

## Human gut homeostasis and regeneration: the role of the gut microbiota and its metabolites

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## Abstract

The healthy human gut is a balanced ecosystem where host cells and representatives of the gut microbiota interact and communicate in a bidirectional manner at the gut epithelium. As a result of these interactions, many local and systemic processes necessary for host functionality, and ultimately health, take place. Impairment of the integrity of the gut epithelium diminishes its ability to act as an effective gut barrier, can contribute to conditions associated to inflammation processes and can have other negative consequences. Pathogens and pathobionts have been linked with damage of the integrity of the gut epithelium, but other components of the gut microbiota and some of their metabolites can contribute to its repair and regeneration. Here, we review what is known about the effect of bacterial metabolites on the gut epithelium and, more specifically, on the regulation of repair by intestinal stem cells and the regulation of the immune system in the gut. Additionally, we explore the potential therapeutic use of targeted modulation of the gut microbiota to maintain and improve gut homeostasis as a mean to improve health outcomes.

**Keywords:** gut microbiota, host-microbiota interactions, stem cells, wound healing, tissue regeneration, metabolites.

## Main text

### 1. Introduction

The gut microbiota encompasses the bacteria, eukaryotes, archaea and viruses that inhabit the gastrointestinal (GI) tract [1]. The levels of microorganisms in our gut, estimated to be present at a ratio of 1.3:1 relative to the total number of human cells in the body, and our dependency on these microbes, has led to the suggestion that humans should be considered as superorganisms, with the gut microbiota being an associated hidden organ [2–4]. The recent “omics” technological revolution has provided an ever-greater insight into the role that these communities play in human physiology and health. Evidence suggests that gut microbiota contributes to health in a broad variety of different ways, including through the gut-brain axis, a gut-liver axis and a gut-skin axis, among others [5–8]. The corollary is also true in that an unbalanced gut microbiota has been linked to metabolic syndrome [9], obesity [10], irritable bowel syndrome (IBS) [11], inflammatory bowel disease (IBD) [12], colorectal cancer [13], cardiovascular diseases [14], skin conditions [15], as well as conditions associated to the central nervous system, such as Parkinson’s disease [16], Alzheimer’s disease [17], multiple sclerosis (MS) [18], autism spectrum disorder (ASD) [19], anxiety and depression [20].

The gut microbiota interacts with the host directly or indirectly via secreted metabolites. These interactions take place in the microenvironment generated at the interface between the human gut epithelium and the lumen, where microorganisms or microbial metabolites can trigger reactions that will generate responses at local or systemic levels. These include immune, metabolic, or neuroactive impacts. For instance, fermentation of dietary fibre by the gut microbiota will produce butyric acid, a short-chain fatty acid (SCFA) that diffuses through the mucous layer generated by

the gut epithelial cells and, via a cascade of interactions, causes an impulse in the vagus nerve that is processed in the central nervous system generating a satiety response [6].

In order for relevant processes to be functional, it is necessary that the microenvironment where these interactions take place is well preserved. The intestinal epithelium is exposed to the intestinal lumen and, therefore, to mechanical and chemical impacts by circulating matter that can damage it. Indeed, conditions and diseases associated to the gut, such as colorectal cancer, have been linked with a compromised ability to regenerate the gut epithelium [21]. Symptoms associated with other systemic conditions, such as ASD, have been associated with inflammatory processes triggered by undesirable compounds traversing the gut epithelium due to increased permeability [22]. Thus, for the human body is reliant on efficient strategies to protect and maintain a healthy gut epithelium. One of these strategies involves the removal and replacement of damaged cells, a process regulated by intestinal stem cells (ISCs). The ISCs are a pool of stem cells, some of which are LGR5+, actively proliferative and necessary to maintain gut homeostasis through the turnover of the intestinal epithelium; others are quiescent and help to replenish the stem cell pool during cell damage [23].

Mechanisms associated with the regeneration of the gut epithelium are yet to be fully understood. However, increasing evidence suggests that the gut microbiota and its metabolites play an important role. Moreover, metabolites produced by the gut microbiota have been found to be involved in different tissue healing processes, by improving nutrient provision to damaged tissue, through the induction of hepatotropic factors or calcium absorption, in a manner than contributes to skin, liver and bone regeneration [24]. Understanding the role of microbial metabolites in stem cell-mediated regeneration could facilitate the development of new gut microbiota-associated therapeutic interventions.

In this review, we summarise the impact that the gut microbiota and its microbial metabolites have on the gut epithelium and, more specifically, their role on the regeneration processes mediated by ISCs, while evaluating the potential to harness gut microbiota targeted interventions for therapeutic benefits.

## **2. The microenvironment of the gut epithelium**

### **2.1. The gut epithelium**

The gut consists of four concentric layers. The outer layer is the adventitia or serosa, followed by the muscular layer, the submucosa and the inner layer, the mucosa, which is exposed to the lumen. The mucosa layer can be further divided into the muscularis mucosae, formed by the smooth muscle involved in peristalsis, the lamina propria of connective tissue, and the epithelium, the part of the mucosa that is in contact with the lumen and responsible for secretory and absorptive functions as well as the maintenance of a barrier [25].

The human gut epithelium consists of a single layer of cells structured in small protuberances named villi, which play a key role in absorbance. These are connected by invaginations known as crypts, where the ISCs are found (Figure 1).

Different types of cell junctions connect the epithelial cells: gap junctions, desmosomes, adherent junctions, and tight junctions. These cell junctions are of key importance to the maintenance of barrier function and, therefore, gut health. In addition, a number of other cell types within the human gut epithelium contribute to absorption or secretion. Absorptive cells include enterocytes and microfold cells, commonly known as M cells. Enterocytes are the most numerous cells and their main function is to absorb nutrients. M cells are involved in the immune response in the gut, and in particular the transport of antigens from the lumen to the lymphoid follicles (similar to lymph nodes) that are grouped in the Peyer's patches, located below the epithelium [26]. Secretory cells include enteroendocrine cells, Paneth cells, goblet cells and tuft cells. Enteroendocrine cells secrete hormones and enzymes into the blood stream in response to intestinal stimulations. Paneth cells are intimately related to ISCs [27]. They are responsible of the protection of the stem cells by secreting antimicrobial, and immunomodulatory proteins and peptides [28, 29]. Goblet cells are responsible of the production of the mucus layer and is the most abundant secretory cell type [30, 31]. Tuft cells, on the other hand, are much less abundant and their functions are yet to be fully understood, although it has been suggested that they are also involved in immune activities [32]. As noted earlier, stem cells are located at the base of the crypts. These cells are constantly dividing, proliferating and differentiating into the other epithelial cell types, moving gradually, pushed by the youngest cells, towards the upper parts of the villi, from where they are eventually shed into the lumen and washed out after an average lifespan of 4-5 days [33]. However, this epithelial cell turnover is not constant over the whole human life. In newborn primate mammals, gut epithelial cells and gut barrier are immature and have a lower turnover rate, enhancing transfer over the intestines and displaying a high non-selective endocytic capacity, which results in increased exposure to microbial and food antigens and it is believed to stimulate the maturation of the immune system [34]. This period is characterised by the epithelial expression of fragment crystallisable receptor (FcRn), which mediates the transfer of immunoglobulin G. The intestinal maturation involves the stimulation of crypt-cell proliferation, reducing the expression of FcRn and reducing the endocytic capacity, at the same time that increases selectivity and cell turnover in adult-type epithelial enterocytes [34]. Therefore, the exposition to the early colonisation of the gut microbiota will play a key role in the maturation of the gut epithelium, the gut barrier and the immune system. Once the gut barrier function is established, the immune system becomes more restrictive to transfer elements that will trigger inflammatory responses and will increase the epithelial turnover rate. Pathogenic infection and malnutrition can increase this turnover rate by stimulating caspases in epithelial cells, like in the case of the protozoan infection of *Cryptosporidium parvum* in a murine model [35]. Ultimately, the gut epithelium, the immune system and the gut microbiota interact intimately with each other, modulating their responses.

## **2.2. The gut microbiota**

The gut harbours the greatest number of microorganisms in the human body, with the colon containing approximately 70% and containing around 22 million genes, which in combination are referred to as the gut microbiome [3, 36–38]. The gut microbiota is different for each individual and, indeed, can change within an individual over time in response to different modulators. The first microbial communities are established at birth and are influenced by delivery mode, either vaginal or C-section [39]. During the first 1-3 years, the gut microbiota increases in abundance and complexity, continues to gain diversity during adulthood at lower pace until about 40 years, time after which it stabilises [40]. Eventually, the diversity of the gut microbiota decreases late in life, a phenomenon that has been linked to negative health impacts [41].

The gut microbiota is shaped by lifestyle and diet, and is relatively resilient to acute stressors such as antibiotic use [42]. However, the impact of these stressors can be dramatically different depending on the developmental stage of the gut microbiota when they take place. Thus, antibiotics have a more extensive and prolonged effect when are provided to the infant population whose gut microbiota is not fully established and mature, interrupting its assembly and development and preventing the establishment of commensal microbiota that might be difficult to regain afterwards [43]. Ultimately, this will have an effect on the microbial diversity of the individuals, contributing to the development of certain health conditions, such as allergies or metabolic syndrome, as microbial diversity is considered to be a major influence in the resilience and plasticity of the gut microbiota [44, 45]. Its variability across individuals reflects a functional redundancy, which ensures that key metabolic functions are performed across populations that are composed of different species. For this reason, it has not yet been possible to define what constitutes a healthy gut microbiota [46]. However, despite that, it is generally accepted that high levels of complexity and richness, both in genes and species, is desirable [46].

The main bacterial phyla present in the human gut are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia. Additionally, we can find other microorganisms in the gut, such as fungi, other microeukaryotes, archaea and viruses (including bacteriophage [phage]). Although the importance of these other microorganisms is less well understood, some are already known to have important roles in influencing population dynamics within the gut. For example, methanogenic archaea of the order Methanobacteriales decrease partial pressures of H<sub>2</sub>, increasing the energetic efficiency of primary fermenters and the production of SCFAs [47]. Other archaea of the order Methanomassiliicoccales use methylated amines, such as trimethylamine, in methane production [48]. The archaeal community in the gut is known as archaeome, but its study is yet to be further expanded [49, 50].

The enteric virome comprises the viral community within the gut, and it is also understudied. However, there has been a change of paradigm in the last 20 years. Viruses in the gut are not seen as a simple cause of disease anymore, but as a component that interacts and regulates other microbe populations [51]. Thus, phage have been proposed as bacteriome modulators for therapeutic applications (phage therapy) [52]. Additionally, the concept of commensal viruses is becoming more accepted at the same time that the expansion of sequencing technology shows diverse and abundant populations that seem to coexist in the human gut in an innocuous manner [51, 53]. Similarly to the bacteriome, the presence of

viruses depends on the diet and the environment, with critical changes occurring in the first two years, previous to stabilisation [53]. Moreover, it has been suggested that commensal viruses could have a similar physiological role to commensal bacteria in maintaining gut homeostasis [54, 55]. The most abundant viruses in the gut include phages, CrAss-like phage, Microviridae, Siphoviridae, Myoviridae and Podoviridae, but also eukaryotic DNA and RNA viruses like adenovirus, astrovirus, rotavirus, bocavirus, picornavirus, anellovirus and picobirnavirus [51, 56]. Other viruses, like enteroviruses or noroviruses are not considered commensal, but can remain in the gut in a chronic way for immunocompromised or people on immune modulation medication [51].

Fungi are also thought to influence the dynamics of the bacteria in the gut and vice versa. Most notably, the reduced bacterial diversity associated with certain conditions has been accompanied by an altered fungi composition in the gut, e.g., enrichment of *Candida* spp. in ASD and cystic fibrosis [57]. The most abundant fungal genera found in the faeces of healthy humans are *Saccharomyces*, *Malassezia* and *Candida* [58–60].

Ultimately, the vast majority of information about the microorganisms in the human gut relies on bacterial communities and their functionality. Different bacterial taxa are distributed across the gut in a manner that is influenced by the variety of micro-environments that exist. The main abiotic regulators of bacterial composition are water activity, gas composition, pH and the presence of molecules with antimicrobial activity that are secreted by the human cells or by other members of the gut microbiota, e.g., bile salts or antimicrobial peptides [61]. The higher levels of oxygen and bile salts in the small intestine relative to the large intestine results in the former being dominated by facultative anaerobes such as those from the former *Lactobacillus* genus and other specific representatives from the phyla Firmicutes and Proteobacteria that have the ability to metabolise bile salts [60]. Bacterial density is higher in the large intestine, especially the colon, with strict anaerobes and fermentative bacteria dominating due to the absence of oxygen and the presence of compounds that are not degraded in the upper gut, such as dietary fibres and polyphenols. Despite these general patterns, it has been difficult to characterise in great depth the distribution of microbial taxa along the gut, with most studies relying on samples of human faeces as a proxy. This is despite the fact that, it has been established that the microbiota in different parts of the gastrointestinal tract and mucosa only partially correlates with faeces [62]).

From a transversal perspective, the small and large intestines also differ, including with respect to the structure of the mucus layer. The small intestine mucus layer is attached strongly to the epithelium and can be considered a single layer, whereas the colonic one can be structured in two separated layers, an inner and dense layer attached to the epithelium, and an outer, more loose layer exposed to the lumen [63]. Bacterial groups are also heterogeneously distributed throughout the mucus layers. The inner layer is less densely populated than the outer layer and microbes associated with the mucus layers differ from the microbiota of the lumen [63, 64]. Ultimately, faecal samples do not reflect the microenvironment surrounding the stem cells placed in the villi crypts. However, human colon biopsies have shown an enrichment in Actinobacteria and Proteobacteria in the mucus layer in comparison to the lumen [65]. *Akkermansia*

*A. muciniphila*, a representative of the phylum Verrucomicrobia, is a mucin-degrader bacterium enriched in the human colonic mucus layer that has been associated with a healthy phenotype, i.e., lower adiposity and improved metabolic health [66]. It has been suggested that certain dietary elements, such as fermented foods or polyphenols, can boost the abundance of *A. muciniphila* and improve gut barrier integrity [67–70]. Moreover, some microorganisms inhabiting mucin crypts, such as *Bacteroides fragilis*, might benefit from strategies that allow evasion of the host immune response, while also being capable of modulating the host immune system and the use of host-derived nutrients [71].

Given the challenges that gut bacteria need to overcome to establish a physical contact with the human epithelium, secreted metabolites may represent a more important mechanism of communication. Bacteria can metabolise and produce a wide range of compounds with a variety of functions. Some of the most studied compounds in the gut are of bacterial origin and their activity can trigger effects far beyond the gut, being involved in complex systemic processes, such as cognitive or immune modulation. Among the different compounds of relevance produced by the gut bacteria are neurotransmitters, endocrine hormones, histamine, bile-derived molecules, branched-chained amino acids, vitamins, antimicrobials, SCFAs and bacterial components such as lipopolysaccharide (LPS) [72].

### **3. The gut epithelium homeostasis, damage and regeneration**

As mentioned above, the microenvironments generated in the gut, including at the gut epithelium, play a key role in determining which representatives of the gut microbiota dominate in that area. In health, the gut microbiota and human cells interact to form a homeostatic system, where there is “a balance between molecular damage and repair in a chemically reactive environment” [73]. When this relationship is disrupted, this homeostasis can be damaged and one of the key activities that can be affected is the regeneration capabilities of the gut epithelium, with resultant impacts on the integrity and function of the gut barrier. A better understanding of these interactions can help us to rationally promote the development or maintenance of homeostasis and, in turn, optimise the regeneration process.

Cell death displays an important role in gut homeostasis, as tissue development requires the elimination of aged, superfluous or damaged cells, being an important mechanism to limit the propagation of the inflammatory stimuli produced by an acute or chronic insult, ultimately promoting regeneration and limiting loss functionality [74]. Cell death can be programmed (apoptosis, necroptosis and pyroptosis) and unregulated (necrosis). Necrosis is an uncontrolled process characterised by the breakdown of the cell membrane and the release of the intracellular compounds into the media, and can be triggered by external factors, like toxins or bacterial and viral infections [74]. Apoptosis relies on caspase activation and is considered as a result of a self-check in the cell, when detects a problem, such as a mutation or microbial inhibition, and triggers the mechanisms to die [75]. Necroptosis also relies on caspase activation, but in this case is negatively regulated by them, and depends on kinase activity of receptor-interacting proteins (RIP). It is characterised by a membrane breakdown, like necrosis, but controlled, releasing damage-associated molecular patterns and heat shock

proteins, among other compounds, that would fuel an inflammatory response [76]. Pyroptosis is a newly described mechanism, also dependent on caspases, originally identified in cells on antimicrobial response as a result of the interaction with pathogen-associated molecular patterns that trigger inflammatory molecules within the cell [77]. Overall, the different mechanisms will eliminate cells that need to be replaced in order to maintain the stability of the gut epithelium and the gut barrier, although some will have a stronger inflammatory impact than others.

Key to this regeneration process are ISCs, characterised by their ability of self-renew, to persist throughout life and to generate other cell types, a function also known as multipotency [73]. In the gut epithelium, these characteristics were thought to be fulfilled by the LGR5+ crypt base columnar (CBC) cells and the +4 cells, which can also regenerate LGR5+ CBC cells and were thus considered as the ultimate reserves for tissue repair [73, 78, 79]. These cell types contain different potential markers, e.g., in the case of +4 cells is the expression of the genes *Lrig1*, *Tert*, *Bmi1* and *Hopx* [80]. However, recent studies have suggested that the regenerative process could be far more complex, as committed cells that have already undergone differentiation, such as enteroendocrine and tuft cells, can revert and de-differentiate into LGR5+ CBC during regeneration of damaged tissue [81]. This suggests that there are different redundant mechanisms to maintain the regenerative function in the gut epithelium. Moreover, it has been suggested that the niche environment might impact the activation of the different processes.

The redundant regenerative mechanisms imply the involvement of a vast spectrum of regulatory and signalling pathways. Indeed, Wnt, Notch, epidermal growth factor receptor (EGFR) and BMP have been found to play major roles in the regulation of the stem cell activity [48]. Paneth and stromal cells are intimately involved in this regard as Paneth cells produce WNT signals, Notch ligands and epidermal growth factor (EGF), while BMP signals are produced by stromal cells. The Wnt pathway is considered the main player with respect to stem cell maintenance in the intestine, whereas the Notch pathway regulates the fate of the ISCs [82]. It is also known that blocking of EGF signalling induces quiescence in LGR5+ CBC cells [81], while BMP signals can remove the multipotency of stem cells, inducing their differentiation in the villi [73]. However, these pathways are not isolated/independent from the environment, and it has been found that some bacterial metabolites can act as inducers and inhibitors at different levels of these pathways and, therefore, act as regulators of stem cell differentiation and, ultimately, gut epithelium regeneration.

#### **4. Control of human gut regeneration by microbiota-derived metabolites**

The gut microbiota can interact with the gut epithelium by direct contact [83] or through secreted bacterial metabolites [23, 84]. It has been reported that microorganisms can interact directly with the ISCs by outer membrane vesicles produced by Gram-negative bacteria [85–88] and could act to modify ISC gene expression patterns [83]. While this interaction has not been tested on ISCs, the uptake of these vesicles by other intestinal epithelial cells has been reported [88, 89]. This crosstalk underpins gut homeostasis and gut regeneration, via the regulation of the differentiation

and proliferation of the ISCs [23]. The aforementioned Wnt, Notch, EGFR and BMP pathways have been described as crucial with respect to the renewal of the gut epithelium, not only through the normal physiological process but also in response to stress or injury [90].

As mentioned above, a variety of microorganisms are located in different proportions along the intestinal tissue. Indeed, a subset of strictly aerobic, non-fermentative microbes, that together are referred to as the crypt-specific core microbiota, might live within the crypt, suggesting a possible direct influence of the microbiota on the ISCs and Paneth cells [83] and, therefore, epithelial regeneration [91]. This crypt-specific core microbiota is thought to have coevolved with mammals under selective conditions to provide a protective and homeostatic role in the gut [92]. From a limited number of studies in mice, it has been reported that the crypt-specific core microbiota would consist of aerotolerant Proteobacteria, including the genus *Acinetobacter* and lower levels of representatives of the genera *Delftia*, *Comamonas* and *Stenotrophomonas* [93–95]. Using proximal colonic organoids [96], it was tested that the LPS of the species living in the crypt would help in the maintenance of local homeostasis and epithelial regeneration by controlling the proliferation of the intestinal epithelium through the necroptosis of the stem cells and the epithelial differentiation process [95]. Also, using the worm moth *Spodoptera litura*, it has been shown how *Acinetobacter* helps to counter the inflammatory effect of a midgut damaging agent (dextran sulfate sodium salt), allowing a better recovery of the tissue [97]. However, studies testing the role of this genus on the control of the mammal's immune system in the context of gut regeneration are needed. It is important to keep in mind, however, that few studies have been published on the microbiota-gut stem cell interaction. All microbes reported above are known to be common contaminants of laboratories and reagents matching water- and soil-associated bacteria [98]. Further studies are needed to elucidate whether or not there exists a crypt-specific core microbiota and its role in tissue regeneration. In addition, the molecular and cellular mechanisms underpinning the microbiota-host cell interaction have yet to be elucidated to understand to what extent it is needed to support the gut stem cell niche, or if other signals could cover the absence of the microbiota in the crypts.

On the other hand, the crosstalk between the gut epithelium and the microbiota can be mediated by the secreted bacterial metabolites. The microbiota has different metabolic roles in the gut such as the fermentation of non-digestible dietary residues or host-derived substrates (e.g., bile acids) and the production of metabolites from the fermentation of carbohydrates producing SCFAs and intermediate metabolites, like lactate and succinate [23, 99], which provide an important source of nutrients for the gut epithelium and act as signalling molecules [23]. SCFAs are important to sustain the proliferation of the ISCs, as revealed by murine and in vitro models [100, 101]. However, some authors have shown that SCFAs may have a negative effect on the proliferation of ISCs proliferation. For example, Kaiko et al. [102] using mice, zebrafish and primary intestinal epithelial cell culture discovered that butyrate suppress the proliferation of the ISCs by the HDAC inhibition which led to the activation of the cell-cycle regulator Foxo3. This effect is dose dependent, where doses of 1 mM had a reversible effect but at doses of 3–10 mM the effect became permanent [102]. In the mammalian colonic lumen, the concentration of butyrate is around 70 mM [103], which led the authors to hypothesize that the colonocytes located in the surface of the gut epithelium metabolise the majority of the butyrate allowing lower concentration of this

metabolite to reach the crypt. This idea was forward proved by Lee et al. [12] by showing how the differentiated colonocytes metabolise butyrate, preventing it from reaching the ISCs in the crypt, which leave open a field to understand how are the gradients of these metabolites inside of the mammalian gut epithelium architecture.

The bacterial components and metabolites responsible for the crosstalk of the microbiota with the gut epithelium are recognised by a diverse plethora of receptors such as the pattern recognition receptors (PRRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and some G-protein coupled receptors (GPCRs) [104]. Also, some cell subtypes express specific receptors for gut microbiota metabolites. For instance, Lee et al. [12] showed that mice fed with lactic acid derived from *Bifidobacterium* and representatives of the genus formerly known as *Lactobacillus* are capable of signalling through GPR81 on Paneth cells, increasing their proliferation. Other cells express receptors that modulate the type 2 immune response. This is the case of intestinal tuft cells, which express *Sucnr1* and *Ffar3*, receptors for the microbial-derived metabolites succinate and SCFAs, respectively [105]. The enteroendocrine cells express receptors for different kinds of metabolites such as SCFAs [106], indole [85], secondary bile acids [93] and structural components of microbial membranes such as LPS [107]. Also, using organoid models, it has been demonstrated how bacterial compounds like muramyl dipeptide stimulate ISCs to confer cytoprotective properties through the expression of the *Nod2* receptor, which is a cytosolic innate immune sensor expressed by LGR5+ stem cells of the intestinal crypt [91]. Additionally, the gut microbiota interacts with the ISCs to stimulate their proliferation through the production of reactive oxygen species (ROS) and pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs) [91, 108].

Despite the increasing knowledge related to the regulatory effects of bacterial metabolites, more studies are needed to understand the molecular mechanisms triggered by these metabolites in the ISCs. In Table 1 are listed some of the microbiota-derived metabolites that have been reported to have an effect on the activation of signal pathways that could modulate key biological processes to induce a regenerative response such as cell proliferation, cell differentiation and control of the immune system.

## **5. Control of inflammation, a key process for human gut homeostasis and regeneration**

The gut is constantly exposed to different environmental conditions and toxins that can alter the homeostasis of this tissue and damage its morphology. Antibiotics, diet, alcohol and a variety of other factors can contribute to a gut injury and an impairing of the mucosal layer that protects the gut, including enteric pathogens, like bacteria, viruses, fungi or protozoa [109]. One of the most important biological processes after tissue damage is wound healing, a key component of tissue regeneration that requires the activation of different molecular and cellular mechanisms. Arguably, the control of the inflammation is one of the most important associated activities, as it determines if the wound is repaired or regenerated. If the wound is repaired, a fibrotic tissue is formed, which might lead to impairment of the tissue function. On the other hand, if the tissue is regenerated, it will have restored its architecture and functionality. The gut, like the skin, the liver or the hematopoietic tissue, has the physiological capacity to regenerate [110]. The intestinal wound healing occurs via three phases [111]: first, the barrier reestablishment lead by post-mitotic epithelial cells, called wound-

associated epithelial cells, and neutrophils migration to the wound area, which secretes pro-repair cytokines, chemokines, and growth factors, [112, 113]; second, the wound channel formation, characterised by the proliferation of the ISCs adjacent to the wound bed; third, the crypt regeneration, where Wnt5a is expressed by the mesenchymal cells, which stop proliferation to allow the fission of the wound channel into the crypts [12]. In the case of mucosal tissue, wound repair is regulated by different cell types, including epithelial, inflammatory, endothelial and mesenchymal cells [104]. However, increasing evidence suggests that the gut microbiota could also be involved in the regulation of the inflammation process, via different metabolites, such as PAMPs/MAMPs or ROS. In *Aedes albopictus*, the intestinal stem cells showed a regenerative response mediated by JAK/STAT (immune-related) and EGFR pathways in response of local gut damages induced either by chemical compounds or by enteric bacterial infections, showing that the response to *Serratia* feeding causes a higher activation of transcription factors related with the EGFR pathway and JAK/STAT, which are required for ISCs proliferation and differentiation. However, the metabolites derived by this microorganism that allow the activation of these pathways were not reported by the authors [114]. Moreover, in *Drosophila*, infection by *Pseudomonas entomophila* blocks these gut repair pathways because it triggers high production of ROS, which modulates stress pathways such as the mTOR pathway, which further leads to failed epithelium renewal [115, 116]. Viruses could be important regulators of the immune response, modulating the microenvironment of the intestinal epithelial cells, helping in the homeostasis of this tissue in the absences of commensal bacteria [117]. However, to our knowledge, the description of the mechanism of how the commensal viruses could trigger regenerative responses have not been reported. Here, we summarise some of the most relevant of these regeneration processes involving the gut microbiota (Figure 2); for deeper reading in the crosstalk immune system-ISCs-microbiota, see reference [118].

It is well known that the microbiota-derived metabolites have a role in the modulation of the immune system [119] and also the resident microbiota of the gut can exert an immunomodulatory effect during wound healing via the indirect activation of interleukin 22 (IL-22), which is a key factor in the promotion of ISC-mediated epithelial regeneration [104]. Some of the derived microbiota metabolites that can activate indirectly IL-22 are tryptophan, indole-3-aldehyde and indole-3-acetic acid [104]. These metabolites are ligands of the aryl hydrocarbon receptor (AHR), a transcriptional factor and key regulator of ILC3, which produces IL-22 [120]. Indeed, *Lactobacillus*-derived indole metabolites can, for example, induce ISCs proliferation through the activation of the AHR [121]. The bacterial metabolite deoxycholate (DCA), a secondary bile acid, has also been shown to play a crucial role in the transition through repair phases of the gut via PGE2 regulation, showing how the proper timing in the expression of this metabolite is crucial for intestinal epithelium healing [81]. During barrier re-establishment, DCA decreases locally in the wound to allow the expression of PGE2. However, during the wound channel formation, DCA increases to inhibit PGE2, leading to crypt regeneration by the activation of the classic innate TLR2/MyD88 signalling cascade. This experiment was conducted using *Clostridium scindens*, which has the capacity to generate secondary bile acids. When another strain not involved in DCA metabolism, such as *C. clostridioforme*,

is used, wound healing is not triggered. Notably, this phenotype is rare, with only 0.0001% of the colonic microbiota being capable of transforming primary into secondary bile acids [122].

Polyamines, such as putrescine and spermidine, are also involved in tissue regeneration and gut homeostasis [123–125]. Despite the control of the cellular response is not clear, Nakamura et al. [126] reported a symbiotic metabolic interaction where *E. coli* increases intracellular polyamine levels in colonic epithelial cells, producing spermidine and promoting gut epithelium renewal by the induction of the epithelium proliferation and differentiation of macrophages, especially the anti-inflammatory macrophages, involved in the resolution of induced colitis in a mouse model.

Representatives of the gut microbiota can also generate PAMPs/MAMPs [127], which can trigger an immunomodulatory response and are recognised by the TLRs and the PPRs. The chemical nature of PAMPs/MAMPs is very diverse, composed by nucleic acids, proteins, peptides, glycoconjugates or lipids [128]. Most of the time, PAMPs/MAMPs trigger a pro-inflammatory response, although this is not necessarily negative as it has been reported that the interaction between the gut microbiota and the intestinal epithelial cells is crucial to maintaining homeostasis in the tissue through a low-level PPRs activation [129, 130]. Moreover, there is a connection between the activation of PAMPs/MAMPs and the release of ROS [131], which is a crucial modulator of the immune response [132] and have been associated as an important molecule during regenerative responses [133, 134]. Besides, ROS have been reported as important modulators of the gut epithelium cells during motility, inflammation and proliferation [108, 135] via pathways such as Wnt and Notch [136]. ROS can be triggered by outer membrane vesicles, which secrete annexin A1, a ligand of the N-formyl peptide receptors that can trigger the expression of Nox1, promoting mucosal wound repair [137]. Also, some commensal gut bacteria generate physiological levels of ROS. Jones et al. [138] reported that *Lactobacillus acidophilus*, *L. casei* and *Lactococcus lactis* stimulate NADPH oxidase 1 (Nox1)-dependent ROS generation in mammalian Caco-2 cells. Ingestion of *L. plantarum* GG induced Nox1-dependent cell proliferation in the murine gut epithelium. Also, *L. plantarum* GG triggered cellular proliferation of ISCs in the intestine of *Drosophila* and mice. Lee et al. [12] tested that lactate produced by *Lactobacillus plantarum subsp. plantarum* strain ATCC 14917 (now renamed as *Lactiplantibacillus*) stimulated ISCs proliferation through Wnt/ $\beta$ -catenin signals of Paneth cells and intestinal stromal cells. This represents an important conserved mechanism during symbiotic interactions, suggesting the potential of *Lactobacillus* species to modulate the homeostasis and regeneration of the gut epithelium.

## 6. Models for the study of the gut microbiota-mediated gut epithelial regeneration

*In vitro*, *ex vivo* and *in vivo* models are used to study the mechanisms of gut epithelial regeneration dependent on gut microbiota actions. Despite that *in vitro* models, like the cell culture of Caco-2, T84 or HT29-MTX cells, offer the possibility of controlling many parameters, they can have important limitations, i.e., not being able to incorporate a faecal community because it would overgrowth and contaminate the cell or tissue culture. Cell culture can also limit the cells positional information, limiting the biological representability of the results.

Some of these limitations can be overcome by the use of gut organoids, which aim to reproduce the complexity and functionality of the *in vivo* tissue, and are arising as a reliable strategy to study host-microbe interactions, both in health and disease models [139]. Organoids include stem and differentiated cells that self-organise in a 3D structure. However, gut organoid behaviour can be limited for the study of gut epithelial regeneration, as they can elicit a regenerative response when damaged, something that has been argued against these models, as they are not fully representative of the *in vivo* response [140]. Recent work has allowed to improve these limitations and further discovery of more epigenetic modulators of regeneration, like VPA and EPZ6438 [140]. Additionally, organoids can be constructed for *ex vivo* studies, when harvested from patients, which would allow a more biologically relevant information and, potentially, personalised studies and treatments.

However, the main difficulty of these organoid models to the study of the interactions with the gut microbiota might be the introduction of the gut microbiota itself, due to the anaerobic nature of some of their representatives. At the moment, specific anaerobic bacteria, like *Clostridium difficile* or *Bifidobacterium adolescentis* [141], and viruses [142, 143]. More work needs to be done to introduce a complex faecal community to study these interactions as realistically as possible.

*In vivo* models can help to address some of the questions that *in vitro* models fall short at the moment. There are several animal models that are used to study the interactions between gut microbiota and gut regeneration (Table 2). These models allow to study complex levels of host-microbiota interactions, since an immune system is present [144]. For this reason, they are used for pathogenic or dietary studies. Rodent models are very commonly used to study human physiology, as their guts have similarities to those of humans. However, they differ in anatomy and gut microbiota composition, and there are physiological conditions, like inflammation, a key process in the study of gut epithelial injuries and regeneration, which are poorly mimicked [51]. Therefore, it would be beneficial to choose a model depending on how relevant its characteristics are to the question that we aim to answer. In Table 2 we have summarised some of the characteristics of each model and some of their advantages or disadvantages.

Overall, *in vitro*, *ex vivo* and *in vivo* models can help identifying key gut microbiota elements involved in the regulation of the gut epithelium regeneration and assess potential interventions to improve health conditions associated with gut epithelial damage.

## **7. Clinical significance and applications of gut microbiota modulation for regeneration purposes**

Understanding the role that the gut microbiota and its metabolites have on the regulation of regeneration allows the possibility of using targeted microbiome modulation as a therapeutic application. There are different strategies that can be followed to modify the resident gut microbiota (Figure 3). Here, we summarise some of them.

As mentioned earlier, lifestyle and dietary habits are important factors shaping the composition and structure of the gut microbiota. Evidence suggests that some foods might be associated with poor health outcomes and that gut microbiota patterns are linked to the consumption of those foods, e.g. Western diets are associated to metabolic conditions and the enrichment of taxonomic groups associated with inflammation [145, 146]. On the other hand, the gut microbiota associated with the consumption of a Mediterranean diet, characterised by high content on dietary fibre and polyphenols, is typically associated to the presence of bacterial groups related to lower inflammatory biomarkers and frailty [147–149].

Despite the fact that the influence of diet on gut regeneration has been difficult to investigate in humans, some studies conducted in mouse models indicate that diet can act as a regulator of epithelial regeneration mediated by ISCs (Table 3). In this way, chow and other modified diets were observed to induce distinct vulnerabilities to gut epithelial injury, microbial alternation and ISC dysfunction in chronically restrained mice [150]. The chow diet enriched the presence of *Lactobacillus reuteri*, which rescued stress-triggered epithelial injury and metabolised raffinose to fructose, feeding a metabolic loop and favouring ISCs maintenance during stress. This study proved that this metabolised fructose increased and engaged glycolysis, ultimately fuelling ISC proliferation. Similarly, other studies with mice suggest that a low-calorie diet favours intestinal regeneration after injuries [151]. A recent work corroborates the idea by reporting that an excess of dietary sugar impairs colonic epithelial regeneration after damage, both *in vitro* and *in vivo* [152]. Transcriptomic analyses showed that dietary sugar impeded the proliferative potential of ISCs by uncoupling glycolysis and the tricarboxylic acid cycle. Ultimately, understanding on dietary processes might be used to prevent injuries or support better treatments for gut epithelial regeneration.

More specific dietary interventions have focused on the use of prebiotics, probiotics or synbiotics, which could be an alternative to improve the gut wound healing and regeneration. Prebiotics are defined as “a substrate that is selectively utilised by host microorganisms conferring a health benefit” [153]. Prebiotics typically associated to positive health outcomes are polyphenols and fibre, linked to production of SCFAs that reduce inflammation biomarkers and improve gut barrier integrity, i.e., by increasing the presence of groups such as Lachnospiraceae, capable of modulating the colorectal cancer microenvironment and reduce its tumorigenesis. Other prebiotics act directly on the gut epithelium, like the polysaccharide glucomannan from *Aloe vera* gel (AGP), which can alleviate epithelial damage [154]. In a mice model, AGP both increased the number of LGR5+ ISCs and induced differentiation in epithelial cells, via Wnt/ $\beta$ -catenin signalling, facilitating the regeneration of epithelial cells and alleviating colitis. Amino acids can also promote intestinal epithelial cell proliferation. That is the case of the tandem L-alanyl-L-glutamine dipeptide that showed proliferative and crypt regeneration properties in a mice model via LGR5+ cells, suggesting the importance of nutritional regulation on intestinal epithelial homeostasis [155]. Thus, there are certain microbial and metabolic profiles associated to different diets and health outcomes that could be modulated through dietary changes and interventions with probiotics (“live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [156]), synbiotics

("mixture, comprising live microorganisms and substrate(s) selectively utilized by host microorganisms, that confers a health benefit on the host" [157]) or postbiotics ("preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [158]). Recently, it was found that live *L. reuteri* D8 was able to colonise the intestinal mucosa, having a protective effect on the integrity of the intestinal barrier, both in organoids and in a mice model [159]. *L. reuteri* D8 produced indole-3-aldehyde, which stimulated the production of IL-22, ultimately accelerating proliferation of intestinal epithelium and recovering the damaged intestinal mucosa. Additionally, LGR5+ and lysozyme+ cells, a staining technique used to discriminate between healthy and diseased cells, increased in numbers in both models, suggesting that *L. reuteri* D8 might be used as a probiotic, either by itself or in fermented foods, to treat intestinal inflammation. Other experiments in mouse models have shown that feeding lactic acid bacteria (LAB) increased the expansion and proliferation of ISCs, Paneth and goblet cells through the lactate stimulated Wnt/ $\beta$ -catenin signals [12]. It was observed that using a *L. plantarum* strain, which was not capable of producing lactate, ISC proliferation was reduced. Moreover, the lack of lactate G-protein-coupled receptor, GPR81, was associated with impaired epithelial development mediated by ISC. Interestingly, a pre-treatment with LAB showed protection in gut injury derived from chemotherapy and radiation as cancer treatments, suggesting further applications for epithelial regeneration. Overall, the evidence suggests that diet could be used to modulate the gut microbiota in a way that groups of microbes associated with beneficial health outcomes are enriched and the metabolite production profile is improved and can rebalance the villi microenvironment, where the epithelium regeneration process takes place [160, 161].

Despite the extended research performed on probiotic interventions, this approach would not be suitable in certain circumstances, like in immunocompromised patients or when the intestinal barrier is impaired. In these situations, an alternative intervention, like the use of extracellular vesicles (EVs) derived from probiotics, can be explored, as they can diffuse through the mucus layer, interacting with the host without inducing sepsis [162]. Thus, EVs from Gram-negative and Gram-positive probiotics have shown to trigger anti-inflammatory cytokines and down-regulate pro-inflammatory ones and interact with epithelial cells promoting the expression of tight junctions, which enhances the intestinal barrier function and reduces inflammatory activity [163, 164]. Therefore, bacterial EVs could be used to modulate gut epithelium inflammation in a safely manner.

Other interventions to modulate the gut microbiota need a more clinical approach, such as the faecal microbiota transplantation (FMT) and the microbial transfer therapy (MTT). FMT consists in the delivery of faecal microbiota from a healthy individual to a patient with a sub-optimal gut microbiota. It has been very efficient in the treatment of *C. difficile* infections, IBD, IBS and other disorders [165–167]. Moreover, it has been observed that the IL-10 production by the mucosal immune cells favours the control of inflammation and facilitates epithelial regeneration, both in a murine colitis model and in FMT human trials in ulcerative colitis [168–170]. However, there are concerns regarding its safety, as its use can display a number of side effects such as diarrhoea, low-grade fever or abdominal cramps [171]. MTT is a modified FMT protocol that includes an antibiotic treatment for 14 days and a bowel cleansing before the administration of a

standardised human gut microbiota (SHGM) for 7-8 weeks [172]. MTT showed improvement of gastrointestinal and ASD symptoms in children with ASD after receiving faecal transplantation of neurotypical control children [172]. Moreover, a follow-up study was conducted two years after the treatment was completed, reporting that most improvements in GI symptoms were maintained, and ASD-related symptoms improved and remained significantly correlated. The changes in gut microbiota composition at the end of the treatment remained at follow-up, including significant increases in bacterial diversity and relative abundances of *Bifidobacterium* and *Prevotella*. However, despite that the *Desulfovibrio* relative abundance decreased after MTT, at the two-year follow-up it was still slightly higher in comparison to the baseline and higher than in not-treated controls. Additionally, further studies suggested that p-cresol sulfate levels were inversely correlated with *Desulfovibrio* abundance, suggesting a potential role of *Desulfovibrio* on p-cresol sulfate modulation [173].

Other interventions considered for clinical purposes include targeted modulation of the gut microbiota, like the use of phage therapy [174]. Phages can regulate the immune responses within the intestinal mucosa and serve as antimicrobials, limiting the presence of pathogenic bacteria in the deeper mucus layer [175]. For this reason, phage therapy is explored as another strategy not only to treat *C. difficile* infections associated to ulcerative colitis, but also other inflammatory bacteria-mediated infections of the gut epithelium, like invasive adherent *E. coli* in Crohn's disease [174]. To our knowledge, direct regulation of phages in the ISCs have not been reported yet. However, it is well known that phages interact with the epithelial cells from different tissues, including the gut [176, 177], which could help maintain immune homeostasis [178]. It is reported that phages adhere to mucus surfaces and could be used to control epithelial inflammation, which is a common factor in gut disorders [179]. Also, phages like T4 induce the expression of the *DEFB4A* gene, which is an antimicrobial peptide with an immunomodulatory effect regulating the wound healing response [180]. It would be necessary to understand the effect of phages in the proliferation, migration or differentiation of the ISCs in order to identify if phages regulate the immune environment in the gut or have direct interaction with the ISCs.

Ultimately, this is a developing field, and phages are provided in cocktails to improve their efficacy. Moreover, future direction of the field involves using phage-specific components, like endolysins, to improve efficacy and limit undesirable interactions [181]. However, if we are interested in removing a specific pathogenic bacterium from the gut microbiota, recent studies have proposed that it could be done using antibodies that recognise specific cell surface elements on that organism [175]. Since any bacteria presenting specific proteins in its surface could be susceptible of being depleted from the gut microbiota, this strategy could help to address conditions linked to the specific actions of a gut microbiota representative. Furthermore, this targeted strategy could also be used to introduce specific microbiota elements, like probiotic species, for beneficial outcomes. Similarly, CRISPR-Cas9 systems have been proposed as tools to edit the gut microbiome to eliminate specific members of the gut microbiota which might be harmful and induce inflammation and dysbiosis, such as opportunistic fungi like *Candida* sp. or parasitic protozoa like *Blastocystis* sp. [182]. Additionally, CRISPR-Cas9 technology can be used to control gene expression and modulate the production of metabolites that ultimately can

act as regulators of the regeneration pathways. In conclusion, targeted approaches could improve gut homeostasis by modulating the microbiota in a very specific manner if a disruptive element is clearly identified.

Other strategies use advances of tissue engineering, including the insertion of genetic modified bacteria into 3D scaffolds, such as hydrogels. Duraj-Thatte et al. tested an engineered living material, which produce a curli fibre matrix, in the intestinal tract of mice, which helped in the recovery of damage tissue [183]. One important characteristic of this matrix is that it persisted for several days in the mouse tissue, which helped in the wound healing of the affected area. This kind of advances led to think in the possibility to incorporate metabolites or specific bacterial strains in delivery systems, which might help to restore the gut microenvironment [183].

Other *in vitro* technologies might add value to the clinical understanding of tissue regeneration in the gut. Thus, microfluidics systems such as those used for organ-on-a-chip models could help to investigate the interactions between diet components, the gut microbiota and the epithelial cells under controlled conditions [184]. These devices could facilitate the study of the interactions between specific bacterial species that inhabit the mucus layer and crypts and are involved in tissue regeneration, with the epithelial cells, helping us to understand how gut microbiota representatives regulate gene expression in cells [99, 185]. This strategy can also be used to test the effect of potentially beneficial microbial strains on the dynamics of regeneration. Moreover, organs-on-a-chip offer the possibility of using cells obtain from patients' biopsies, opening the door to personalised medicine and treatments.

Overall, our deepening understanding on the dynamic interaction between the gut microbiota and gut epithelial regeneration allow us to develop and adapt new strategies and technologies, which ultimately will impact treatments to ameliorate and improve gut health.

## **8. Conclusions and future perspectives**

Gut microbiota-derived metabolites are crucial regulators of the microenvironment of the gut. They help in the control of key signalling pathways important for the homeostasis and maintenance of the ISCs, where the immune system response is crucial to keep a regenerative environment. This process allows healing gut wounds without forming fibrotic tissue and keeping at bay the release of pro-inflammatory factors that hamper the renewal of the gut epithelium. However, the study of the multiple roles that different metabolites produced by the great diversity of gut microbes have on different sections of the intestine is certainly at its infancy. Studies to understand the crosstalk of these metabolites with different cell populations of the gut are needed to seek targeted therapeutic strategies. The advances in intestinal organoids and the microfluidic systems organ-on-a-chip are promising models to introduce complex anaerobic communities to explore the interaction of the intestinal microbiota, their metabolites and ISCs. In these state-of-the-art systems, the incorporation of other cell types, such as immune cells, could help to understand the regulation by the

microbiota and its metabolites on the intestinal immunological environment that allow gut epithelium regeneration. Also, a better understanding of the triad food-microbiota-health would contribute to better modulate the gut environment, for instance, by incorporating specific strains of microbes that secrete or induce the production of specific metabolites. The advances in gene manipulation techniques, such as CRISPR-Cas9, could contribute to this aim as this system permits stable genetic modifications to add or remove microbial strains or to control gene expression from complex communities. This, in turn, could modulate the production of bacterial metabolites that would further modulate the ISCs. All this will grant a better understanding of the microenvironment needed to intestinal homeostasis and successful gut epithelium regeneration.

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Figure 1-3 created with Biorender.com. Authors would like to thank Amy Fitzpatrick for fruitful discussions.

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### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Availability of data and material**

Not applicable

### **Author contributions**

CMA-G and EG-G conceived and designed the manuscript. CMA-G and EG-G wrote the manuscript. JSE and PDC critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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## List of figure legends

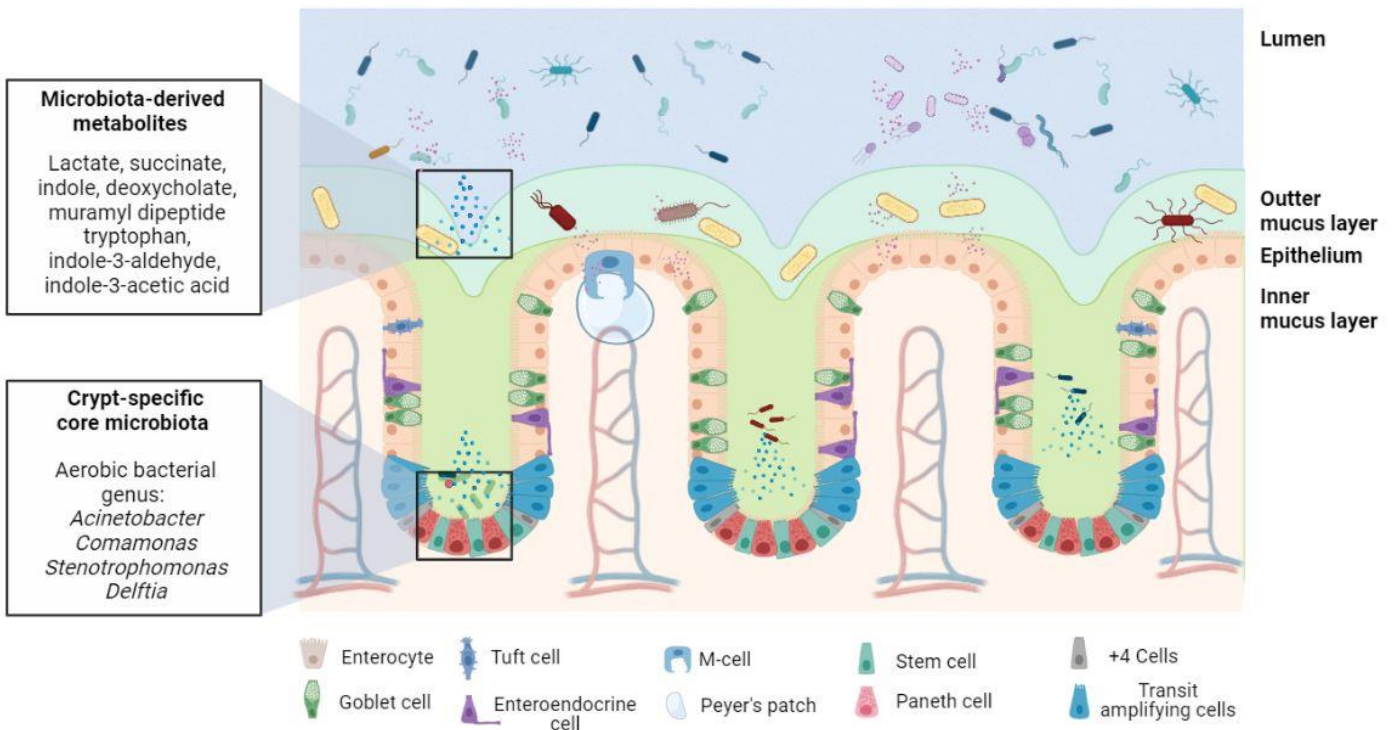
**Figure 1. Representation of a colonic section.** A high and diverse gut microbiota is present in the lumen. The outer mucus layer harbours mucin-degrading bacteria, such as *Akkermansia muciniphila* and others of health relevance, like *Bifidobacterium* sp. or *Bacteroides* sp. The inner layer is less densely populated by microorganisms (crypt-specific core microbiota), which are in contact with the stem cells (LGR5+ cells) and differentiated stem cells of the gut epithelium. The transit amplifying cells undergo a limited number of cell divisions before differentiating into other intestinal cells.

**Figure 2. Some immune system signals triggered by gut microbiota molecules during gut wound healing.** The gut epithelium could be damaged by different physical and chemical inputs leading to the death of colonocytes, after a gut wound the post-mitotic colonocytes start to migrate and neutrophils secrete cytokines and other growth factors that help to start a wound healing response, also, the local microbiota secretes different metabolites and expresses PAMPs/MAMPs, both can generate an immunomodulatory response that could control gut tissue regeneration. Other pathways related with the immune response like the EGFR and JAK/STAT are important regulators of ISC proliferation and differentiation and are triggered by bacteria.

**Figure 3.** Summary of the different strategies for regeneration interventions and studies based on the effect of the gut microbiota. For targeted therapy, see references 174, 178, 179, 180, 182 ; for in vitro and microphysiological systems, see references 183, 185 ; for clinical transfers, see references 165, 166, 168, 169, 170, 172; for postbiotics and extracellular vesicles, see references 12, 159, 162, 163; and for dietary interventions, see references 147, 148, 149, 151, 154, 155.

## List of figures

### Figure 1



ACCEPT

Figure 2

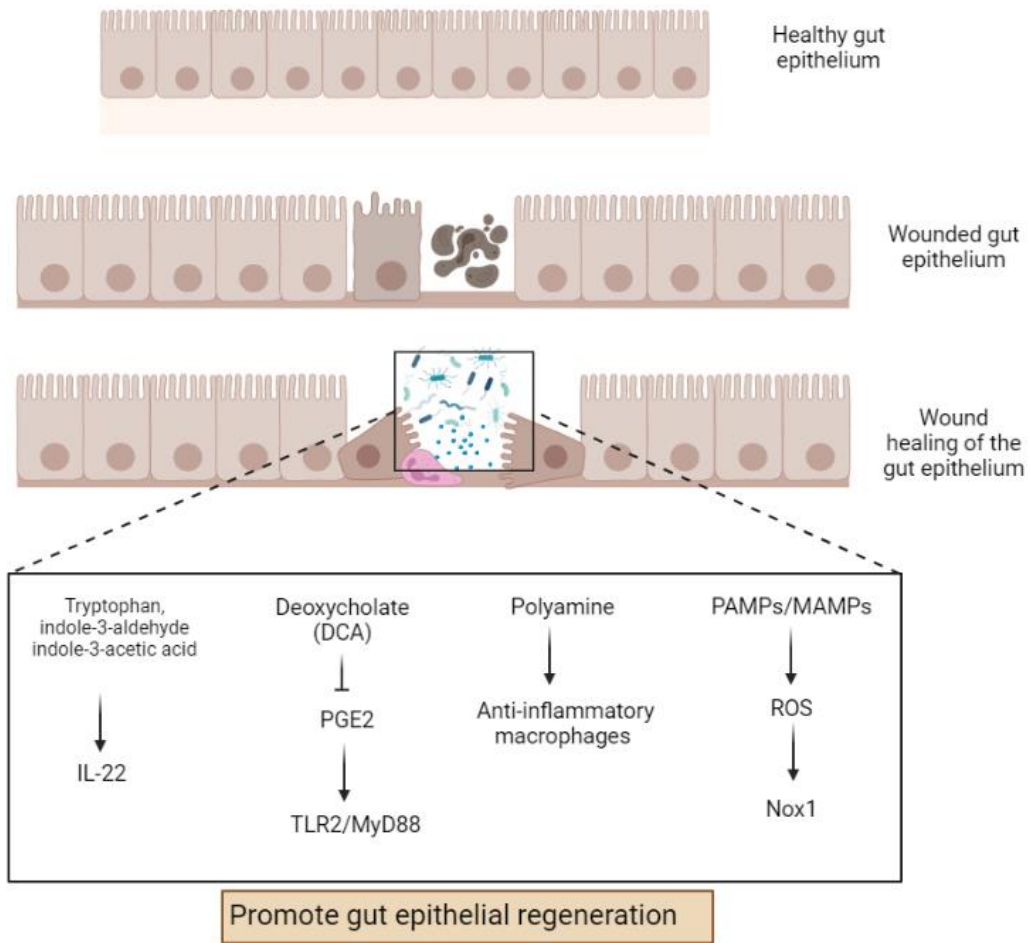
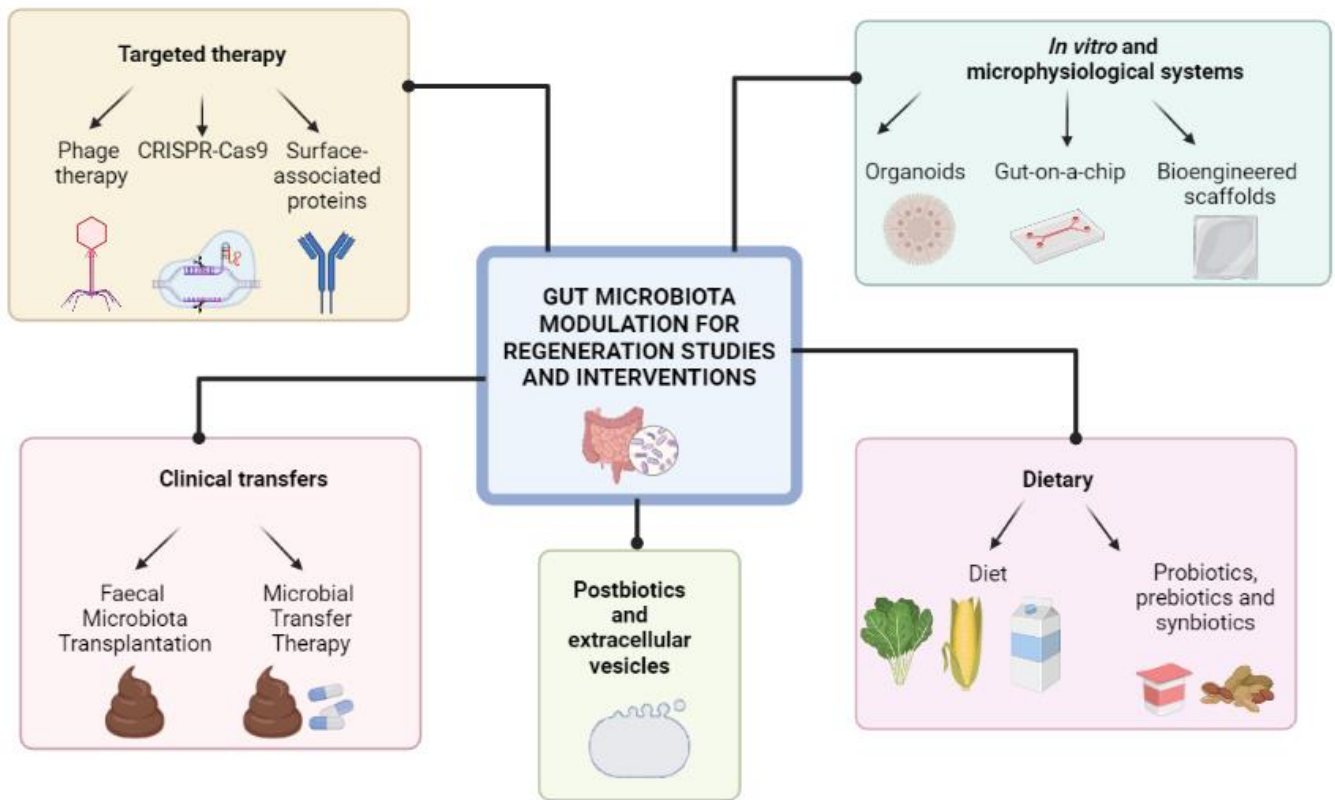


Figure 3.

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## List of tables

**Table 1.** Metabolites derived by the gut microbiota discussed in this review that could be important for gut regeneration and homeostasis

Metabolites	Examples of gut microbes capable of producing the metabolites [84]	Pathways activated by the metabolite	Biological process activated important for gut regeneration	Ref
Short-chain fatty acids	<i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Campylobacter jejuni</i> , <i>Staphylococcus aureus</i> , <i>Bifidobacterium</i> , <i>Coprococcus</i> , <i>Clostridium</i> , <i>Roseburia</i> , <i>Faecalibacterium</i>	G protein-coupled receptors (GPCRs), mitogen-activated protein kinase (MAPK), Keap1-Nrf2	<ul style="list-style-type: none"> <li>- Anti-inflammatory effects by the inhibition of the expression of IL-6, IL-1<math>\beta</math> and TNF<math>\alpha</math>.</li> <li>- Promote the release of anti-inflammatory IL-10 from regulatory T (Treg) cells.</li> <li>- Epigenetic regulation by the HDAC inhibition which promotes chromatin acetylation and target gene transcription.</li> <li>- Antioxidant properties</li> </ul>	[186–192]
Indole metabolites	<i>Escherchia coli</i> , <i>Clostridium sporogenes</i>	AhR and/or PXR-based signalling	<ul style="list-style-type: none"> <li>- Increase expression of IL-22 and IL-10 levels which enhance mucosal healing by activating innate and adaptive lymphoid</li> <li>- Immunomodulation via stabilization of Treg cells, they can be exploited as microbial-metabolite-derived therapies</li> </ul>	[12, 24]
Bile acids	<i>Bifidobacterium</i> , <i>Bacteroides</i> , <i>Clostridium</i> , <i>Lactobacillus</i> , <i>Enterobacter</i>	G-protein-coupled receptor. nuclear farnesoid X. Receptor (FXR) or the G protein-coupled receptor (TGR5)	<ul style="list-style-type: none"> <li>- Modulate host metabolism regulation of glucose homeostasis, lipid metabolism</li> <li>- Regulate immune responses and regulate the polarization of macrophages</li> </ul>	[12, 24, 193]
Polyamines	<i>Campylobacter jejuni</i> , <i>Clostridium saccharolyticum</i>	Mitogen-activated protein kinase (MAPK)	Cellular proliferation and differentiation processes	[194]

Table 2. Summary of advantages and disadvantages of different models for the study of gut regeneration mediated by gut microbiota.

Models		Relevance for human physiology	Advantages	Disadvantages	Ref.
<b><i>In vitro</i> and <i>ex vivo</i> studies</b>	<b>Cell culture</b>	<ul style="list-style-type: none"> <li>-Contain multiple cell types (co-culture system)</li> <li>-Cancer cell lines: HT-29, Caco-2, T84, SW480 (accumulated mutations)</li> </ul>	<ul style="list-style-type: none"> <li>-Controlled conditions</li> <li>-Cost effective</li> <li>-Reproducibility</li> <li>-High throughput</li> </ul>	<ul style="list-style-type: none"> <li>-Monolayers do not represent the whole diversity of the tissue</li> <li>- Not fully representative of normal cells physiology)</li> <li>-Does not allow co-culture with a complex gut microbiota system</li> <li>-Cannot apply mechanical stimuli (peristalsis)</li> </ul>	[195, 196]
	<b>Organoids</b>	<ul style="list-style-type: none"> <li>-Similar function exertion to</li> <li>-Patient-derived cells from biopsies</li> <li>-Contain all epithelial cell types, transporters and pattern recognition receptors</li> </ul>	<ul style="list-style-type: none"> <li>-Controlled conditions</li> <li>-Possibility of personalised treatments</li> </ul>	<ul style="list-style-type: none"> <li>-Human samples difficult to obtain</li> <li>-Expensive</li> <li>-Difficult to polarise cells</li> <li>-Does not allow co-culture with a complex gut microbiota system</li> <li>-Difficulty to track certain biomarkers</li> </ul>	[195, 197]
<b>Animal models</b>	<b><i>Drosophila melanogaster</i></b>	<ul style="list-style-type: none"> <li>-The turnover rate is of one week approx.</li> <li>- It has cells with similar functions: enterocytes ISCs and enteroendocrine cells</li> <li>-Mucus barrier</li> </ul>	<ul style="list-style-type: none"> <li>-Generation of numerous progeny</li> <li>-Genetic manipulation</li> <li>-Cost effective</li> <li>-Immune response</li> <li>-Easy sample collection</li> <li>-Easy control over diet and genetics</li> </ul>	<ul style="list-style-type: none"> <li>-Stem cell proliferation (turnover of one week)</li> <li>-No adaptive immune system</li> </ul>	[198, 199]
	<b><i>Danio rerio</i></b>	<ul style="list-style-type: none"> <li>-Intestinal morphology similar to vertebrates</li> <li>-Several gut functions and immune genes conserved between zebrafish and mammals</li> <li>-Innate and adaptive immunity</li> </ul>	<ul style="list-style-type: none"> <li>- Transparent early in life (&lt;2 weeks) and develop ex utero from a fertilized egg, allowing fully monitorisation</li> <li>-Generation of numerous progeny</li> <li>-Immune response</li> </ul>	<ul style="list-style-type: none"> <li>-Simple digestive tract morphology</li> <li>-Logistics limitations (Aquatic facility)</li> </ul>	[187,196]

			-Easy sample collection -Easy control over diet -Genetic manipulation		
	<b><i>Caenorhabditis elegans</i></b>	- Less human relevance	-Microbe colonisation can be easily monitored in whole animals  -Naturally engage in interactions with bacteria -Genetic manipulation -Cost effective	Only has enterocytes	[176, 177]
	<b>Mice</b>	-Cell spectrum similar to human -Mucus layer -Gut structure and anatomy differs from human	-Easy sample collection -Easy control over diet and genetics	-Ethical considerations -Expensive	[187,197]

Table 3. Summary of examples of different dietary interventions and their impact on gut regeneration mechanisms.

Interventions		Gut environment effect	Gut regeneration input	Ref
<b>PREBIOTIC</b>	Chow diet	Increased <i>Lactobacillus reuteri</i> , which metabolised raffinose to fructose	Metabolised fructose increased glycolysis contributing to ISC proliferation	[124]
	Polyphenols and fibre	Increased Lachnospiraceae to production of SCFAs	Reduced inflammation and improved gut barrier integrity	[63,69,183]
	<i>Aloe vera</i> gel	Increased polysaccharide glucomannan	Increased the number of LGR5+ ISCs and induced differentiation in epithelial cells, via Wnt/ $\beta$ -catenin signalling, facilitating the regeneration of epithelial cells and alleviating colitis.	[154]
	Amino acids L-alanyl-L-glutamine dipeptide	Promoted intestinal epithelial cell proliferation	Proliferative and crypt regeneration properties in a mice model via LGR5+ cells	[155]
	<i>L. reuteri</i> d8	Produced indole-3-aldehyde	Stimulated the production of IL-22, ultimately accelerating proliferation of intestinal	[159]

<b>PROBIOTIC</b>			epithelium and recovering the damaged intestinal mucosa	
	Lactic acid bacteria ( <i>Bifidobacterim</i> and <i>Lactobacillus</i> spp)	Pre-treatment with LAB-type symbionts or lactate protected mice in response to gut injury	Increased the expansion and proliferation of ISCs, Paneth and goblet cells through the lactate stimulated Wnt/ $\beta$ -catenin signals	[12]
	De Simone formulation (mixture of highly concentrated lyophilized living bacteria)	Therapeutic effects against stomach erosions	Regeneration of the stomach wall associated with an increase in stem cell proliferation and enhanced production of protective factors such as mucus	[134]

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