for dietary interventions. [34,82-85]

2. Gastrointestinal *in vitro* model systems

Studies that establish an association between a specific microbiota composition and a disease phenotype provide incomplete information about possible underlying mechanisms. [86] *In vitro* studies are often required to give more mechanistic insight. The complex interactions between human gut microbiota, epithelial cells and immune cells are difficult to mimic in *in vitro* models, and also other factors, such as variable oxygen levels and gut peristalsis should be included. A major advantage of *in vitro* models is that they can be tightly controlled under reproducible conditions. Also, they allow detailed

mechanistic analysis; have limited ethical restrains and require no expensive and time-consuming ethical approval procedures (as required for human clinical trials or animal studies). Furthermore, since pharmaceutical procedures and dietary research usually take many years, a representative *in vitro* model may considerably accelerate these procedures. Altogether, this makes the development of *in vitro* models that closely resemble the conditions in the gastrointestinal tract highly relevant.

Exactly mimicking the gastrointestinal situation *in vitro* seems hardly possible; some parameters will typically be omitted in the development of a model that is suitable to answer specific questions. Thus, the research questions to be answered largely determine which *in vitro* model is most appropriate to use, although all currently available systems have their specific limitations as well. Ideally, the *in vitro* model should allow the analysis of the direct interactions between host cells and microbes, as it exists in the gut. Direct host-microbe interactions may be more relevant in the small intestine, with a rather thin mucus layer compared to the colon where the much thicker mucus layer is a more prominent physical barrier. The gut lumen is almost completely anaerobic. Only minute amounts of oxygen will penetrate from the epithelium into the lumen. Thus, the gut microbiome consists of facultative and (predominantly) strict anaerobic bacteria. An *in vitro* model of the gut therefore preferably establishes true anaerobic conditions for the microbes, while the host cells are cultured under aerobic conditions. Ideally, an *in vitro* gut model allows the analysis of parameters that differentiate between health and disease, as well as the effect of (dietary) interventions. Host parameters that are considered to be important are cell viability, proliferation and differentiation, epithelial permeability

(barrier function) and cytokine production. On the luminal side, microbial parameters, such as bacterial fitness, bacterial composition, substrate utilization and metabolite production (such as SCFAs) are important to analyze. The currently available *in vitro* models of the human gastrointestinal tract are discussed in the following sections. These models can be divided into models that enable the study of isolated components of the gut ecosystem, such as gut epithelium cells and mucosa **(section 2.1)** or models that study the gut microbiota in isolation **(section 2.2)**. However, to truly mimic the mutual communication between human gut (epithelial) cells and the gut bacteria, systems are needed that allow co-culturing of both in one system, which are reviewed in **section 2.3**.

2.1 Models for gut epithelium and mucosa

Intestinal cell lines, such as Caco-2, HT-29, T-84 and DLD-1, are frequently used as representatives of the human gastrointestinal epithelium, however, they originate from gastrointestinal tumors. Their true epithelial characteristics are often compromised. Still, epithelial cell lines can be used in Ussing chamber experiments, in which properties like transport of substances and permeability through the epithelial cell layer can be assessed. Intestinal explants have the advantage that the integrity of the intestinal mucosa layer remains intact. [87,88] Also, precision-cut intestinal tissue slices (PCIS) are an *ex vivo* model used for drug metabolism studies. [89,90] All cell types from the gut are present in PCIS and this model also allows study of diseased tissue. [91] More recently, intestinal organoids or 'mini guts' are being established as models of the human intestinal epithelium that contain all main types of epithelial cells, e.g. enterocytes, goblet cells, enteroendocrine cells and Paneth cells. [92] These gut organoids can be grown *in vitro* from resident stem

cells in the gut and remain genetically stable in culture for many cell divisions (over months to years). [93] Also, the gut organoids maintain their location-specific characteristics, so a differentiation can be made between colonic, ileal, jejunal and duodenal primary human intestinal epithelium. [94] Models using epithelial cells can be exposed to bacteria or bacterial extracts or products secreted by bacteria. However, this is different from a co-culture device, in which different cell types are grown (and remain viable) for a certain time period. Also, a potential effect of the epithelial cells towards the bacteria cannot be studied in such a cell model system.

2.2 Models for gut bacteria

Examples of systems that are used to study the human gut microbiota in isolation are the TNO dynamic *in vitro* model of the human large intestine (TIM-2), the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), the "Three stage continuous culture system", the Lacroix model and the fecal minibioreactor arrays (MBRAs). [95-98] The TIM-2 is designed to simulate the conditions found in the proximal colon. [99] Accumulation of metabolites in the lumen is prevented by constant and active removal of these metabolites by means of a dialysis system. In addition, peristalsis, temperature and pH are controlled in this system to mimic the *in vivo* human situation. The TIM-2 system allows for the analysis of fermentation patterns and effects of prebiotic and probiotic supplement intake on microbial composition. [100-105] The SHIME contains five connected vessels that are designed to closely mimic the bacterial compartment of the gastrointestinal tract of an adult human. [106] Each reactor simulates a different part of the GI-tract: stomach, small intestine, ascending colon, transverse colon and descending colon. In this model, the

295 'intraluminal content' is continuously stirred and pH-controlled. In addition, pancreatic
296 enzymes and bile are added to more closely resemble the *in vivo* situation. In this model
297 the fermentation patterns of four polysaccharides were shown to be similar to the
298 fermentation pattern that occurs *in vivo*. [107] The SHIME is relevant for intervention
299 studies, such as supplementation studies of different probiotic strains or prebiotics. [108-110]
300 The "Three stage continuous culture system" comprises three culture vessels, simulating
301 the ascending, transverse and descending colon. This system simulates the nutritional and
302 environmental conditions in the human large intestine. Oxygen-free conditions, pH
303 control and transit time closely resemble the *in vivo* situation. [95,111,112] The Lacroix model
304 is also a three stage continuous culture system, which uses immobilized fecal microbiota
305 and is used to simulate fermentation of the infant colon. [97,113] Finally, the fecal
306 minibioreactor array (MBRA) is another *in vitro* system used to cultivate and investigate
307 fecal microbiota communities. In these bioreactors, consisting of six single vessel
308 chambers in an anaerobic chamber, the diluted feces of multiple human donors is used. In
309 one study this system is used to test competition between different ribotypes of *C.*
310 *difficile*. [98]
311
312 The systems described above may generate valuable information about the response of
313 the gut mucosa to bacterial (products) or direct effects of nutritional factors to the
314 composition of the gut microbiota. However, they do not allow the analysis of the mutual
315 communication between the gut bacteria and the intestinal epithelium or simulate disease
316 conditions of the host. For such systems, an additional barrier needs to be taken and that

317 is to co-culture bacteria under anaerobic conditions while gut (epithelial) cells are
318 provided with sufficient oxygen.
319
320 **2.3 Models for gut host-microbe interactions**
321 An *in vitro* gut host-microbe co-culture system would have many advantages for
322 unraveling the direct role of gut bacteria in intestinal health, provided that it is robust and
323 truly simulates the gut ecosystem. A schematic figure of the host-microbe interaction at
324 the aerobe-anaerobe interphase is shown in **Figure 1A**. Below, we give a concise
325 overview of recently developed systems that enable the co-culture of (anaerobic) gut
326 bacteria and (oxygen-requiring) epithelial cells (also see **Table 1** for a comparative
327 overview).
328 **I)** Transwell co-culture models are examples of systems that are used to study cell-cell
329 interaction. These Transwell co-culture systems seem to be particular useful to study the
330 interaction between bacteria, mucosal immune cells and intestinal epithelial cells under
331 static conditions, but are more frequently used under aerobic conditions. [114-116] Recently,
332 a custom-made variant of such a Transwell co-culture system was developed that allows
333 the analysis of host-microbe interactions between oxygen-requiring Caco-2 cells and
334 anaerobic *F. prausnitzii* bacteria for up to 8 h. [117] The Transwell **'apical anaerobic**
335 **model of the intestinal epithelial barrier'** chamber (see **Figure 1B**) contains oxygen-
336 containing medium in the bottom compartment. Caco-2 cells pre-grown on the filter of an
337 insert are placed in the chamber. Subsequently, anaerobic culture medium, with or
338 without *F. prausnitzii*, is added in the insert allowing direct contact with the Caco-2 cells.
339 After this, the whole system is placed in an anaerobic workstation. Dissolved oxygen

levels remained high in the bottom compartment and low in the upper compartment over a 12 h incubation period. *F. prausnitzii* bacteria pre-grown to stationary phase were added in anaerobic host cell culture medium (M199) to the upper compartment. The number of viable *F. prausnitzii* remained relatively stable, but still dropped approximately 10-fold after an 8 h co-culture period with Caco-2 cells. In comparison, viability of *F. prausnitzii* dropped over 10,000-fold when cultured for 30 min in oxygen-containing M199. During 8 h of co-culturing, Caco-2-dependent transepithelial electrical resistance (TEER) was slightly enhanced by *F. prausnitzii* compared to control conditions without bacteria. The ^{3}H-mannitol flux across the Caco-2 monolayer was not affected by *F. prausnitzii* during the first 6 h of co-culture, after which it increased in comparison to control conditions without bacteria. Global gene expression analysis of Caco-2 cells exposed for 4 h to either live or UV-killed *F. prausnitzii* revealed that live bacteria suppress cellular pathways involved in inflammatory response and immune cell trafficking much stronger than dead bacteria. The most pronounced findings were the increase in IL-10 and a decrease in NF-κB signaling. Thus, the 'apical anaerobic model of the intestinal epithelial barrier' maintains (sufficient) viability of host cells and microbes for up to 8 h, allowing real time measurements of TEER. In addition, it shows that the metabolic activity of *F. prausnitzii* is required to acquire its maximum anti-inflammatory capacity.

II) The Host-Microbiota Interaction (HMI^{TM}) module is a custom-made co-culture system consisting of two compartments, a "luminal" compartment containing gut bacteria and a "host" compartment containing the "enterocytes", e.g. Caco-2 cells (see **Figure 1C**). [118] An important difference with the above-described Transwell co-culture system is

363 that these two compartments have (semi-)continuous flow of fluid and are separated by a
364 functional double layer (a semi-permeable membrane and an artificially added mucus
365 layer). The HMI module was designed to be connected to an adapted version of the
366 SHIME, containing only the first 3 reactors that simulate the stomach, the small intestine
367 and the ascending colon. The SHIME was inoculated with a fecal sample of a healthy
368 individual and after passing the 3 reactors the effluent, consisting of a complex mixture
369 of intestinal bacteria, flows through the "luminal" compartment of the HMI module. The
370 "host" compartment containing Caco-2 cells receives semi-continuous flow of cell
371 culture medium in the opposite direction. The separating layer (semi-permeable
372 polyamide membrane with 0.2-µm pore size coated with a mucus layer) was shown to be
373 permeable for FITC-dextran of up to 150 kDa in size, but obviously does not allow direct
374 interaction between bacteria and host cells. In this co-culture system, important features
375 of the gastrointestinal tract, such as shear stress, permeability, oxygen diffusion and the
376 possibility of the microbiota to colonize the mucus layer are taken into account to closely
377 mimic the human *in vivo* situation. In addition, a dietary intervention using the dried
378 fermentation products of baker's yeast (*Saccharomyces cerevisiae*) was studied in this
379 system. Caco-2 cells appeared very sensitive to direct exposure to the effluent of the
380 adapted SHIME leading to a 80% reduction in cell viability after 2 h. In contrast, Caco-2
381 cells remained viable for up to 48 h when cultured in the HMI module downstream of the
382 SHIME. The SHIME-HMI combined system was used to study the effect on the luminal
383 and mucosa-associated microbiota, as well as on Caco-2-mediated cytokine production
384 upon treatment with fermentation products of *S. cerevisiae*. The presence of Caco-2 cells
385 in the HMI module did not strongly affect the number and relative abundance of different

bacterial groups in the luminal samples, although a consistent trend of reduced bacterial numbers was observed in time (comparing 0, 24 and 48 h co-culturing). The treatment with *S. cerevisiae* fermentation products significantly enhanced the levels of SCFAs in the SHIME effluent entering the HMI module. Remarkably, this was associated with a lower total number of luminal bacteria, similar for all four groups tested. Passing the *S. cerevisiae*-treated effluent through the Caco-2-containing HMI module resulted in a significant increase in the abundance of luminal Bacteroidetes, Firmicutes and bifidobacteria. Interestingly, Caco-2 cells produced significant amounts of pro-inflammatory IL-8 at the end of the 48 h co-culturing with the normal SHIME effluent, which was completely suppressed by the treatment with *S. cerevisiae* fermentation products, indicating an anti-inflammatory response induced by this "intervention". This is in line with immune modulating / anti-inflammatory properties of this product that have previously been demonstrated in *in vivo* studies. [119-121] A reduction of pro-inflammatory IL-8 production was correlated with an increased butyrate production in the SHIME. [122] Interestingly, this intervention resulted in a 31% increase in butyrate production in the ascending colon of the HMI module. Simultaneously, the HMI module allows for the analysis of the bacterial colonization of the mucus layer. While the strict anaerobic bifidobacteria colonized the upper side of the mucus layer (facing the luminal compartment), *F. prausnitzii* was mainly detected in the lower parts of the mucus (facing the "host" compartment) as observed in the human gut *in vivo*. This may be due to the capability of *F. prausnitzii* to survive microaerophilic conditions in the abundant presence of flavins and/or thiols.

III) The 3rd system that aims to simulate host-microbe interactions occurring at the oxic-anoxic interphase of the (human) gut is the **'Human oxygen Bacteria anaerobic' (HoxBan) co-culturing system** (see **Figure 1D**). In contrast to the previously described "apical anaerobic model of the intestinal epithelial barrier" and HMI module, the HoxBan system does not require specialized (e.g. custom-made) equipment. The HoxBan system consists of an anaerobic and an aerobic compartment that are created in a 50 mL plastic tube. The bottom compartment contains the anaerobic bacteria of interest in specific culture medium solidified with 1% agar. The top compartment contains the oxygen-requiring epithelial cells on a glass coverslip (cells facing down), covered with cell culture medium. Oxygen is penetrating in the agar from the top compartment, creating an oxygen gradient, resembling the steep gradient across the human intestinal epithelium. Obligate anaerobic bacteria in the lower compartment are protected from oxygen by the agar and can grow at the lower end of the gradient. [123] In practice, the liquid (hand-warm) agar broth is inoculated with *F. prausnitzii* in an anaerobic workstation, aliquoted (40 mL each) in 50 mL plastic tubes and allowed to solidify. Subsequently, the HoxBan tubes are transferred to a cell culture cabinet and Caco-2 cells, pre-grown on coverslips to 80-100% confluency, are placed upside-down on the bacteria-containing agar medium. The tubes are filled to the top with cell culture Dulbecco's Modified Eagle Medium (DMEM). Subsequently, the tubes are placed in a standard humidified cell culture incubator at 37^{o}C and 5% CO_2 for up to 18-36 h. No reduction in viability of Caco-2 cells was observed when co-cultured with *F. prausnitzii* for 24 h. In fact, this analysis showed for the first time that mutualism is observed between oxygen-requiring intestinal epithelial (Caco-2) cells and anaerobic *F. prausnitzii* bacteria. A remarkable enhancement of *F. prausnitzii*

growth was observed directly below the Caco-2-containing coverslips. Interestingly, this was not seen when *F. prausnitzii* was co-cultured with non-intestinal cells, like the human liver cancer cell line HepG2, indicating that this effect is (intestinal) cell type-specific. Moreover, Caco-2-*F. prausnitzii* co-cultures in the HoxBan system confirmed the anti-inflammatory and anti-oxidative stress effects of live *F. prausnitzii* on Caco-2 cells. The HoxBan setup allowed analyses of the consumption and production of metabolites (the "exo-metabolome", including SCFAs, hydrocarbons, lipids and amino acids) in the liquid cell culture medium after 18 h of co-culture. These analyses revealed that levels of formate are strongly increased if *F. prausnitzii* is co-cultured with Caco-2 cells, while butyrate levels are not changed (compared to *F. prausnitzii* without Caco-2 cells). The selective effect on the levels of these SCFAs requires further study, but could be a result of the selective use of butyrate by the "enterocytes". Currently, research in additional applications of the HoxBan system is being performed. These include studies assessing the effect of prebiotic and vitamin interventions on host-microbiota interplay and adaptation of this system to a disease model for IBD. The results observed in the HoxBan model correspond with previously performed *in vivo* studies. Anti-inflammatory effects of this bacteria were demonstrated in a murine TNBS-induced (chemical induced) colitis model, in which administration of *F. prausnitzii* and its supernatant had a protective effect. [124] Also a beneficial effect of *F. prausnitzii* on intestinal epithelial barrier function has been described in a murine model of low-grade inflammation. [125] Furthermore, a large meta-analysis in 2014 showed that the abundance of *F. prausnitzii* is reduced in IBD patients when compared with healthy subjects. [36]

IV) A 4th system that is relevant for host-microbe interaction studies is the **human gut-on-a-chip** (see **Figure 1E**). However, in contrast to the previously described systems, its use for co-culturing human cells with strict anaerobic gut bacteria has not been performed yet and it may be technically very challenging to maintain both aerobic and (strict) anaerobic conditions in this system. Still, very interesting results were obtained when co-culturing Caco-2 cells with oxygen-tolerant gut bacteria, which may be relevant for further development of true aerobic-anaerobic co-culturing systems. The gut-on-a-chip consists of two microchannels, simulating the gut lumen and the blood compartment, separated by a porous flexible membrane coated with extracellular matrix (ECM) and lined by Caco-2 cells. [126] Apart from continuous medium flow providing low shear stress to Caco-2 cells, this system is unique because of the fact that it can also mimic peristalsis-like motions by stretching and relaxing the ECM-coated porous membrane. This membrane is attached to two hollow side chambers that are rhythmically inflated/deflated. Especially promoted by the peristalsis-like motions, Caco-2 cells differentiate into a complex intestinal epithelium consisting of four types of intestinal epithelial cells, i.e. absorptive enterocytes, mucus-secreting goblet cells, enteroendocrine cells and Paneth cells. Moreover, 3D villi-like structures are formed. [126,127] The gut-on-a chip allows the analysis of TEER, which increased more rapidly compared to monocultured Caco-2 cells in transwell cultures. Gut-on-a-chip allows the long-term (days up to two weeks) co-culture with bacteria. Probiotic *Lactobacillus rhamnosus* GG (LGG) formed microcolonies on the surface of Caco-2 cells and increased the TEER compared to Caco-2 cells not exposed to LGG. Co-culturing of Caco-2 cells with a formulation of probiotic bacteria (VSL#3, containing 6 bacterial strains originally

476 isolated from the human gut microbiome) for 72 h induced transcriptome changes in
477 Caco-2 cells that more closely resemble the human ileum, as compared to monocultured
478 Caco-2 cells in the gut-on-a-chip. Moreover, VSL#3, as well as antibiotic therapies, were
479 shown to suppress villus injury and loss of TEER was induced by pathogenic Entero-
480 invasive *E. coli* (EIEC) bacteria. Interestingly, exposure to LPS isolated from pathogenic
481 *E. coli* did not directly affect TEER or villus injury in Caco-2 cells in the gut-on-a-chip.
482 Only when human peripheral blood mononuclear cells (PBMCs) were also included in
483 the lower capillary channel (simulating the blood compartment), both loss of TEER and
484 villus injury were induced by LPS. Moreover, inclusion of PBMCs and LPS in the gut-
485 on-a-chip resulted in the polarized secretion of inflammatory cytokines (IL-1β, IL-6 and
486 TNFα) to the "blood compartment". Finally, the manipulation of peristaltic motions
487 appeared to be highly relevant for host-microbe interactions, where the absence of such
488 cyclic mechanical deformations increased the levels of *E. coli* colonizing the enterocyte
489 surface, a process that might resemble bacterial overgrowth. As highlighted before, strict
490 anaerobic bacteria have not been co-cultured with Caco-2 cells in the gut-on-a-chip and
491 given the small diameters of the channels it may be technically impossible to maintain
492 anaerobic conditions in the "luminal compartment".
493 **V)** The 5th and most recently described aerobic-anaerobic co-culture system is the
494 **HuMiX (human-microbial crosstalk) modular microfluidic device**. [128] This device is
495 composed of a modular stacked assembly of elastomeric gaskets sandwiched between
496 two polycarbonate enclosures (see **Figure 1F**). Each gasket defines a distinct spiral-
497 shaped microchannel. The upper compartment is the 'Microbial microchamber' and is
498 separated from the middle compartment: 'the Epithelial cell microchamber' by a

Nanoporous membrane (pore diameter 50 nm). The 'Epithelial cell microchamber' contains the oxygen-requiring Caco-2 cells, forming the epithelial cell barrier. The bottom microchannel is the 'perfusion microchamber', which is separated from the 'Epithelial cell microchamber' by a Microporous membrane (pore diameter 1 µm). In this device, Caco-2 cells are first cultured and grown for 7 days to form a well-differentiated layer of epithelial cells. Monocultured Caco-2 cells established significantly higher TEER in the HuMiX as compared to Caco-2 cells cultured in a similar set-up in a Transwell device. Moreover, clear expression of the tight junction protein occludin at the cellular membrane was demonstrated by immunofluorescence microscopy. Subsequently, bacteria were inoculated in the Microbial microchamber and co-cultured for an additional 24 hours. Following co-culture, all individual cell contingents can easily be accessed and evaluated. In this study, the researchers first inoculated the commensal facultative anaerobe *Lactobacillus rhamnosus* GG (LGG), which was also studied in the gut-on-a chip (see above). Both the oxygen-requiring Caco-2 cells and the facultative anaerobe LGG remain viable during co-culture. Integrated oxygen sensors in this device allow the real time monitoring of dissolved oxygen concentrations. Clearly different oxygen levels were detected between the "perfusion microchamber" and the "microbial microchamber", though the latter was not completely devoid of oxygen. Still, the authors show that this device can also be used to study the effect of obligate anaerobic bacteria in co-culture with Caco-2 cells. The obligate anaerobic strain *Bacteroides caccae* (of the phylum Bacteroidetes) inoculated in combination with LGG remained viable and a relative increase in number of *B. caccae* compared to LGG was detected after a 24 hour co-culture period with Caco-2 cells. However, absolute numbers of both bacteria before and

after co-culture were not shown. Moreover, the potential difference in growth rate between these two bacteria (in the absence of Caco-2 cells) was not established. So a potential selectivity of Caco-2 cells towards specific bacteria cannot be concluded from these experiments. Importantly, this device allows the additional inclusion of immune cells (i.e. CD4+ T cells) to the perfusion chamber, to help further clarify specific immunological research questions. Finally, the authors validate the HuMiX in relation to previously performed *in vivo* studies. They show that the transcriptional responses of the epithelial cells co-cultured with LGG in the HuMiX are in line with *in vivo* expression data obtained from human and piglet studies. [129-131] This study nicely demonstrates that it is crucial to establish (near) anaerobic conditions for the microbiota in a representative gastrointestinal co-culture device, since clear differences in transcriptional responses between LGG grown under aerobic and anaerobic conditions were shown.

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Table 1. Characteristics and applications of recently developed (anaerobic) bacterial-epithelial gut co-culture models.

	A. Transwell 'apical anaerobic model of the intestinal epithelial barrier'	**B. Host-Microbiota Interaction (HMI™) module**	**C. HoxBan co-culture system**	**D. Human gut-on-a-chip**	**E. The HuMiX model**
Human gut epithelium model (cell type)	*Caco-2*	*Caco-2*	*Caco-2, DLD-1*	*Caco-2*	*Caco-2*
Direct contact bacteria and host cells	*Yes*	*No (separated by mucus and microporous membrane)*	*Yes*	*Yes*	*No (separated by Nanoporous membrane)*
Mucus layer	*No*	*Yes (artificially added)*	*Yes (artificially added)*	*Yes (mucus production)*	*Yes (mucin layer)*
"Gut epithelial cells" grown in: (during co-culturing)	*M199 + 10% FBS*	*DMEM + 10% FBS*	*DMEM + 10% FBS*	*DMEM + 20% FBS*	*DMEM + 20% FBS*
(Anaerobic) bacteria grown in: (during co-culturing)	*Anaerobic M199 (-FBS)*	*Mixed carbon-source bacterial broth for SHIME*	*YCFAG (Anaerobic F. prausnitzii broth)*	*DMEM + 20% FBS*	*Anoxic DMEM medium*
Host-Microbe co-culture time	*Up to 8 h*	*Up to 48 h connected to SHIME*	*Up to 36 h*	*1-2 week*	*24 h*
Static or fluid flow (shear stress)	*Static*	*Fluid flow (6.5 mL min^{-1} = 3 dyne cm^{-2})*	*Static*	*Fluid flow (30 uL h^{-1} = 0.02 dyne cm^{-2})*	*Flow rate: 25 µl min^{-1}*
Simulation of peristalsis	*No*	*No*	*No*	*Yes*	*No*
Co-culture with strict anaerobic bacteria	*Yes (i.e. F. prausnitzii)*	*Yes (SHIME effluent, including F. prausnitzii)*	*Yes (i.e. F. prausnitzii)*	*Not described*	*Yes (Bacteroides caccae)*
Mixed bacterial cultures	*Not described*	*Yes (fecal inoculum from healthy human in SHIME)*	*Not described*	*Yes (VSL#3)*	*LGG and B. caccae*
Combination with other types of (human) cells	*Not described*	*Not described*	*Not described*	*Yes (PBMCs, endothelial cells)*	*Yes CD4+ T cells*
Analysis of epithelial barrier function	*Yes (TEER, ^{3}H-mannitol flux, IF-staining for TJ proteins)*	*Yes (bilateral diffusion of 4-20-150 kDa FITC dextran)*	*Yes (staining for TJ proteins)*	*Yes (TEER)*	*Yes (HuMiX-TEER device and Staining for TJ protein occludin)*
Intervention studies (diet, medication, etc)	*Not described*	*Yes (S. cerevisiae fermentation products)*	*Yes (prebiotics, vitamins)*	*Yes (probiotic VSL#3 and antibiotic mixture)*	*LGG is used as a probiotic treatment*
Model of disease	*Not described*	*Not described*	*Yes (induction of inflammatory state in epithelial cells)*	*Yes (bacterial overgrowth and inflammation)*	*Not described*

***Abbreviations**: Caco-2: human colon epithelial cell line. DLD-1: human colon epithelial cell line. M199: medium 199. DMEM: Dulbecco's Modified Eagle Medium. YCFAG: medium containing yeast extract, casitone, fatty acids and glucose. FBS: fetal bovine serum. H: hours. PBMCs: peripheral blood mononuclear cells. TEER: transepithelial electrical resistance. TJ proteins: tight junctions proteins. LGG: Lactobacillus rhamnosus GG.*

Concluding remarks

Dysbiosis of the gut microbiota is associated with many common diseases, however limited tools are available to determine what is the cause or consequence of this phenomenon. *In vitro* models for host-microbe interactions occurring in the (largely anaerobic) gut are instrumental to analyze the molecular and cellular mechanisms involved. Several (anaerobic) bacteria-gut epithelial co-culture systems models have recently been developed. A comparative overview of the characteristics and applications of these systems is given in **Table 1**. Each of these systems has its own pros and cons, and the specific research question will largely determine which system is most suitable to use. Key factors to consider are 1) whether a strict anaerobic compartment for gut bacteria is required; 2) whether single or complex mixtures of bacteria need to be analyzed; 3) whether direct contact with bacteria and gut epithelial cells is important, 4) whether analysis of the barrier function (such as TEER) is needed; 5) whether effects on both gut epithelia, as well as bacterial metabolism will be analyzed; and maybe at least as important 6) whether the equipment and infrastructure is available to perform such experiments. A major "weakness" of all systems so far is that they all rely on the use of Caco-2 cells as representative of the human gut epithelium. Still, Caco-2 cells originate from heterogeneous human epithelial colorectal adenocarcinoma and may therefore behave quite differently as compared to true human gut epithelium. Recent advancements in generating primary human epithelium from intestinal stem cells hold great promise for "upgrading" these host-microbe co-culturing systems with location-specific and/or disease-specific human gut epithelium. Thus, co-culturing oxygen-requiring human gut epithelial cells with anaerobic gut bacteria is technically feasible, however, the individual

563 systems need further refinement to help us unravel the complex functional links between

564 disease and gut microbiome dysbiosis.

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FIGURE 1

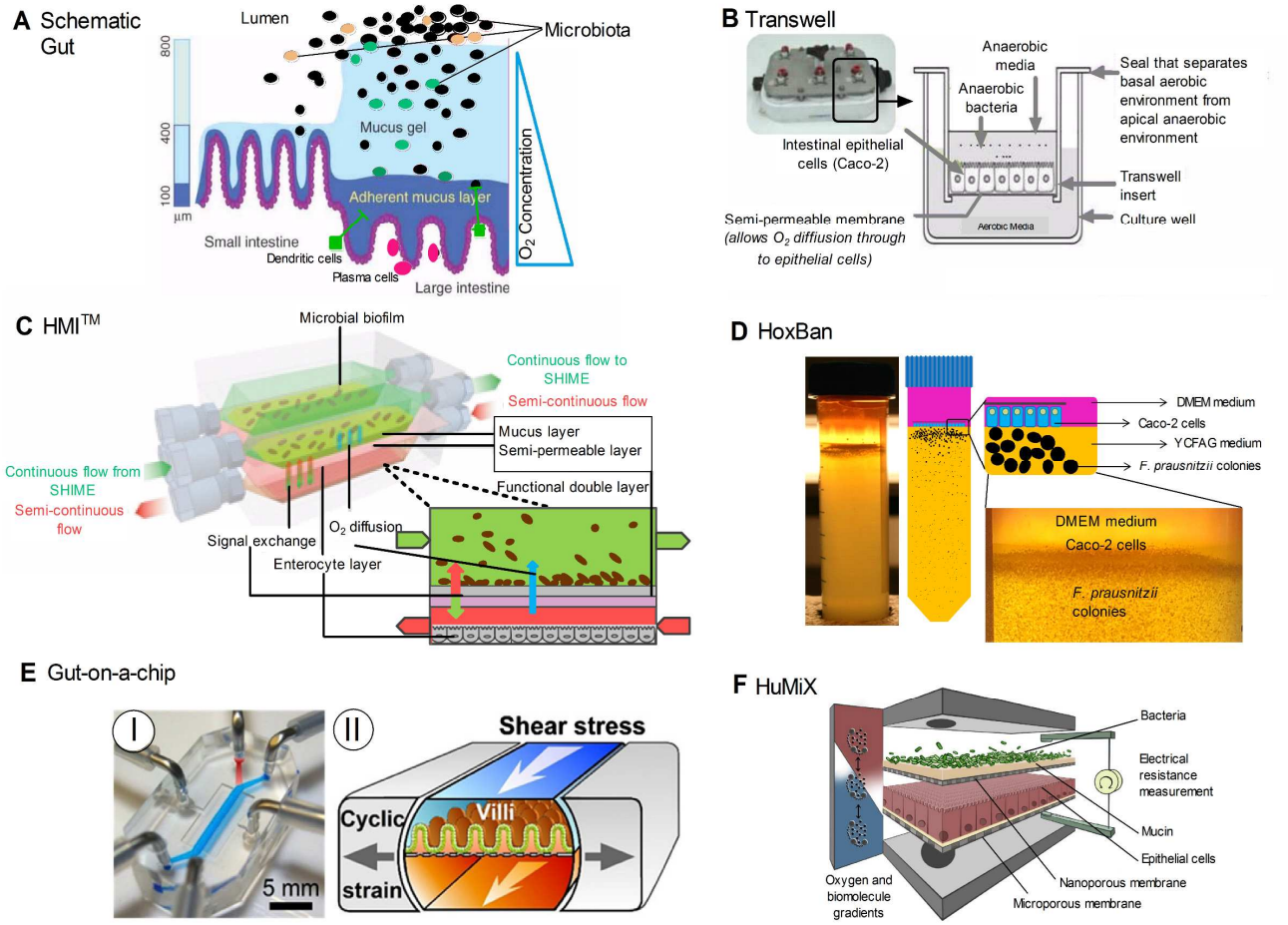

Figure 1. Recently developed (anaerobic) bacterial-epithelial gut co-culture models. A) Schematic figure of the aerobe-anaerobe interphase of the human gut (adapted from *Barbosa T. et al.; Wiley Interdiscip Rev Syst Biol Med, 2010)* [132] ; B) The Transwell 'apical anaerobic model of the intestinal epithelial barrier' [117] ; C) The Host Microbiota Interaction module (HMI™ module) [118] ; D) The Human Oxygen-Bacteria anaerobic (HoxBan) co-culture system [123] ; E) The human gut-on-a-chip microdevice [127] and F) The HuMiX device. [128] See main text for detailed description. All models are shown with permission of the authors when this is required.

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