Trends in Endocrinology & Metabolism

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REVIEWS

Review

Intestinal Organoids: A Tool for Modelling Diet–Microbiome–Host Interactions

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Dietary patterns, microbiome dysbiosis, and gut microbial metabolites (GMMs) have a pivotal role in the homeostasis of intestinal epithelial cells and in disease progression, such as that of colorectal cancer (CRC). Although GMMs and microorganisms have crucial roles in many biological activities, models for deciphering diet–microbiome–host relationships are largely limited to animal models. Thus, intestinal organoids (IOs) have provided unprecedented opportunities for the generation of *in vitro* platforms with the sufficient level of complexity to model physiological and pathological diet–microbiome–host conditions. Overall, IO responses to GMM metabolites and microorganisms can provide new insights into the mechanisms by which those agents may prevent or trigger diseases, significantly extending our knowledge of diet–microbiome–host interactions.

Highlights

Dietary patterns modulate the gut microbiota and alter its functions by modulating the production of GMMs, which are capable of regulating homeostasis and the risk of disease.

Complex interkingdom regulatory networks and crosstalk occur between the host, its gut microbiota, and its diet.

Immortalized cancer cell lines grown in 2D monolayers differ genetically, metabolically, and phenotypically from *in vivo* cells. However, 3D IOs can mirror structural alterations, mutational signatures, and gene expression changes between patient and patient-derived organoids.

IOs provide new opportunities to study how the gut microbiota , or its products, interact with intestinal epithelial cells. In this review, we discuss recent publications using IOs to study the nutrient–microbiome axis in gastrointestinal homeostasis and disease. We also highlight an array of novel approaches by which the nutrient–microbiota–gut epithelium triangle can be further understood and mechanisms governing gastrointestinal diseases better deciphered.

Diet, Microbiome, and Gut Microbial Metabolites

Are we really what we eat? Apart from its obvious nutritional value, food can also promote health and prevent [1–6] or trigger [7,8] disease. Although diet exhibits a strong impact on health, complex interrelationships with the **gut microbiota** (see Glossary), host genetics, and other environmental factors are also needed for the propagation of disease [7]. After ingestion, food is digested into a multitude of different small molecules in the gastrointestinal (GI) tract, some of which are absorbed by the intestinal wall while others are further processed by the gut microbiome (Box 1). The gut microbiota comprises trillions of microorganisms that inhabit the human GI tract, with low counts in the stomach and proximal part of the small intestine compared with at least 10^{11} cells/g in the colon. These microorganisms shape the chemical structure, lifespan, bioavailability, and biological activities of most of the compounds ingested via diet, pharmaceuticals, and xenobiotics [9]. However, the relevance of dietary patterns, gut microbiota composition, and **GMMs** in tissue homeostasis and organ physiology are poorly understood and we are only just beginning to understand the complex symbiosis between the gut and these microorganisms [7,10–15].

The European Prospective Investigation into Cancer and Nutrition (EPIC) study, one of the largest cohort studies in the world, has established associations between fruit, vegetable, and **fiber** consumption and a decreased risk of developing different forms of cancer [33]. High intake of dietary **phytochemicals**, such as polyphenols, fiber, and antioxidant compounds, among others, have been frequently associated with a reduced risk of GI cancers [2,7,34,35]. However, there are unresolved questions regarding the mechanisms by which certain native phytochemical or GMMs exert specific bioactivities.

To date, the most common approach to studying the effect of food components on intestinal inflammation [36], toxicological interactions [37], microbiome research [38], or the potential bioactivity of compounds, such as polyphenols against cancer [4,16,36,39], stems from the administration of such native molecules to a variety of human 2D cell lines. However, such studies face two major criticisms: (i) as mentioned earlier, it is increasingly clear that the biological effects of

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Box 1. From Farm to Fork, and beyond

Diets rich in phytochemicals and fiber, commonly derived from several types of fruit and vegetable, have been associated with a reduced risk of chronic diseases, such as cardiovascular and neurodegenerative diseases, obesity, diabetes, and GI cancers [3,13,16–20]. However, the biological effects of these compounds cannot be directly linked to the native molecules as they occur in the plant. Most nutrients are not metabolized in the oral cavity, and are resistant to the acidic conditions in the stomach. For example, flavonoid glucosides are hydrolyzed in the brush border of the small intestine, removing the attached sugar and releasing aglycone, which may then enter epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane [21]. Before circulation into the blood stream, aglycone reaches the liver, where it is conjugated, releasing sulfate, glucuronide, and methylated metabolites.

In most cases, phytochemicals, such as polymeric proanthocyanidins (PACs) reach the colon nearly intact [16,22], where, together with nondigestible polysaccharides [23], they cross their destiny with the gut microbiota [22]. Here, polyphenols and fiber undergo extensive microbial bioconversion (see Figure 1 in the main text), producing GMMs derived from polyphenols and short-chain fatty acids (SCFAs) [23,24]. GMMs can act either locally by exerting their bioactivity on intestinal epithelial cells while concomitantly modulating the gut microbiota itself [9,20,22], or systemically, once absorbed, where some are conjugated in the liver and then released in the bloodstream to target different organs, including the central nervous system [25–27].

By contrast, a Western dietary pattern is characterized by high-sugar and high-fat foods, including highly processed foods as a principal player. As a result, this dietary pattern can promote colonic inflammation and CRC [7,8]. For example, after the consumption of a high-fat meal, bile acids are released into the duodenum, facilitating the emulsification and absorption of dietary lipids and fat-soluble vitamins. These cholesterol-derived metabolites are then metabolized in the intestine by the gut microbiota, producing altered levels of secondary bile acids that may promote CRC [28,29]. Western dietary patterns have been also linked to high levels of trimethylamine (TMA) and trimethylamine N-oxide (TMAO), microbiome-derived metabolites produced by the metabolism of dietary carnitine and choline, and associated with CRC [30,31]. The International Agency for Research on Cancer (IARC) concluded that processed meat was carcinogenic to humans on the basis of sufficient evidence from studies of CRC [32]. Carcinogenic chemicals, such as *N*-nitroso-compounds (NOC), polycyclic aromatic hydrocarbons (PAHs), and heterocyclic aromatic amines (HAA), may reach the colon and cause increased levels of DNA adducts and DNA damage. The diet–gut microbiota–host triangle evolves as a promising avenue in the prevention of GI diseases, such as CRC, but more research is required for a full understanding of gut homeostasis, and disease prevention and propagation.

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several classes of molecule cannot be directly linked to native compounds, but rather to their metabolites [40–42]; and (ii) immortalized and cancer cell lines grown in 2D monolayers on plastic minimally recapitulate key cellular complexities, topographies, and molecular signalling associated with 3D tissue architecture [38,43]. Although these observations are common to most bioactive compounds and organ–diseases of interest, they are particularly relevant in GI diseases given: (i) the precise cellular topography of the intestinal epithelium; (ii) the fact that dietary metabolites constantly influence intestinal epithelium; and (iii) the complex interaction among bacteria and intestinal epithelial cells [17,22]. To overcome these issues, different animal models have been utilized , offering a superior complexity over 2D models [27,44]. However, metabolic control along with gut microbiota in these organisms is not the same as in humans [45,46], and humanized models do not necessarily reflect the real relationships seen in humans either. In the latter case, the gut microbiota is transplanted into a host with which it has not coevolved, and ecological factors, such as diet and disease genotype, that have initially driven the dysbiosis in humans, are not present in rodent recipients [47].

Intestinal Organoid Cultures Reveal the Role of Phytochemicals in Tissue Homeostasis and Diseases

Recently, protocols for establishing **IOs** have demonstrated that cells derived from adult tissue biopsies and resection have the ability to survive, proliferate, and self-organize into 3D structures *in vitro* (Box 2) that closely recapitulate the tissue of origin [48]. Thus, 3D IOs have proven to be valuable model systems in studies aimed at not only deciphering normal tissue homeostasis, but also investigating molecular mechanisms associated with disease onset, progression, and response to therapy [49–54].

Box 2. Establishment of Human Intestinal Organoids

Organoids can be derived from two fundamentally different sources: (i) using methods replicating human development, it is possible to derive IOs from pluripotent stem cells (PSCs), including both embryonic stem cells and induced PSCs; and (ii) from tissue material, it is possible to extract organ-specific adult stem cells (ASCs) from both normal and diseased tissues (Figure I). Both IO cultures can reflect the architecture, regional specification, and cell composition of the epithelium *in vivo*. On the one hand, PSC-derived organoids are able to generate adjacent stromal cells and offer a more physiologically accurate model for studying the high complexity of mucosal interactions. However, PSC-derived organoids do not necessarily reflect a specific regional identity. These 3D organoids recapitulate early stages of cellular proliferation and are used for studying development processes and fetal infections. On the other hand, ASC-derived organoids may be better suited for modelling diseases since they are genetically stable. Thus, ASC-derived organoids represent a promising system for studying the intestinal epithelium in homeostasis and disease. For example, ASCs can be isolated from small clinical specimens from normal colon and CRC for establishing organoids that can be expanded to mimic the epithelial part of either the normal colon or the cancer, respectively.

First, tissue resections and biopsies (Figure I) are kept in ice-cold phosphate buffered saline (PBS) until processing. Second, human crypts are then isolated, whereby healthy and/or cancerous tissues are dissected into ~5-mm^3 pieces and washed repeatedly with cold PBS. Subsequently, intestinal tissues are incubated in a chelation solution supplemented with EDTA. After shaking, crypts, fragments of epithelium, or single cells are embedded in hydrogel. The hydrogel is a gelatinous protein mixture derived from mouse tumour cells, and polymerizes rapidly at 22–35°C. The medium contains tissue-specific growth factors as described elsewhere [48,50,55]. Importantly, IO cultures preserve the *in vivo* cellular diversity [48], covering most, if not all, of the cellular lineages existing in the native intestinal tissue. Furthermore, identical culture techniques can be applied to both normal tissue and neoplastic tissue and constitute a method for generating comprehensive panels of patient lines, thereby embracing aspects of human genetic variation. However, important aspects to consider are that the work associated with deriving and maintaining panels of patient line is labor intense and that, although IOs maintain the cellular complement of the epithelium, they lack immune cells, a functional nervous system, and a mesenchymal niche.

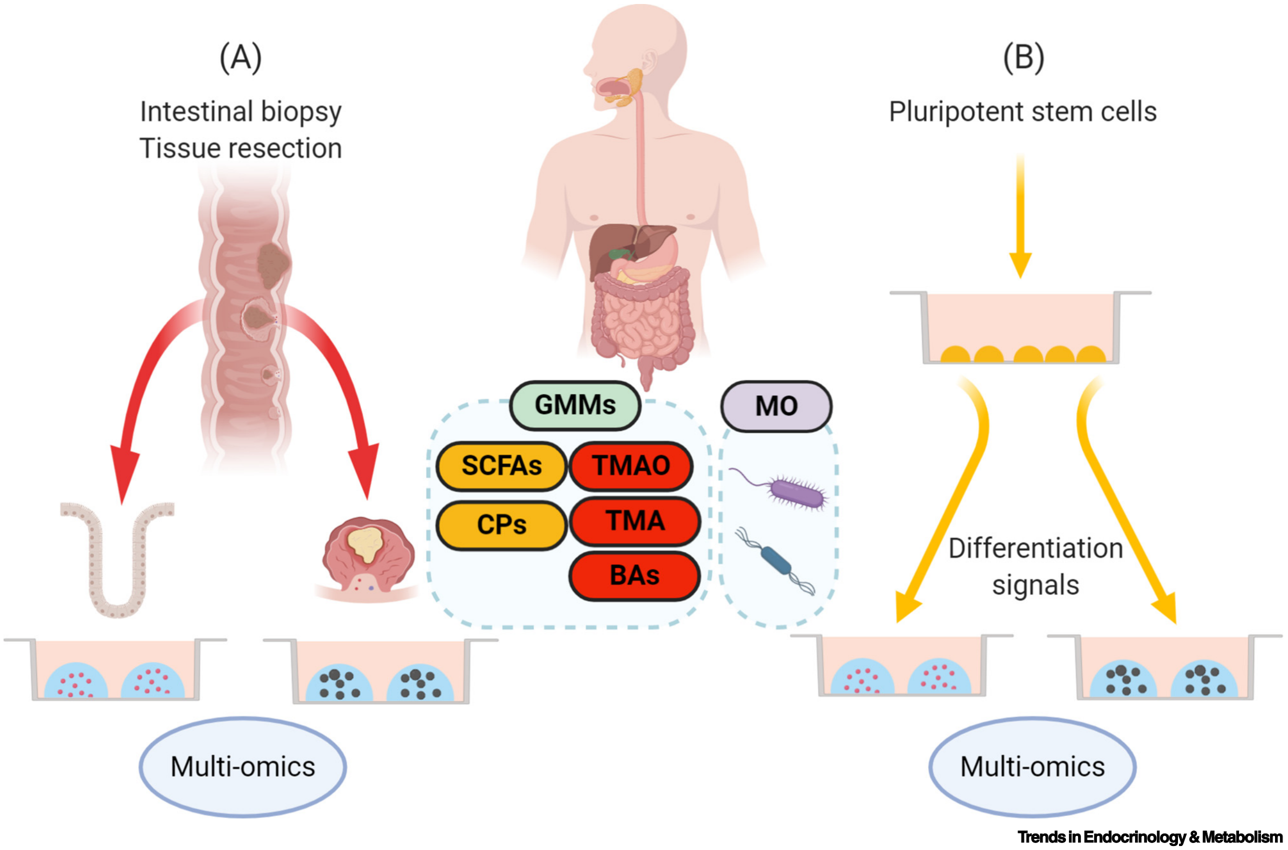


Figure I. Modelling Diet–Microbiome–Host Interactions *in vitro*. Schematic diagram summarizing the generation of intestinal organoids (IOs) from adult stem cells (A) and pluripotent stem cells (PSCs) (B). On the one hand, intestinal crypts and singe cells separated from healthy and carcinogenic tissues are embedded in hydrogel and the media containing growth factors is then added (A). On the other hand, PSC-derived IOs (B) can be differentiated following normal developmental stages to generate intestinal epithelium. Afterwards, human IO and tumoroid responses to gut microbial metabolites (GMMs), such as short-chain fatty acids (SCFAs), microbial catabolism of phytochemicals (CPs), secondary bile acids (BAs), trimethylamine N-oxide (TMAO), and trimethylamine (TMA), or microorganisms (MO) can be evaluated by, among others, multi-omics approaches (see Box 3).

The intestinal epithelium is a highly organized, self-renewing tissue with a proliferative crypt compartment and a differentiated villus [56]. The continuous cellular turnover of the intestinal epithelium is conserved by stem cells at the bottom of the crypt, which generate transit-amplifying cells, which then differentiate into various intestinal epithelial cells types, such as Paneth cells, goblet cells, enteroendocrine cells, and enterocytes, among others. To evaluate the effect of GMMs or microorganisms on gut health, an ideal model should preserve *in vivo* cellular diversity and retain basic physiological functions of the intestinal epithelium.

In one benchmark study, Zietek and colleagues showed that organoids established from the small intestine of mice preserved the main features of the intestinal epithelium in culture and could be used for studying nutrient transport, nutrient sensing, and hormone secretion [57]. Consecutively, human IOs were used to emulate nutrient transport physiology during digestion [58], and murine IOs determined intestinal mechanisms for dietary fat absorption [59]. At this point, several groups began studying various chemicals and dietary components that can promote or affect intestinal epithelium health. Cai and co-authors investigated the effects of different dietary constituents on IO growth [60]. The authors observed that several dietary constituents did not significantly affect IO growth. However, caffeic acid inhibited organoid growth in a concentration-dependent manner. The higher the concentration of caffeic acid, the fewer crypt-like structures could be seen, and these results were consistent with other *in vitro* research [61]. However, the results with other compounds, such as monosodium glutamate and chlorogenic acid, were not in agreement with previous studies [60,62]. Thus, these results highlight that the use of organoids for testing phytochemicals is still in its infancy. Future studies must carefully develop the experimental design and consider not only that results may provide valuable insights related to *in vivo* models, rather than *in vitro* 2D cell lines, but also that the observed responses are limited to the epithelial component of the intestine.

Several observational studies have reported significant associations between a high intake of **cruciferous vegetables** and lower risk of several types of GI cancer [63,64]. The potential health benefits of consuming cruciferous vegetables are attributed to compounds such as indole-3-carbinol (I3C), which was recently studied in small intestine mouse organoids [65]. The results provided robust evidence that I3C regulates Wnt and Notch signalling, with an important role in maintaining normal cell fate and, in turn, goblet cell differentiation. However, the acidic environment of the stomach can merge I3C molecules with each other to form a complex mixture of polycyclic aromatic compounds, known as acid condensation products, such as 3,3'-diindolylmethane (DIM), and the biological activities of such products may differ from those of I3C. By contrast, during the transit of glucosinolates, formation of I3C may still occur, but to a lesser degree, in the large intestine, due to the myrosinase activity of colonic bacteria [63,66]. Thus, the low, temporal amount of I3C expected to reach the intestine could have a marginal impact on Wnt and Notch pathways. This static and multicellular system may be an alternative strategy to animal models for the prescreening of GMMs. Nevertheless, relevant findings must ultimately be validated in animals.

Besides the role of diet in the regulation of the intestinal epithelium homeostasis, organoids derived from malignant colorectal lesions are opening new windows of opportunity to investigate the impact of diet on tumorigenesis. Recently, Toden and colleagues reported a potent chemoprotective role of flavan-3-ols (a commercial grape seed extract dissolved in DMSO, comprising monomers, dimers, and trimers) in CRC by studying IOs as a preclinical model system [67]. Flavan-3-ols consistently suppressed the formation and growth of both IOs derived from APC^{Min} mouse and undeclared clinicopathological characteristics of human CRC tumoroids by inhibiting the cell cycle and inducing programmed cell death. From a mechanistic point of view, gene expression profiling revealed the suppression of prosurvival and self-renewal pathways,

Glossary

Cruciferous vegetables: many commonly consumed cruciferous vegetables come from the *Brassica* genus, including broccoli, Brussels sprouts, cabbage, cauliflower, collard greens, kale, kohlrabi, mustard, rutabaga, turnips, bok choy, and Chinese cabbage. Brassicaceae (also named Cruciferae) is a medium-sized and economically important family of flowering plants. They are mostly annual, biennial, or perennial herbaceous plants and, therefore, are available throughout the year. Similar to other vegetables, cruciferous vegetables contain a large number of phytochemicals, including folate, carotenoids, chlorophyll, as well as fiber. However, cruciferous vegetables are unique because they are also rich sources of glucosinolates, sulfur-containing compounds that are responsible for their pungent aromas, and spicy/bitter taste. Apart from glucosinolates, cruciferous vegetables are a rich source of nutrients produced by the hydrolysis of glucosinolates, such as indoles and isothiocyanates.

Fiber: parts of fruits and vegetables containing substances such as cellulose, lignin, and pectin-containing carbohydrate polymers that are resistant to endogenous digestive enzymes. Dietary fiber can be considered a key ancestral compound that preserves gut ecology, regulating macronutrients and host physiology.

Gut microbial metabolites (GMM): humans rely on the microbiome to break down dietary components, such as fiber and phytochemicals, or release metabolites, such as bile acids. GMMs are bacterial fermentation products, and these biochemical transformations shape the chemical structures of such compounds, thus modifying their lifespan and bioavailability, and providing different biological activities.

Gut microbiota: all microorganisms found in the GI tract, including bacteria, viruses, and fungi, with a fundamental role in many host processes; it helps the body to digest certain foods and with the production of vitamins or bioactive metabolites. It also has a key role combating infection, and supporting the immune system.

Intestinal organoids (IOs): human or mouse IOs are 3D *in vitro* tissue models that incorporate several physiologically relevant features of the *in vivo* gut epithelium, such as a polarized epithelial including Hippo signalling, in organoids treated with flavan-3-ols. Despite these promising findings, from a nutritional point of view, **proanthocyanidins (PACs)** are subject to extensive metabolism once introduced into the GI tract. These compounds can reach the distal GI tract almost intact, where they are efficiently transformed into low-molecular-weight phenolic compounds by the colonic microbiota [22,23,30,68,69]. Therefore, flavan-3-ol monomers, dimers, and trimers reaching the colon become available to the gut microbiota. Then, microbial catabolism begins, producing hydroxy-phenyl-γ-valerolactones (PVLs) and, to a lesser extent, their derived hydroxy-phenylvaleric acids (PVAs), with only a small percentage of unmetabolized PACs remaining [16,70]; for example, Choy and colleagues recovered only 11% of ingested PACs in pig feces [71].

A formal demonstration of the influence of PAC catabolism in tumorigenesis was provided by Ravindranathan and colleagues [72]. To test the potential benefit of combining PACs and curcumin in the prevention of CRC, CRC cell lines were first treated with curcumin or PACs either as single agents or in combination. The combined treatment of PACs and curcumin consistently decreased the mRNA levels of the proliferation marker Cyclin D1, and the expression of *PDE3B*, a gene associated with peroxisome proliferator-activated receptors (PPARs) and with inhibition of proliferation and crosstalk between insulin signalling pathways. To strengthen their findings, authors evaluated the combination of curcumin and PACs *in vivo* following the growth of subcutaneous xenografts of HCT116 cells in athymic mice. Interestingly, PACs efficiently decreased the expression of both Cyclin D1 and PDE3B when administered as single agents. This suggest that those responses were not likely to be modulated by PACs but rather by the release of GMMs, which were shaped by the mouse microbial catabolism. However, the combination of curcumin and PACs decreased the expression of Cyclin D1 and PDE3B and attenuated tumour growth *in vivo* to a greater extent than curcumin or PACs administered as single agents. Lastly, the authors established patient-derived tumoroids (stages IIA, IIB, and IIC) to confirm the trends observed using 2D cell lines and mice models. The expression level of the genes encoding Cyclin D1 and PDE3B was downregulated by the combination of curcumin and PACs. However, mRNA levels of Cyclin D1 and PDE3B did not decrease when colon tumoroids were treated with native PACs alone.

To date, most studies have investigated the role of phytochemicals on IOs modelling homeostasis and carcinogenesis. However, to the best of our knowledge, cancer initiation and propagation by means of diet-related metabolites (Box 1) or the protective effects of GMMs in presence or absence of carcinogenic agents have not yet been explored (see Outstanding Questions). Overall, IOs derived from either normal or diseased tissue provide a complementary mechanism for studying the impact of dietary components on cell behaviour. Even though this technology represents an important tool for deciphering different pathological processes affecting humans and, in turn, potentially allow us to identify mechanisms that can counteract diseases, experimental designs should be readapted taking into account: (i) the digestion and microbial catabolism of native dietary constituents to understand their metabolism and determine those compounds that reach the gut epithelium; (ii) ideally, healthy and disease-related IO responses should be evaluated; and (iii) the correct polarity of the gut should be considered for experiments with metabolites and dietary components to target either the apicobasal membrane for mimicking systemic exposure or the apical membrane for luminal exposure (see later).

Investigating the Structural and Functional Changes Induced by the Microbiome at the Gut Epithelium

Recent microbiome studies have expanded beyond simply profiling microbiota compositions, and are increasingly characterizing microbial functions by using functional meta-omics approaches

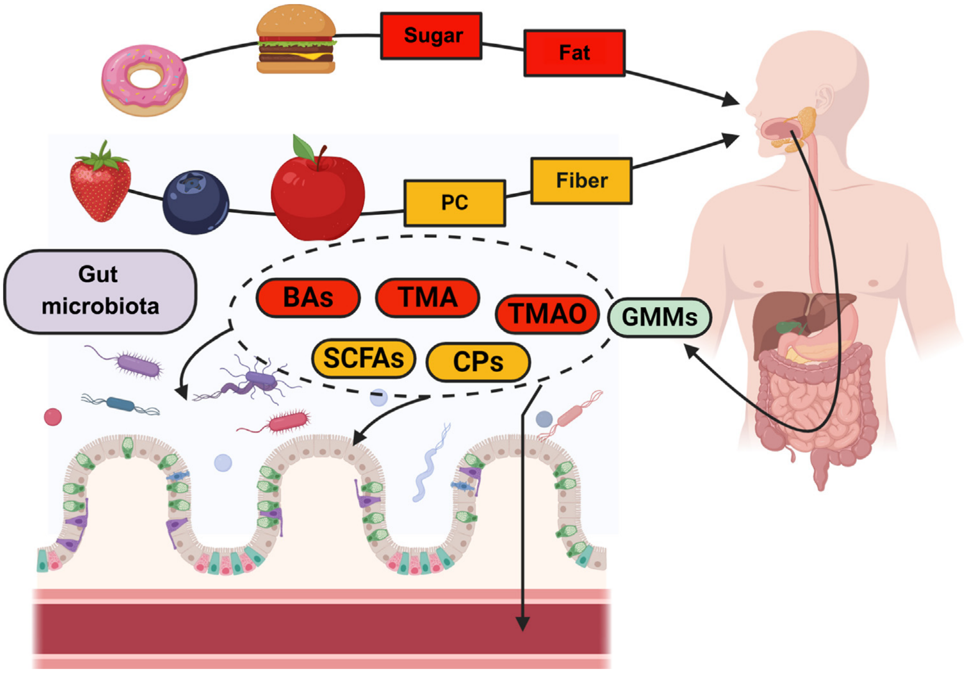
layer containing multiple cell types and a functional lumen replicating real-life conditions. IOs enable the study of gut epithelial cells, investigating responses to GMMs, microorganisms, or modelling homeostasis and diseases. However, IOs do not mimic the complexity of the *in vivo* situation due to the lack of the immune and nervous systems, and the mesenchymal niche.

Phytochemicals: plant-derived chemicals that occur in fruits, vegetables, whole grains, nuts, seeds, and legumes, being responsible for color, taste, and smell, among other characteristics.

Proanthocyanidins (PACs): present in flowers, nuts, fruits, bark, and seeds of various plants as a defense against biotic and abiotic stressors; their astringency protects the plant from pathogens and predators. PACs are oligomeric and polymeric products of the flavonoid biosynthetic pathway. The building blocks of PACs include the flavan-3-ols catechin and epicatechin.

[73]. By combining meta-omics approaches, a functional profile can be obtained to the extent that these techniques can provide strain-level taxonomic resolution, assess the potential functions encoded, and quantify the metabolic activities occurring within a complex microbiome. As an example, a shotgun metagenomic sequencing of bacterial DNA and metabolomics in cecal contents in rats with type 2 diabetes mellitus (UCD-T2DM) supported the idea that diabetes-specific host signals affect the ecology and GMMs of the gut microbiome when controlling for diet, age, and housing environment [74]. The strength of analyzing both the activity and microorganisms is that it revealed significant associations between the gut microbiome and human disease [11,31,73,75].

The gut epithelium is the principal site for detecting GMMs and microorganism, and both can act locally (Figure 1) by exerting their bioactivity on intestinal epithelial cells. For example, fiber-derived compounds, such as short-chain fatty acids (SCFAs), are fatty acids with saturated aliphatic tails between two and six carbons long and have implications for both host health and disease [8,69,76,77]. These GMMs have been commonly monitored in several biological fluids [24,78]. Although the importance of studying these microbial and diet-related metabolites has increased [77,79], their role in the human gut epithelium remains challenging to determine. Schilderink and co-authors examined whether SCFAs induce the secretion of paracrine factors influencing epithelial homeostasis [80]. Interestingly, butyric acid enhanced ALDH1A1 and ALDH1A3 expression in human and mouse IO cultures, respectively. The expression of ALDH1A1–3 is critical for the epithelial conversion of retinol to retinoic acid. This work demonstrated the importance of IOs in deciphering the physiological interaction between the gut epithelium and microbiome in



Trends in Endocrinology & Metabolism

Figure 1. Linking Gut Microbial Metabolites (GMMs) with the Gut Microbiota and Diet. The digestion and microbial catabolism of a diet rich in phytochemicals (PCs) and fiber (yellow boxes) produce short-chain fatty acids (SCFAs). By contrast, a Western dietary pattern (red boxes) increases secondary bile acids (BAs) and levels of trimethylamine N-oxide (TMAO) and trimethylamine (TMA), among others. This figure illustrates how food GMMs, with their metabolized forms, may act either locally on intestinal epithelial cells, concomitantly modulating the gut microbiota, or systemically once absorbed through the gastrointestinal tract.

healthy conditions and identified a new mechanism by which butyrate, through induction of retinoic acid synthesis, can contribute to maintain gut homeostasis. Serotonergic enterochromaffin (EC) cells have been suggested to fulfil the role of chemosensors in the gut epithelium and, together with tuft cells, they transduce chemosensory information to the nervous system [81–83]. Bellono and co-authors explored the applicability of IOs to decipher the role of certain GMMs [82]. They demonstrated that EC cells express specific chemosensory receptors, are electrically excitable, and modulate serotonin-sensitive primary afferent nerve fibers via synaptic connections, enabling them to detect and transduce environmental, metabolic, and homeostatic information from the gut directly to the nervous system. Allyl isothiocyanate, isovalarate, dopamine, epinephrine, and norepinephrine also specifically and consistently activated EC cells. By contrast, SCFAs elicited small, but consistent responses to Ca^{2+} transients. In light of these recent findings, IOs represent an excellent biological system to explore chemical signals produced by the gut microbiota.

Exploring the influence of microorganisms on the intestinal epithelium is more complex than studying the impact of metabolites. The gut epithelium is where microorganisms interact with the host and, therefore, mirroring a real-life scenario means that microorganisms and, ideally, complex mixtures of microorganisms have to be introduced into the lumen of the organoid. However, this is technically challenging. Human and mouse IOs accurately mimic the gut architecture, luminal accessibility, and tissue polarity [79], and three approaches have been used so far for introducing microorganism into organoids: (i) disrupted organoids; (ii) 2D cultures derived from IOs; and (iii) microinjections. For the first option, once organoids are disrupted, they expose the apical side and, at this point, dissociated cells may interact with microorganism [84]. For example, a study jointly co-cultured IOs and lamina propria lymphocytes (LPLs) to explore the protective effect of *Lactobacillus reuteri* D8 [85], which is considered a key player able to protect the integrity of intestinal mucosa, although little is known regarding its effects on the stem cell niche. The authors revealed that *L. reuteri* D8 promoted the growth of IOs, and protected organoid morphology upon tumor necrosis factor alpha (TNF-α) treatment. Although elevated levels of cells expressing Lgr5 were also observed, the antibody still needs to be validated using tissue from knockout animals. The authors argued that *L. reuteri* D8 stimulates LPLs to secret IL-22 through aryl hydrocarbon receptors (AhRs), which activates STAT3 phosphorylation to accelerate the regeneration of intestinal stem cells. To expose the apical part, another option is to dissociate organoids into single cells and seed these cells onto an extracellular matrix or coated dish, and then to add microorganisms directly into the culture media, allowing interaction between the microorganisms and the host cell monolayer [86,87]. More recently, a new technique has been developed that reverses IO polarity, whereby the apical surface everts to face the media [88]. This emergent and effective model can probe barrier integrity, nutrient uptake, and could open new possibilities for studying diet–microbiome–gut epithelium interactions.

The approaches mentioned here have mainly been performed with aerobic bacteria; however, most gut microbiota are anaerobic. To overcome the challenge of studying anaerobic microbiota using IOs, which grow under normal oxygen concentrations, microinjection of microorganisms into the lumen of IOs (estimated 10% O_2) has been used [89]. In this study, the authors took advantage of a high-throughput microinjection device, which facilitated efficient and reproducible injections into the lumen of gut organoids. As well as this technological advance, this research showed that, after fecal transplant, aerobic and anaerobic communities could be transferred into the IO lumen and cultivated over 4 days, with little change in the relative composition of microbial communities. The number of cells and size of organoids differ from one IO to the other, and, thus the microbial cargo should be normalized taking into account dimensional parameters. This technical advance can open new horizons to investigate complex microbial communities. Nevertheless, it will be important to align such studies with *in vivo* studies to ascertain whether the observations truly recapitulate what happens inside the human gut.

Strategies to Evaluate Intestinal Organoid Responses to Microorganisms and GMM Interactions: From Bulk Tissues to Single Cells

Cellular and molecular assessments have confirmed IO responses to GMMs and microorganisms. However, to understand the mechanisms that underscore these interactions with, and responses in, epithelial cells, a combination of multi-omics approaches is crucial (Box 3). Omics approaches have traditionally been performed on biological fluids, homogenized tissues, or homogenized cells, measuring the average gene expression, proteome, or metabolome [6,90,91]. Focusing on small molecules, the exo- and intrametabolome of IOs can elucidate significant metabolic processes affecting IOs and tumoroids, because metabolites represent both the downstream output of the genome and the upstream input from the environment. However, bulk omics approaches, such as proteomics and metabolomics, eliminate all spatial information, morphology, and heterogeneity, which are vital to disentangle the essence of such complex eukaryotic–prokaryotic networks. To overcome these limitations, mass spectrometry imaging (MSI) has emerged as a novel potent tool to assess the heterogeneity of a tissue at a single cell resolution [92–95]. Thus, by determining the spatial distribution and abundance of known or unknown molecular species, MSI can identify the cellular distribution of specific GMMs in the IO epithelium, the biotransformation of such compounds in metabolites, and the metabolic and/or proteomic response of intestinal cells to the compound [96].

By performing 'bulk' 'omics approaches, the variability in cell type composition can significantly confound analyses of these data, since different biological processes continuously occur at the single cell level and responses to chemical microenvironments may differ. Genomics, transcriptomics, epigenomics, proteomics, and metabolomics are now increasingly focused on the characterization of individual cells [97,98]. For example, single-cell RNA sequencing was used to reveal adjustments in cell populations in IO cultured with different growth factors [48], as well as the proportions of the different cell types and their responses to bacterial infections [99], such as *Salmonella*. However, such emergent techniques are expensive and technologically challenging, and MS approaches in particular may require a superior sensitivity, and extended linear dynamic range and resolving power. However, they could clearly open new horizons in the near future, enabling us to determine how GMMs and/or microorganisms

Box 3. Multi-Omics Approaches

A multi-omics approach was recently used to obtain a holistic view of molecular mechanisms in mouse IOs cultured in different defined mediums [100]. The research by Lindeboom and co-authors resulted in a novel workflow to investigate metabolites and lipids of IOs and can be used to explain the potential of lipidomics and metabolomics approaches aim at studying chemical signatures on IOs for diet-microbiome-host interactions. To isolate those compounds, the authors used a two-phase extraction system that provided relevant biological signatures. As a result, lipidomics highlighted that the metabolism of lipoproteins and lipids, such as glycerophospholipid biosynthesis and phospholipid metabolism, were upregulated. In addition, metabolomics analysis revealed that amino acids were downregulated in stem cell-depleted IOs and upregulated in stem cell-enriched IOs.

Thus, this study illustrated the potential of multi-omics approaches to provide valuable new insights into the differentiation mechanisms. Extrapolating from this research to the topic of the current review, the combination of IOs and multi-omics approaches, particularly comprehensive lipidomics and metabolomics, could provide new insights into the mechanisms by which nutrient–gene or microbiome–gut epithelium interactions may influence the intestinal stem cell niche. This could unlock new possibilities for understanding the role of GMMs and microorganisms in personalized nutrition as well as the initiation, propagation, and prevention of GI diseases.

affect the distribution of cell types, transcriptional factors, and the proteome and metabolome in 3D IOs at the single cell level.

Concluding Remarks and Future Perspectives

New insights are rapidly being gained into the field of 'nutrition and gut microbiota'. Several studies have determined that, after the ingestion of phytochemicals and fiber, the gut microbiome starts a complex microbiota catabolism that releases important GMMs. Overall, nutrients, GMMs, and the microbial community maintain a healthy gut epithelium. By contrast, a Western dietary pattern promotes microorganisms and GMMs that negatively affect the gut epithelium and may accelerate diseases. In most cases, studies have separately described the microorganisms present in the gut community and proteins and/or metabolites in different biological fluids. Although recent multi-omics approaches have revealed significant associations between the gut microbiome and human GI diseases, the mechanisms by which GMM–gene interactions influence the stem cell niche has received little attention thus far.

Recently, IO culture models have been demonstrated to be powerful tools to mirror the behaviour of epithelial cells. On this basis, healthy IOs and tumoroids offer several particular advantages: (i) the ability to investigate cell-type intrinsic mechanisms in normal and diseased tissue-derived organoids; and (ii) the possibility to explore the expression, localization, and activity of proteins and intracellular signalling processes led by several diet-related compounds, GMMs, and microorganisms.

A proper experimental design, as described earlier, and the combination of IOs, GMMs, and microorganisms will help answer unresolved questions related to the mechanisms by which GMMs result in disease prevention and initiation, as well as microbiome–gut epithelium crosstalk. We envision that the field might see many applications of IOs in the future; such approaches will clarify the mechanisms responsible for diet–microbiome–host interactions, and will open new possibilities for understanding, and treating, GI diseases.

Outstanding Questions

Dietary patterns are associated with health outcomes, but there are unresolved questions regarding the mechanisms by which microorganisms or GMMs may exert health benefits. Can IOs provide support for the results from epidemiological and dietary observational studies and also provide mechanistic insights?

The type, quantity, and biological activity of GMMs produced in humans depend on the composition of gut microbiota. Can IOs reveal the mechanistic responses to these subtle changes?

Can GMMs target the colonocyte epigenome as a promising strategy for reprogramming aberrant processes associated with GI diseases, such as CRC, at the early stages of the disease? Subsequently, can GMMs be identified and then associated with the native phytochemicals and microorganisms responsible for microbial catabolism?

Can the negative health effects driven by GMMs, such as trimethylamine (TMA), trimethylamine N-oxide (TMAO), and secondary bile acids, be neutralized by the positive effects of others, such as SCFAs or polyphenol catabolism? At the same time, can IOs reveal these complex interactions?

IOs are continuously being improved. However, can they be co-cultured to include other cellular components that are present *in vivo*, such as nerves, immune cells, and muscles?

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