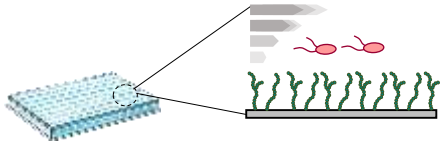
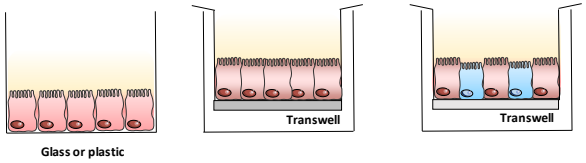




Polar monolayer of enterocytes secreting mucins

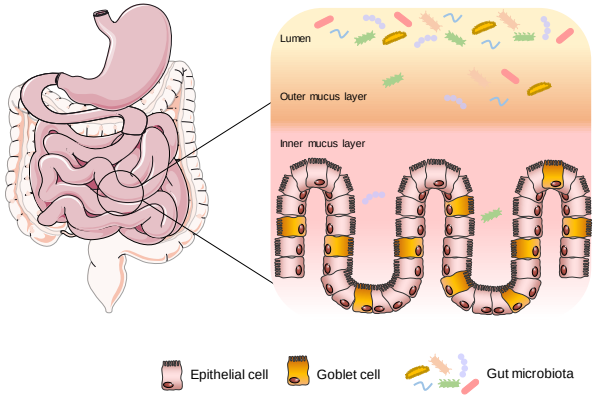
Co-culture of Caco-2 and HT29-MTX

***In vitro* mucus/mucin binding assays**

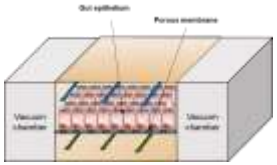
***In vitro* cell models**

Intestinal organoids

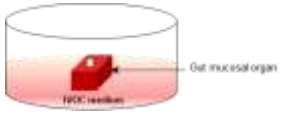
***In vivo* animal models**



***Ex-vivo* organ cultures**

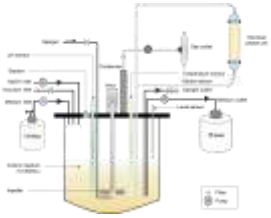


Gut on Chip

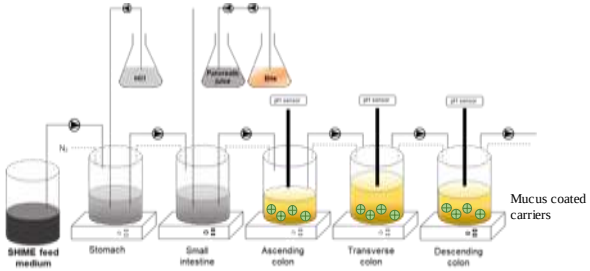


In vitro organ culture

***In vivo* digestive models with mucosal phase**



M-ARCOL



M-SHIME

Experimental models to study intestinal microbes-mucus interactions in health and disease

Sentence summary: The review summarises the state of the art for studying gut microbes-mucus interactions using *in vitro*, *ex vivo* and *in vivo* experimental models.

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ABSTRACT

A close symbiotic relationship exists between the intestinal microbiota and its host. A critical component of gut homeostasis is the presence of a mucus layer covering the gastrointestinal tract. Mucus is a viscoelastic gel at the interface between the luminal content and the host tissue that provides a habitat to the gut microbiota and protects the intestinal epithelium. The review starts by setting up the biological context underpinning the need for experimental models to study gut bacteria-mucus interactions in the digestive environment. We provide an overview of the structure and function of intestinal mucus and mucins, their interactions with intestinal bacteria (including commensal, probiotics and pathogenic microorganisms) and their role in modulating health and disease states. We then describe the characteristics and potentials of experimental models currently available to study the mechanisms underpinning the interaction of mucus with gut microbes, including *in vitro*, *ex vivo* and *in vivo* models. We then discuss the limitations and challenges facing this field of research.

Keywords: intestinal mucus, gut microbiota, experimental models, mucin *O*-glycosylation

INTRODUCTION

The human gastrointestinal (GI) tract harbours a complex and diverse community of microbes, including 10 trillion of microorganisms, collectively referred to as the gut microbiota (Sender *et al.*, 2016). Several regulatory mechanisms cooperate to maintain intestinal homeostasis and a disturbance of the relationship between the gut microbiota and the host can results in several disorders including chronic inflammatory diseases and metabolic syndromes (Rooks & Garrett, 2016). While the intestinal microbiota provides important benefits to the host, such as calorie extraction and immune system maturation, it also holds the power to activate various innate and adaptive immune signalling which can lead to uncontrolled and deleterious intestinal inflammation (Pickard *et al.*, 2017). A key component in maintaining a beneficial relationship between the commensal microbes inhabiting the intestine and the host is the presence of an appropriate barrier that prevents bacteria to reach and persist on the epithelial surface (Johansson & Hansson, 2016, Sicard *et al.*, 2017, Bretin *et al.*, 2018). It is well acknowledged that intestinal epithelial cells (IECs) provide a physical and biochemical barrier that prevents the translocation of commensal bacteria to the underlying host tissue. In addition, there is an emerging paradigm that the mucus layer is an important modulator of human health in mediating the homeostatic relationship between the gut microbiota and the host. On the luminal side, the mucus layer provides the first physical, chemical, and biological line of defence against large particles, including commensal bacteria and invading pathogens, segregating them from IECs (Turner, 2009, Peterson & Artis, 2014). Furthermore, mucus provides a biological niche for a microbial community, referred to as mucus-associated microbiota, which is likely to have a major influence on human health (Martens *et al.*, 2018). However, advances in this field of research have been hampered by the lack of suitable model systems recapitulating all the interactions occurring at the mucosal interface. This review provides an overview of currently

available experimental models to study the interplay between gut bacteria and intestinal mucus at a mechanistic level, and summarizes their main applications and the challenges remaining in this field of research.

1. Overview of mucus structure and function in the gastrointestinal (GI) tract

1.1. Mucus structural organisation

Mucus structure. Mucus is a highly hydrated gel made up of more than 98% water that makes it totally transparent, microscopically invisible and difficult to study. This aqueous viscoelastic secretion also contains electrolytes, lipids and various proteins (Bansil & Turner, 2018). Mucus is found throughout the entire GI tract from the stomach to the large intestine, with its thickness and structure varying depending on the location, reflecting its various protective functions.

The mucus in the small intestine consists of one layer, while the stomach and colon have a bi-layered mucus. In human stomach, the mucus is about 200–400 μm in thickness and consists of an inner layer loosely attached to the epithelial surface, keeping the surface neutral (pH 7) while the gastric lumen pH is acidic (pH 2), and an outer layer which is mobile on the luminal side. Only few bacteria have evolved strategies to colonise the stomach, among which *Helicobacter pylori* is a specialist (Atuma *et al.*, 2001, Juge, 2012). In the small intestine, mucus fills up the space between the villi but is not attached to the epithelium and is somewhat permeable to bacteria (Atuma *et al.*, 2001). In the colon, the two layers mediate opposite interactions with the microbiota; whereas the outer layer (up to 800 μm) is densely colonised by an important microbial biomass, the inner layer (>200 μm in humans) is virtually devoid of bacteria leaving a space virtually free of microbes (commensals and or pathogens) leaving a space virtually free of microbes above the epithelium (Johansson *et al.*, 2013). However, single-cell imaging at tissue scale in mice revealed the presence of bacteria in close proximity of the epithelium (Earle *et al.*, 2015). Among commensal microorganisms, Segmented Filamentous Bacteria (SFB) are immunomodulatory commensals with the ability

105 to adhere to IECs and to invade this mucus layer without invading the host (Hedblom *et al.*,
106 2018, Ladinsky *et al.*, 2019). Of note, a recent study revealed differences in mucus
107 organization between the proximal and distal colon of rodents (Kamphuis *et al.*, 2017): in the
108 later, the mucus layer is attached to the faecal pellet and absent from the surface of the
109 epithelium (Kamphuis *et al.*, 2017).

110 Other studies demonstrated that the mucus thickens as the microbiota become more
111 diverse, as particularly evident in the colon (Jakobsson *et al.*, 2015). This is also supported by
112 studies using germ free mice showing an impairment in mucus structure (Johansson *et al.*,
113 2008, Johansson *et al.*, 2013, Jakobsson *et al.*, 2015). Gnotobiotic mice colonized with human
114 faecal microbiota present a mucus layer structure resembling that of conventional mice by day
115 7 post-colonization (Hayes *et al.*, 2018). Animals housed in distinct rooms of the same animal
116 facility exhibit distinct microbiota profiles that are associated with large differences in the
117 inner colon mucus layer, thereby affecting mucus barrier properties (Jakobsson *et al.*, 2015).
118 Also, it has been demonstrated in mice that mucus becomes thinner with age (Elderman *et al.*,
119 2017). Variations in the mucus thickness and spatial organisation of the gut microbiota in
120 mice were also found to be dependent of the diet (Earle *et al.*, 2015). Interestingly, the
121 thickness of the mucus layer has been shown to undergo circadian fluctuations, with highest
122 microbial proximity to the mucosal surface during the dark phase (Thaiss *et al.*, 2016).
123
124 ***Mucus secretion.*** The mucus is produced and secreted by specialized cells namely goblet
125 cells located in the crypt in the small intestine and in higher numbers in the upper crypt in the
126 colon (Johansson & Hansson, 2013, Johansson & Hansson, 2016, Sicard *et al.*, 2017). Before
127 secretion in the gut lumen, mucin polymers are stored in mucin granulae within the goblet
128 cells (Johansson *et al.*, 2011, Johansson *et al.*, 2013). The function of goblet cells varies
129 depending on their localisation in the small intestinal or colonic crypts (Pelaseyed *et al.*,

2014). Apart from their role in secreting mucus, small intestinal goblet cells can play a role in delivering luminal material to the immune system (Pelaseyed *et al.*, 2014). Interestingly, a study from Gunnar Hanson's laboratory identified a subpopulation of goblet cells called "sentinels" goblet cells (Birchenough *et al.*, 2016). These cells are able to sense Toll-like receptor (TLR) microbial ligands at the entrance of colonic crypts and trigger the activation of NLRP6 inflammasome, leading to mucus secretion from neighbouring goblet cells to defend the colon against bacterial invasion (Birchenough *et al.*, 2016).

Renewal of the mucus is an important factor to preserve epithelial damage and bacterial exposure. The colonic mucus has a rapid turnover, since the inner mucus layer is renewed within 1 hour (Johansson, 2012), while the gut epithelium renewal takes around 4-5 days (De Weirdt & Van de Wiele, 2015).

Gastro-intestinal mucins. The main structural components of mucus are large glycoproteins called mucins. The protein sequences of mucin domains share a common core structure rich in the amino acids prolin (P), threonine (T) and serine (S) called the PTS domain. These domains are then decorated by *O*-linked glycans made up of *N* acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), galactose (Gal) and usually terminated by sialic acid and fucose (Juge, 2012, Johansson & Hansson, 2016, Sicard *et al.*, 2017). These *O*-glycans render the mucin domains highly resistant to protease degradation and confer mucins their high-water binding capacity.

Mucins are produced as transmembrane mucins or secreted gel forming mucins (Juge, 2012, Johansson et al., 2013). In the stomach, MUC1 and MUC5AC are produced by the superficial epithelium, while MUC6 are secreted by the stomach glands (Johansson *et al.*, 2013, Johansson & Hansson, 2016). In the small intestine and colon, mucus is structurally built around the mucin-2 glycoprotein (MUC2). The folding and dimerization of MUC2 is a

demanding process owing to the large number of disulfide bonds, and a defect during this process may affect the structure and function of intestinal mucus (Johansson *et al.*, 2011). Proteolytic cleavages of MUC2 catalysed by the host as well as bacteria enzymes favour the transition from firm to loose layer form and allow bacteria to penetrate into the mucin net-like structure of the outer mucus layer (Johansson *et al.*, 2008). In addition to this proteolytic activity, the degradation of mucin glycan chains by bacterial glycosidases contribute to the establishment of a microbial community in the outer mucus layer (Johansson *et al.*, 2008, Pelaseyed *et al.*, 2014).

Mucin glycosylation. Glycosylation is the most frequent post-translational modification of proteins and can occur in *N*-linked and *O*-linked form, and *O*-glycosylation is the main modification of mucins (Arike & Hansson, 2016). Mucin-type *O*-glycans are built from eight core structures, with core 1, core 2, core 3 and core 4 glycans most commonly found in intestinal mucins (Brockhausen *et al.*, 2009). *O*-glycosylation is initiated in the Golgi apparatus by the addition of a GalNAc residue to the hydroxyl group of serine and threonine of the mucin backbone. Further elongation and branching of the *O*-glycan chains is governed by a large family of glycosyltransferase enzymes (Bennett et al., 2012). The oligosaccharides can be further modified by addition of histo-blood group antigens (ABO, Lewis), secretor (H) epitopes and sialic acids and sulfate (Rossez et al., 2012, Bansil & Turner, 2018). Mucin glycosylation varies along the GI tract (Robbe *et al.*, 2003, Robbe *et al.*, 2004, Holmen Larsson *et al.*, 2013) and is linked to microbial colonization (Juge, 2012, Bergstrom & Xia, 2013, Tailford *et al.*, 2015, Arike *et al.*, 2017). Mounting evidence suggests that mucin glycosylation is critical to the biological and physical role played by mucus in the gut by influencing the physico-chemical properties and penetrability of mucus and by modulating the composition of the associated mucus-associated microbiota (see section 1.3). Not

surprisingly, an alteration of mucin *O*-glycosylation profile has been reported in intestinal diseases associated with an impaired gut barrier function such as inflammatory bowel disease (IBD) and colorectal cancer (Larsson *et al.*, 2011, Theodoratou *et al.*, 2014) as also supported by work in animal models (Bergstrom *et al.*, 2017) (see also section 4.5).

1.2. Mucus function in the gut

For decades, mucus has been considered to act as a simple physical barrier protecting the host, but mounting evidence suggests that mucus plays additional biological and immunological roles in maintaining gut homeostasis. The coating gel of mucus is acting, in concert with the immune system, the intestinal epithelium and the gut microbiota, to provide a physical, biological, and chemical line of defence against potentially harmful invaders while harbouring a distinct microbial community having a major influence on host health.

Throughout the gut, the viscous mucus secretion acts as a lubricant that helps the progress of digestive matter along the GI tract and protects the underlying epithelium from excessive mechanical or chemical stresses. In the stomach, the mucus coating creates a pH gradient that protects the epithelium against the crude acidic gastric environment. Mucus acts as a size exclusion filter for larger compounds while selectively allowing transport of small molecules such as gases, ions, nutrients, and many proteins to reach the enterocytes (De Weirdt & Van de Wiele, 2015), but the mucus lining would prevent digestive enzymes from attacking these cells.

In the colon, the outer mucus layer serves as a biological habitat for various microorganisms. Indeed, the glycan structures in the mucus provide potential binding sites and constitute a carbon and energy source to support the growth of commensal but also pathogenic bacteria (Tailford *et al.*, 2015) (see sections 2.1 and 2.2). It is believed that the

mucin glycosylation patterns along the GI tract contribute to the microbial tropism of certain taxa in the mucus (Tailford *et al.*, 2015).

The mucus layer also helps in the protection of the epithelium and, in association with the immune system, plays a crucial role in intestinal homeostasis. This gel is an important retention matrix for non-mucin proteins with immune regulatory molecules such as antimicrobial molecules (e.g. bactericidal RegIIIγ, α-defensins, secretory immunoglobulins IgAs, etc), therefore limiting the number of bacteria that can reach the epithelium and the underlying immune system (Peterson & Artis, 2014, Johansson & Hansson, 2016). This physical and biological barrier helps to keep the tremendous amount of bacteria that reside in the lumen as well as enteric pathogens at a safe distance from the epithelium (Chassaing *et al.*, 2014, Johansson *et al.*, 2014, Chassaing *et al.*, 2015). However, this system can be subverted and invading pathogens or pathobionts have evolved strategies to circumvent this barrier by e.g. degrading mucins and/or influencing mucin secretion (Rolhion & Chassaing, 2016). In summary, mucus has a dual role in relation to the gut microbiota, it is an ecological niche for bacteria by providing adhesion sites and nutrients, while protecting the underlying epithelium from microbial aggressors that can breach this barrier.

1.3. The mucus-associated microbiota

The gut microbiota composition is known to differ along the longitudinal axis of the GI tract but it also varies transversally from the lumen to the mucosa due differences in key physiological parameters such as nutrient availability or oxygen gradient. The colonic epithelium is made of crypts with specific oxygen conditions and various concentrations of glycans that is a niche for mucin- degrading bacteria such as *Bacteroides fragilis* (Pereira & Berry, 2017). The use of Carnoy fixative to preserve the mucus layer has been a crucial step

for the detection of bacteria in the mucosal environment (Johansson *et al.*, 2008). It is now well appreciated that the faecal microbiota community differs from the luminal, mucosa- or mucus-associated bacterial communities (Swidsinski *et al.*, 2005, Li *et al.*, 2015) .

Studies in humans demonstrated that the abundance of Bacteroidetes appears to be higher in faecal/luminal samples than in the mucosa (Eckburg *et al.*, 2005). Members of Firmicutes phylum and in particular *Clostridium* cluster XIVa are significantly enriched in the mucus layer compared to the lumen (Van den Abbeele *et al.*, 2013). Analysis of human colonic biopsies have also shown a distinct mucosal community enriched in Actinobacteria and Proteobacteria compared to the luminal community (Albenberg *et al.*, 2014). Certain species such as *Bacteroides acidifaciens*, *B. fragilis,* and *Akkermansia muciniphila* are enriched in the outer layer of colon mucus (Derrien *et al.*, 2004, Donaldson *et al.*, 2016).

Similar findings have been observed in animals. Indeed, mice studies have shown that Firmicutes were enriched in the mucosa-associated microbiota, especially members of the Lachnospiraceae and Ruminococcaceae families (Tailford *et al.*, 2015). Bacterial species such as *Bacteroides thetaiotaomicron* or *Escherichia coli* display specific genomic repertoires to persist in the outer mucus layer compared with the same species in the intestinal lumen (Li *et al.*, 2015). This spatial localisation may be reflective of the radial oxygen gradient that shapes the mucus-associated and faecal microbiota, since oxygen can favour or impede certain microorganisms (Albenberg *et al.*, 2014). Moreover, laser capture microdissection (LCM) in combination with metagenomics studies provided new insights into the composition of the mucus-associated microbiota (Wang *et al.*, 2010). The use of LCM in mouse models revealed that this microbial community is especially dominated by *Acinetobacter* in the colonic crypts (Pedron *et al.*, 2012). Using LCM coupled to DNA sequencing–based analysis, Chassaing and Gewirtz recently reported profound differences at the phyla level between the inner mucus

communities comprising 20%–60% Proteobacteria and a concomitantly marked reduction in Bacteroidetes as compared to faecal microbiota (Chassaing & Gewirtz, 2019).

Due to a high polysaccharide content (up to 80% of the mucin biomass), mucus provides an ecological niche for the intestinal microbiota. Mucus-associated bacteria are able to use oligosaccharides from mucins as binding sites through specific bacterial adhesins that promote their colonisation (Section 2.1) or as an energy source to support their growth (Section 2.2). Robbe and colleagues first suggested that the important repertoire of potential ligands and/or carbon sources in mucins could explain the pattern of bacterial colonisation in the different gut regions (Robbe *et al.* 2004). Mucin degradation has been extensively studied in pathogenic bacteria and more recently investigated in commensal bacteria including *A. muciniphila*, *Bacteroides* spp., Bifidobacteria and *Ruminococcus* spp. (Derrien *et al.*, 2004, De Weirdt & Van de Wiele, 2015). A disproportion of bacterial taxa able to invade mucus could further play a role in the development of the dysbiotic microbiota associated with the onset of various intestinal diseases (see section 3).

2. Mucin-bacteria interactions

2.1. Mechanisms of mucin binding by commensal and pathogenic microorganisms in the gut

Cell-surface proteins of pathogens and probiotics/commensal strains have been implicated in mediating the binding of microbes to intestinal mucus (Fig. 1). These include (i) specialized cell-surface adhesins or lectins, (ii) appendages such as pili and flagella or (iii) moonlighting proteins (see (Juge, 2012) for a review). In particular, a considerable amount of

research has been devoted to the characterization of these adhesins in *Lactobacillus* species (as extensively reviewed in (Van Tassell & Miller, 2011, Nishiyama *et al.*, 2016)).

Mucus binding proteins. Mucus-binding proteins (MUBs) containing a variable number of Mub repeats are unique to gut inhabiting Lactobacilli and these proteins have been thoroughly characterised in *Lactobacillus reuteri,* a gram-positive bacterial species inhabiting the GI tract widely used as a probiotic (Frese *et al.*, 2011). MUB from *L. reuteri* ATCC 53608 is one of the best studied examples of mucus adhesins in commensal bacteria. It is a large protein consisting of six type 1 repeats (Mub1) and eight type 2 repeats (Mub2) with each repeat divided into a mucin binding (MucBP) domain and an immunoglobulin binding protein domain (Kuznetsova, 1990, MacKenzie *et al.*, 2009, Etzold *et al.*, 2014). The Mub repeats mediate binding to mucin glycans, through interactions with terminal sialic acid (Etzold *et al.*, 2014, Gunning *et al.*, 2016), and Igs (MacKenzie *et al.*, 2009). MUB has the shape of a long, fibre-like structure, of around 180 nm in length (Etzold *et al.*, 2014), and forms appendices reminiscent to pili found in pathogenic and, more rarely, other commensal bacterial species. However, in contrast to pathogenic pili which adhesin is restricted to the N-terminal tip, MUB interactions with mucin glycans occur through its long and linear multi-repeat structure, as shown by atomic force spectroscopy (Gunning *et al.*, 2016). This multivalent binding would restrict penetration through mucus and limit access of the bacteria to the epithelium surface. In addition, MUB from *L. reuteri* ATCC 53608 was recently shown to modulate inflammatory responses in human monocyte-derived dendritic cells *via* interaction with DC-SIGN (Bene *et al.*, 2017). The presence of mucus adhesins was also shown to mediate the binding of *L. reuteri* strains to both HT-29 and mucus-producing LS174T cells. The binding of *L. reuteri* to mucus led to a decreased enteropathogenic *E. coli* (EPEC) adherence to small intestinal biopsy epithelium (Walsham *et al.*, 2016). Recombinant Mub proteins containing

303 Mubs5s6 domains from Lp-1643 protein of *L. plantarum* Lp9 have been shown to adhere to
304 human intestinal tissue sections (Singh *et al.*, 2017) and inhibited the adhesion of
305 enterotoxigenic *E. coli* (ETEC) to cultured intestinal HT-29 and Caco-2 cell lines, probably
306 through the recognition of cell-surface mucins (Singh *et al.*, 2018). Together, these findings
307 show that the nature and function of these adhesins are strain-specific with the potential to
308 target either the epithelium or the mucus layer and compete with pathogens.
309
310 ***Flagella.*** Several microorganisms have evolved strategies, in particular extracellular
311 appendages such as flagella, pili and fimbriae, to attach to and to penetrate the mucus layer
312 (Juge, 2012). Pili and flagella are large polymeric proteins that form long surface structures
313 involved in bacterial adhesion. Flagella are composed of several thousand copies of flagellin
314 subunits and have been extensively studied in EPEC and enterohemorrhagic *E. coli* (EHEC)
315 for their role in virulence and motility, but their role in mucus binding remains unclear. The
316 adhesive properties of bacterial flagella to mucus were previously reported for *Clostridium*
317 *difficile* where crude flagella, recombinant flagellar FliC and FliD proteins were shown to
318 bind to murine mucus (Tasteyre *et al.*, 2001). In pathogenic *E. coli* strains, the H6 and H7
319 flagella EPEC E2348/69 and EHEC EDL933 and their flagellin monomers were shown to
320 bind to mucins and to bovine mucus (Erdem *et al.*, 2007). Further studies then showed that
321 EPEC and EHEC O157:H7 adherence to HT-29 cells is related to mucin-type core 2 *O*-
322 glycan, facilitating invasion into host cells (Ye *et al.*, 2015, Ye *et al.*, 2015). However,
323 flagella are involved in the ability of these pathogenic strains to cross the mucus layer,
324 conferring a selective advantage in penetrating the mucus layers and reaching the epithelial
325 surface, as demonstrated with Adherent-Invasive *E. coli* (AIEC) LF82 (Khodzhakuliev &
326 Ovezova, 1986). It is therefore tempting to speculate that in EPEC and EHEC, the flagella
327 have a preference for cell-surface mucins rather than secreted mucus, in line with their ability

to penetrate the mucus layer and attach onto the cell surface before invasion. In the probiotic *E. coli* strain Nissle 1917, a direct interaction was observed between isolated flagella from EcN and porcine MUC2 and human mucus but not murine mucus. The mucus component gluconate was identified as one receptor for the binding of EcN flagella (Troge *et al.*, 2012). EcN was therefore proposed to confer the probiotic strain the ability to compete for binding sites on host tissue with bacterial pathogens.

Pili. Pili have been identified in *Lactobacillus rhamnosus* GG where they confer binding to mucus (Kankainen *et al.*, 2009, von Ossowski *et al.*, 2011) and are predicted to exist in other *Lactobacillus* species including *L. casei* and *L. paracasei*, based on genomics analyses (Douillard *et al.*, 2013, Aleksandrzak-Piekarczyk *et al.*, 2015, Nissila *et al.*, 2017). In *L. rhamnosus* GG, these are composed of a three-protein complex SpaCBA, which has been involved in adhesion to mucus, IECs, and immunomodulatory interactions with IEC (Lebeer *et al.*, 2012, von Ossowski *et al.*, 2013, Ganguli *et al.*, 2015, Vargas Garcia *et al.*, 2015, Bene *et al.*, 2017). The mucus-binding pili of *L. rhamnosus* GG shares immunological and functional similarities with those of the clinical *Enterococcus faecium* strain E1165. The binding of *E. faecium* E1165 to mucus could be prevented by the addition of the mucus-binding SpaC protein or antibodies against *L. rhamnosus* GG (Tytgat *et al.*, 2016). Collectively, these studies show the potential of using mucus adhesins from probiotic strains to prevent the binding of enteric pathogens to the host.

Although not a resident member of the gut microbiota, several *Lactococcus lactis* strains have also been shown to exhibit mucus-binding properties through bacterial surface proteins such as mucin-binding proteins and pili (as recently reviewed in (Mercier-Bonin & Chapot-Chartier, 2017)). The mechanisms of adhesion have been extensively studied by atomic force spectroscopy demonstrating a comparable role played by these two surface

353 proteinaceous components in adhesion of *L. lactis* TIL448 to pig gastric mucin (PGM) neutral
354 oligosaccharides under static conditions, whereas a more important contribution of the MUBs
355 than the pili one was observed under shear flow (Le *et al.*, 2013).
356
357 ***Other cell surface proteins***. Other cell surface proteins implicated in the binding of
358 commensal bacteria to mucin include aggregation-promoting factors (APFs) from *L.*
359 *plantarum* NCIMB 8826 (Bolonkin, 1990) or *L. lactis* (Lukic et al., 2012, Lukic et al., 2014),
360 mucus-binding protein A (CmbA) from *L. reuteri* ATCC PTA 6475 (Etzold et al., 2014,
361 Jensen et al., 2014), Lam29 from *L. mucosae* ME-340 (Watanabe *et al.*, 2010), mucus
362 adhesion-promoting protein (MapA) from *L. fermentum/reuteri* 104R (Rojas *et al.*, 2002) , a
363 mucus-binding factor (MBF) from *L. rhamnosus* GG (von Ossowski *et al.*, 2011, Nishiyama
364 *et al.*, 2015), a MucBP-containing mannose-specific adhesin protein (Msa) from *L. plantarum*
365 WCFS-1 (Pretzer *et al.*, 2005), a 32-Mmubp from *L. fermentum* BCS87 (Macias-Rodriguez *et*
366 *al.*, 2009), an extracellular transaldolase (Tal) from *Bifidobacterium bifidum* DSM20456
367 (Gonzalez-Rodriguez *et al.*, 2012) and a recently-characterised serine rich repeat protein
368 (SRRP) from *L. reuteri* ATCC 53608 (Sequeira *et al.*, 2018). It is expected that adhesion of
369 these commensal or probiotic bacteria to mucus may favour their persistence within the gut in
370 order to exert their beneficial effects to the host. Furthermore, it was recently suggested that
371 carbohydrate binding modules (CBMs) appended to glycoside hydrolases could contribute to
372 the tropism of gut bacteria to glycan-rich area of mucins in the colon, as shown for
373 *Ruminococcus gnavus* sialic-acid-specific CBM40 (Owen *et al.*, 2017).
374
375 ***Blood group binding adhesins***. In addition, several human enteric pathogens bind to human
376 histo-blood group antigens (HBGAs) expressed on the gut mucosa, including *Campylobacter*
377 *jejuni*, *Norwalk virus* and *H. pylori*. The role of HBGA recognition to mucin binding has been

extensively studied in the gastric pathogen *H. pylori* where *Helicobacter* adhesins have been reported to play a critical role in the attachment of the pathogen to both the glycosylated gastric epithelial cell surface and to glycosylated mucins. The binding of *H. pylori* to gastric mucins through blood group binding adhesin (BabA) and sialic acid-binding adhesin (SabA) revealed a complex charge/low pH-dependent mechanism involving four modes of *H. pylori* adhesion to MUC5B, MUC7, and MUC5AC mucins (Linden *et al.*, 2008, Skoog *et al.*, 2017). More recently, a novel outer membrane protein adhesin named LabA has been identified in *H. pylori* and shown to bind to LacdiNAc, a structure, which is also expressed on MUC5AC (Rossez *et al.*, 2014). Binding of *H. pylori* to gastric mucins therefore is determined both by the mucin glycosylation and also by the adhesins expressed by individual strains. A chitin-binding protein GbpA from *Vibrio cholerae* shown to bind to N-acetyl-D-glucosamine residues of intestinal mucin has been proposed as an important factor mediating intestinal colonisation and pathogenesis by *V. cholerae* (Bhowmick *et al.*, 2008, Wong *et al.*, 2012).

Moonlighting proteins. Unexpectedly, several primarily cytoplasmic proteins have been reported to play a role in mucin binding. Due to their dual function, these proteins are referred to as moonlighting proteins (Henderson & Martin, 2011, Henderson & Martin, 2013, Henderson, 2014). In *L. acidophilus*, *L. plantarum* and *Mycoplasma genitalium* for instance, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was clearly demonstrated to play a role in bacterial adhesion and bind mucins (Alvarez *et al.*, 2003, Kinoshita *et al.*, 2008, Patel *et al.*, 2016). While the exact domain responsible for mucin binding remains to be elucidated, GAPDH is suggested to play a similar role in other commensal or pathogenic microorganisms (Kinoshita *et al.*, 2013). In *L. reuteri*, elongation factor-Tu (EF-Tu) was found to bind the PGM when exposed at the bacterial cell surface (Nishiyama *et al.*, 2013). Here, the sulfated carbohydrate moieties of mucins were demonstrated to play a significant role in EF-Tu-

mediated bacterial adhesion to PGM and mucosal surfaces (Nishiyama *et al.*, 2013). Proteosurfaceome analyses in a range of microorganisms have revealed a large repertoire of cytoplasmic proteins present at the bacterial cell surface but their implications in binding to various extracellular matrix (ECM) proteins, including mucins, remain to be more systematically investigated (Chagnot *et al.*, 2012, Desvaux *et al.*, 2018).

2.2. Mechanisms of mucin degradation by commensal and pathogenic microorganisms in the gut

Several enzymatic activities are required for the degradation of mucins by pathogens or commensal bacteria including glycoside hydrolases (GHs), sulfatases, or proteases (Fig. 1) as described below.

Glycoside hydrolases. Mucin glycan degradation in bacteria relies on the expression of GHs such as sialidases (GH33), α-fucosidases (GH29, GH95), exo- and endo-β-N-acetylglucosaminidases (GH84 and GH85), β-galactosidases (GH2, GH20, GH42), α-N-acetylglucosaminidases (GH89), endo-β1,4-galactosidases (GH98) and α-N-acetylgalactosaminidases (GH101, GH129) (www.cazy.org). These enzymes have been functionally characterised in resident members of the gut microbiota able to forage on mucins, including *A. muciniphila*, *B. thetaiotaomicron*, *B. bifidum, B. fragilis*, and *R. gnavus*, as recently reviewed (Tailford *et al.*, 2015, Ndeh & Gilbert, 2018). The released mono- or oligosaccharides derived from mucus degradation by these commensal bacteria can be utilised by the bacteria itself or scavenged by other bacteria inhabiting the mucus niche including pathogenic species such as *Salmonella* species*, C. difficile*, diarrhoeagenic *E. coli*, or *Vibrio cholerae* through cross-feeding interactions (Fabich *et al.*, 2008, Abyzov *et al.*, 2012, Ng *et*

al., 2013). In addition, some of these pathogens have the glycolytic potential to release mucus-derived sugars for their own consumption (Mondal *et al.*, 2014, Arabyan *et al.*, 2016).

Sulfatases. Sulfatases are being increasingly investigated for their role in modulating the gut microbial ecosystem in health and disease. Some members of the gut microbiota such as *B. thetaiotaomicron*, *Bacteroides ovatus*, and Prevotella sp. strain RS2 *Bifidobacterium breve* UCC2003, or *B. fragilis* possess mucin- desulfating sulfatases or glycosulfatases (Salyers *et al.*, 1977, Berteau *et al.*, 2006, Benjdia *et al.*, 2011, Egan *et al.*, 2016, Praharaj *et al.*, 2018). Mucin sulfatase activity of these species may provide them a competitive advantage in the infant gut and/or the adult gut. The mucin- desulfating sulfatases that have been characterised so far include sulfatases specific for the - D- galactopyranosyl 3- sulfate, - Dgalactopyranosyl6- sulfate, and 2- acetamido- 2- deoxy- D- glucopyranosyl6- sulfate (6- SO3- GlcNAc) building blocks of the oligosaccharide chains. GlcNAc-6-S can be found in terminal or branched positions of mucin oligosaccharide. The desulfation of mucin by bacterial sulfatases may be a rate-limiting step in mucin degradation mechanism, allowing glycosidases to access and act on the mucins by other members of the gut microbiota. The release of sulfate from mucins may also contribute to the expansion of Sulfate-reducing bacteria (SRB) in the gut (Rey *et al.*, 2013). SRB are able to produce hydrogen sulfide (H_2S) which can reduce disulfide bonds present in the mucus network, leading to mucus erosion and access of bacteria to the epithelium, therefore contributing to epithelial damage and inflammation This mechanism has been proposed to be involved in the aetiology and/or severity of IBD (Ijssennagger *et al.*, 2016). In addition, Hickey and colleagues showed that sulfatases of *B. thetaiotaomicron* are required for its outer membrane vesicles to transit to underlying host immune cells and cause colitis (Chatzidaki-Livanis & Comstock, 2015). Together these data highlight the complex role of bacterial sulfatases in the gut.

453

454 ***Proteases***. Bacterial proteases from commensal or pathogenic *E. coli* have also been
455 implicated in the recognition and degradation of mucins. In EHEC, StcE (secreted protease of
456 C1 esterase inhibitor from EHEC) was originally described as specifically cleaving C1
457 esterase inhibitor (C1-INH) (Lathem *et al.*, 2002, Grys *et al.*, 2006) but later showed to be
458 even more active against MUC7 (Lathem *et al.*, 2002). This soluble enzyme is important in
459 reducing mucin levels. StcE has been suggested to have a dual role during human infection,
460 (i) by promoting the penetration of bacterial cells through the mucus barrier lining the GI tract
461 and thus facilitating the intimate EHEC adherence to IECs, which is an essential step in
462 colonisation (Hews *et al.*, 2017), and (ii) by acting as an anti-inflammatory agent protecting
463 bacterial and host cell surfaces from complement-mediated lysis (Grys *et al.*, 2005, Abreu &
464 Barbosa, 2017). StcE is secreted by a Type II, subtype a, secretion system (T2aSS) (Monteiro
465 *et al.*, 2016, Hay *et al.*, 2018). This mucinase is a metalloprotease belonging to the peptidase
466 M66 family (IPR019503) carrying one zinc atom per protein but no structural calcium, which
467 is a reported feature of metalloproteases (Yu *et al.*, 2012). Recently, EHEC StcE
468 metalloprotease was shown to reduce the inner mucus layer in human colonic mucosal
469 biopsies and the MUC2 glycoprotein levels in mucin-producing LS174T colon carcinoma
470 cells (Hews *et al.*, 2017).

471 Pic (protein involved in intestinal colonisation), also previously known as Shmu
472 (*Shigella* mucinase), is a secreted protease identified in *Shigella flexneri* and
473 enteroaggregative *E. coli* (EAEC) (Henderson *et al.*, 1999). Pic is secreted by a Type V,
474 subtype a, secretion system (T5aSS) and belongs to the subfamily of serine protease
475 autotransporters (SPATEs), with a catalytic domain corresponding to the peptidase S6 family
476 (IPR030396). This enzyme was reported to display proteolytic activity against gelatin as well

as bovine and murine mucin but not hog gastric mucin (Henderson *et al.*, 1999). PicU was also shown to exhibit mucinolytic activity in uropathogenic *E. coli* (Parham *et al.*, 2004).

Hbp (hemoglobin-binding protease), also previously known as Tsh (temperature-sensitive haem-agglutinin), is capable of cleaving bovine submaxillary mucin but not hog gastric mucin, which so far would appear as a feature of mucinolytic serine protease autotransporter of Enterobacteriaceae (SPATE) of the peptidase S6 family (Dutta *et al.*, 2002). In some EHEC strains, a SPATE of the peptidase S6 family exhibiting mucinolytic activity was identified on plasmid pO113, namely EpeA (EHEC plasmid-encoded autotransporter) (Leyton *et al.*, 2003). In AIEC, a Vat (vacuolating autotransporter) homologue belonging to the SPATE of the peptidase S6 family was demonstrated to exhibit a mucinolytic activity (Gibold *et al.*, 2016). Vat-AIEC appears to significantly contribute to the colonisation ability of AIEC by decreasing mucus viscosity as well as enhancing bacterial penetration in mucus and access to IECs (Gibold *et al.*, 2016).

In some non-O157 EHEC strains, a subtilase cytotoxin (SubAB) was identified (Paton *et al.*, 2006, Wang *et al.*, 2007) and appeared to contribute to mucin depletion as shown with a Shiga-toxin encoding *E. coli* (STEC) O113:H21 strain (Gerhardt *et al.*, 2013). While the A subunit harbours the enzymatic activity with a subtilase-like serine protease domain belonging to the peptidase S8/S53 family (IPR000209), the mucinolytic activity of SubAB remains to be clearly established.

Other proteins have been described in *V. cholerae*. Among them, TagA is a secreted protease of *V. cholerae* that specifically cleaves mucin glycoproteins (Szabady *et al.*, 2011). The *V. cholerae* extracellular chitinase ChiA2 secreted in the intestine hydrolyzes intestinal mucin to release GlcNAc, and the released sugar is successfully utilized by *V. cholerae* for growth and survival in the host intestine (Mondal *et al.*, 2014).

SslE (secreted and surface associated lipoprotein), previously known as YghJ, is a secreted and cell-surface lipoprotein degrading the major mucins in the small intestine, namely MUC2 and MUC3, thus facilitating bacterial penetration of the mucus layer and ultimately adhesion to host cells (Luo *et al.*, 2014, Valeri *et al.*, 2015, Tapader *et al.*, 2017). SslE is secreted via a T2aSS and appears inactive against the mucin-like CD43, bovine submaxillary mucin, gelatin, or IgG (Luo *et al.*, 2014). This Zn-metalloprotease, belonging to the peptidase M60 family (IPR031161), is found in pathogenic and commensal *E. coli*, including ETEC, EHEC O104:H4, *E. coli* SE-11 or Nissle 1917 strains. AcfD (accessory colonisation factor D) from *V. cholerae* is homologous to SslE but its putative mucinolytic activity remains to be investigated (Peterson & Mekalanos, 1988). Of note, SslE is also considered as a relevant target for the development of vaccines against intestinal pathogenic *E. coli* (Nesta *et al.*, 2014, Naili *et al.*, 2016, Naili *et al.*, 2017).

3. ***Importance of mucus-bacteria interactions in health and disease***

In the colon, the outer mucus layer offers a niche to commensal bacteria by providing preferential binding sites (Section 2.1) and nutrients (Section 2.2). Due to its proximity to host cells and the immune system, the mucus-associated microbiota, sometimes also referred to as the mucobiome (Belzer *et al.*, 2017), has been proposed as an important modulator of health. The integrity of the mucosa relies on a combination of factors including the gut microbiota composition, the diet and host genetic factors (Fig. 2) (Martens *et al.*, 2018). The mucus and mucus-associated bacterial community play a key role in limiting access of invading pathogens to the underlying epithelial cells and in limiting the progression of intestinal and extra-intestinal diseases (Donaldson *et al.*, 2016).

Effect of bacteria and bacterial products on mucus production. A number of animal studies (using antibiotic-treated, germ-free or gnotobiotic mice) suggest that the presence of bacteria triggers the development of the protective mucus layer. Mice treated with the antibiotic metronidazole, but not streptomycin, display an altered goblet cell function and thinning of the inner mucus layer (Wlodarska *et al.*, 2011). However, another study reported that depletion of the intestinal microbiota following a three week-antibiotic period (cocktail of four antibiotics) did not modify mucus penetrability (Johansson *et al.*, 2015).

Compared to conventionally housed animals, germ-free mice have fewer goblet cells, which are smaller in size (Kandori *et al.*, 1996) and harbour an impaired mucus layer, indicating that the formation of the protective mucus layer depends upon the presence of bacteria (Rodriguez-Pineiro & Johansson, 2015). Johansson and colleagues demonstrated that the mucus of germ-free mice displayed a significant decrease in Muc2 level and was more penetrable to bacterium-size fluorescent beads as compared to conventionally raised mice (Johansson *et al.*, 2015). The gut microbiota composition of germ free animals is normalized two weeks after colonisation in terms of microbiota composition, but up to 8 weeks are needed to reach a normalized mucus phenotype (Johansson *et al.*, 2015, Hayes *et al.*, 2018). In support of this, fortification of the mucus layer and increased diversity of mucin glycosylation was observed within 48 hours of human intestinal organoid colonization with human-derived, non-pathogenic *E. coli* (Hill *et al.*, 2017). Some bacteria, in particular Anaerostipes, have been shown to display mucus-stimulating properties (Jakobsson *et al.*, 2015). *Lactobacillus* species can also stimulate MUC2 production and secretion by the goblet cells in the human gut (Sicard *et al.*, 2017). Representative members of the two main phyla of the gut microbiota, *B. thetaiotaomicron* and *Faecalibacterium prauznitzii* can modulate goblet cell differentiation and thus mucus production (Wrzosek *et al.*, 2013). A recent study showed that *Streptococcus thermophilus*, a transient food-borne bacterium, was able to induce mucus

pathway in gnotobiotic rodents despite its poor capacity for mucus adhesion and mucin glycan degradation *in vitro* (Fernandez *et al.*, 2018). Some of the mechanisms mediating mucin production and secretion by gut bacteria have been elucidated as described below.

Pathogen associated molecular patterns such as lipopolysaccharide (LPS) or peptidoglycan are known to induce mucus production (Petersson *et al.*, 2011). LPS and flagellin purified from Gram-negative bacteria as well as lipoteichoic acid from Gram-positive bacteria have been shown to induce mucin upregulation via the Ras-signalling pathway (McNamara & Basbaum, 2001). LPS also increases the production of interleukin (IL)-8 by goblet cells, which further promotes mucin secretion (Smirnova *et al.*, 2003). TLR family members play an important role in mucus formation. Mice lacking the TLR adaptor protein MyD88 show a decreased production of mucus (Bhinder *et al.*, 2014). Mice engineered to lack the flagellin receptor, TLR5 deficient mice, have a disorganised mucus layer and lack a well-defined inner layer when compared to wild type animals with an increase abundance of Proteobacteria in close contact with the epithelial surfaces (Carvalho *et al.*, 2012, Chassaing *et al.*, 2014, Chassaing *et al.*, 2015). Lastly, it has been shown in vitro using various human-derived cell lines that bacterial metabolites such as short-chain fatty acids (SCFA) and especially butyrate can stimulate MUC2 production in the absence of other energy sources (Willemsen *et al.*, 2003, Gaudier *et al.*, 2004). The effect of butyrate on MUC2 gene expression is mediated by epigenetic modifications (acetylation/methylation of histones) on the MUC2 promoter as demonstrated *in vitro* using human goblet cell-like LS174T cells (Burger-van Paassen *et al.*, 2009). Fernandez and colleagues suggested that lactate produced by *S. thermophilus* in the GI tract could stimulate mucus production via a signalling pathway dependent of KLF4, a transcription factor involved in the differentiation of goblet cells (Fernandez *et al.*, 2018). Some other bacterial effectors have been identified to

mediate mucin expression and glycosylation such as small peptides from *R. gnavus* and *B. thetaiotaomicron* (see section 2).

Interactions of pathogens with mucus. The mucus barrier provides a bulwark against intestinal pathogens (Johansson *et al.*, 2013, Sicard *et al.*, 2017, Martens *et al.*, 2018). The importance of intestinal mucus in controlling enteric infection has been widely documented in *Muc2* knockout mice (*Muc2*$^{-/-}$ mice) (see section 4.5), which do not produce mucus in the small and large intestine, thus leading to a close contact between bacteria and the epithelium. Bergstrom and colleagues reported that *Muc2*$^{-/-}$ mice exhibit an increase susceptibility to murine bacterial pathogen *Citrobacter rodentium* (Bergstrom *et al.*, 2010). Likewise, Muc2 plays a crucial role in controlling *Salmonella* infection (Zarepour *et al.*, 2013). In a similar way, *H. pylori* has evolved mechanisms allowing its residence in the gastric mucus layer (Moore *et al.*, 2011). As previously described for bacteria, *Muc2*$^{-/-}$ mice are also more susceptible to enteric parasitic infection with *Trichuris muris* since they exhibit a delayed expulsion of the parasite compared to wild type animals (Hasnain *et al.*, 2010). Clearance of parasitic infection is associated with exclusion of helminths via a TH2 cell-mediated goblet cell increase and mucus release (Artis & Grencis, 2008). *Entamoeaba histolytica* also possesses lectins binding to mucins and secretes proteases responsible for the cleavage of Muc2, allowing the protozoan to invade the underlying epithelium (Lidell *et al.*, 2006). Recently, a detailed investigation of the cooperative roles for colonic microbiota and Muc2 in mediating innate host defence against *E. histolytica* was carried out using *Muc2*$^{-/-}$ mice, germ free mice and mucus-secreting LS174T cells, demonstrating that mucus secretion and pro-inflammatory responses were microbiota-specific (Leon-Coria *et al.*, 2018). Lastly, as shown with *S. flexneri* and *H. pylori*, some pathogenic bacteria are able to reshape mucin structures

by remodelling their glycosylation pattern in a type III secretion system-dependent manner (Sperandio *et al.*, 2013, Magalhaes *et al.*, 2015).

Emerging data suggest that pathogenic bacteria can benefit from the capacity of commensal microorganisms to release mucin degradation products that can be used to support their proliferation within the mucus niche. For example, *B. thetaiotaomicron* can release free sialic acid from colonic mucus glycans that can be utilized by *C. difficile* and *Salmonella* Typhimurium to promote their own colonisation and persistence in the gut (Ng *et al.*, 2013). Another study indicates that EHEC bacteria colonise the mucus layer within the cooperation of local bacterial communities including *B. thetaiotaomicron* and other anaerobes which are able to cleave host glycan- derived sugar and produce fucose (Pacheco *et al.*, 2012). EHEC then senses fucose produced by *B. thetaiotaomicron* to control expression of its type III secretion system (Pacheco *et al.*, 2012, Cameron *et al.*, 2018).

Mucus-pathogen interactions have also been evidenced in the extra-digestive area. *Pseudomonas aeruginosa*, a Gram-negative flagellated pathogen, is the main causal agent for the development of pneumonia in immunocompromised patients and patients with cystic fibrosis (CF). This infection is associated with a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride and bicarbonate ion channel protein with a key role in protecting the small intestine from bacterial invasion. CF conducts to blockage airway, mucus hypersecretion leading to chronic bacterial lung infections and inflammation. It has been shown that *P. aeruginosa*, via LPS, upregulate MUC2 and MUC5AC gene expression contributing to the excessive mucus production and airway blockage seen in CF (Bellu *et al.*, 2013).

Effect of diet on mucus. Recent evidences have demonstrated that the diet can influence the properties of colonic mucus and thereafter interfere with the gut microbiota.

Fibres. Living in symbiosis with the host, the gut microbiota depends mostly on non-digestible fibres and polysaccharides as energy source. In the absence of fibres in the diet, the gut microbiota shifts towards the utilisation of host glycans such as those provided by mucins, resulting in a thinner protective colonic mucus (Sonnenburg & Sonnenburg, 2014, Earle *et al.*, 2015). Accordingly, Desai and colleagues demonstrated that a low-fibre diet promotes the enrichment of mucin-degrading bacteria and the overexpression of carbohydrate-active enzymes (CAZymes) that degrade the colonic mucus barrier (Desai *et al.*, 2016, Martens *et al.*, 2018). In these mice fed with a deprived-fibre diet, infection with *C. rodentium* promotes greater epithelial access and lethal colitis (Desai *et al.*, 2016).

A lack of fermentable fibres in the diet also leads to a reduction in epithelial cell proliferation resulting in a thin mucosa with encroached bacteria (Chassaing *et al.*, 2015). Further studies in mice reported that inulin supplementation increases the number of colonic goblet cells, which correlates with a thicker mucus layer and an increase proportion of the *Akkermansia* genus (Kleessen *et al.*, 2003, Everard *et al.*, 2013). Similarly, studies in mice showed that a diet enriched in inulin fibre prevents mucus deterioration (Schroeder *et al.*, 2018). It was recently showed that inulin but not cellulose protects against diet-induced obesity by reducing microbiota encroachment in a cytokine IL-22-dependent manner (Zou *et al.*, 2018), demonstrating the importance of dietary factors, especially soluble fibre, in the homeostasis of host-microbiota relationship. Considering the increased mucus foraging activity occurring when mice are fed with a low-fibre diet, a recent study showed that supplementation with probiotic bifidobacteria (*B. longum*) or prebiotic fibre (inulin) could reduce such mucus defect. Notably, administration with *B. longum* was sufficient to restore mucus growth, while administration with inulin could prevent the increase of mucus penetrability in mice fed a western style diet (WSD) (Schroeder *et al.*, 2018).

Western diet and food additives. Besides fibres, other nutrients within a WSD can modulate intestinal barrier function. A WSD is a rich in saturated fats and simple carbohydrates but depleted in dietary fibres. As a result, a diet-induced obesity in mice leads to colon mucosal barrier dysfunction with a thinner mucus layer (as described above) and treatment with *A. muciniphila* appears to counteract this effect by improving mucus thickness (Everard *et al.*, 2013). Similarly, mice fed a high-fat and high-sugar diet exhibit an increased abundance of mucin-degrading species leading to a decrease in mucus thickness (Martinez-Medina *et al.*, 2014). The diet of modern societies has dramatically changed as evidenced by a steady increase in the consumption of processed foods concomitantly with an increase in the use of food additives (Chassaing *et al.*, 2015). Mice treated with dietary emulsifiers (polysorbate 80 or carboxymethylcellulose) show a reduced mucus thickness and increased gut permeability. In these animals, some bacteria appear in close contact with the epithelium. Emulsifier-treated mice have an altered microbial composition associated with increased levels of mucolytic bacteria including *R. gnavus* and a marked reduction in microbial diversity, with a bloom in *Verrucomicrobia* phyla, especially *A. muciniphila* (Chassaing *et al.*, 2015). This may further contribute to the intestinal passage of bacterial constituents such as LPS and flagellin, which participates in the development of low-grade inflammation and metabolic disorders in wild type mice and of colitis in susceptible host animals (Chassaing *et al.*, 2014, Chassaing *et al.*, 2015, Chassaing *et al.*, 2017). Mice fed with diets enriched in maltodextrin, a filler and thickener used in food processing, show a reduction of *Muc2* expression, making the host more sensitive to low-grade inflammation but with no significant change in mucosa-associated microbiota (Laudisi *et al.*, 2018).

Effect of food contaminants on mucus. The intestinal mucosa is increasingly appreciated as a key player in the emerging field of gut toxicology of environmental pollutants, as recently

674 reviewed (Gillois *et al.*, 2018). Human contamination mainly occurs via the oral route through
675 consumption of food but also through polluted water and soil exposure.

676 ***Nanomaterials***. The use of nanotechnology in many common consumer products,
677 especially in food products, is growing. Scarce studies have evaluated the interactions of food
678 nanoparticles with the microbiota and mucus (Mercier-Bonin *et al.*, 2018). It was shown *in*
679 *vitro* that common nanoparticles of Titanium dioxide (TiO_2) are trapped into mucus, leading
680 to areas with a high local concentration (Talbot *et al.*, 2018). Silver nanoparticles are widely
681 used in food industry to colour the surface of confectionary and pastries. Rats fed with these
682 particles exhibit higher numbers of goblet cells and a modification of the glycosylation
683 pattern of mucins with a decreased proportion of sulfated mucins and an increased proportion
684 of sialyated mucins (Jeong *et al.*, 2010). Repeated silver nanoparticle-exposure may therefore
685 produce pathological regions in the lamina propria (Jeong *et al.*, 2010).

686 ***Persistent Organic Pollutants.*** A recent study showed that mice chronically exposed
687 to benzo[a]pyrene (BaP) which is the most toxic member of the polycyclic aromatic
688 hydrocarbons family display significant shifts in the composition and relative abundance of
689 stool and mucosa-associated bacterial communities (decrease of *Verrucomicrobiaceae*,
690 represented by *A. muciniphila*) (Ribiere *et al.*, 2016). Furthermore, exposure to
691 perfluorooctane sulfonate (PFOS, environmental contaminant used as a surfactant and
692 repellent) in a mouse model of *C. rodentium* infection led to a significant reduction in mucin
693 gene expression and a failure to clear the bacterial infection (Suo *et al.*, 2017). Smoke
694 exposure also significantly affects the mucosa-associated bacterial community and alters the
695 expression of mucins in the murine gut (Allais *et al.*, 2016).

696

697 ***Mucus and inflammatory related diseases***.

Inflammatory bowel diseases (IBD). Barrier disturbances including alterations in the thickness or composition of the intestinal mucus layer are recognized to play a crucial role in the onset of GI disorders such as Crohn disease (CD) or ulcerative colitis (UC). The mucus layer in UC patients is thinner and has an altered glycosylation profile making it more penetrable to bacteria (Johansson *et al.*, 2014). To better understand the onset of IBD, several murine models of intestinal inflammation (genetically or chemically induced) have been established. The most common experimental model of colitis relies on the administration of Dextran Sodium Sulfate (DSS) in the drinking water. Mice orally administered with DSS display an inner mucus layer which is more penetrable by bacteria within 12 hours (Johansson *et al.*, 2010). Similarly, *IL-10*$^{-/-}$ and *TLR5*$^{-/-}$ mice that develop spontaneous colitis have a thicker mucus layer and more penetrable inner mucus layer when compared to wild type animals (Johansson *et al.*, 2014). *Muc2*$^{-/-}$ mice develop intestinal inflammation with diarrhoea, rectal bleeding and prolapse (Johansson *et al.*, 2008) and are more susceptible to DSS-induced colitis; these animals exhibit a massive number of bacteria in close contact with host tissues, further promoting inflammation (Van der Sluis *et al.*, 2006). Moreover, abnormal mucin *O*-glycosylation has been associated with an increased inflammation, highlighting the importance of mucin glycans in the maintenance of gut homeostasis (Johansson *et al.*, 2014) (Bergstrom & Xia, 2013, Bergstrom *et al.*, 2016).

These changes in mucus composition were also mirrored by changes in the gut microbiota composition at the mucosal surface. IBD patients exhibit a disproportion of mucin-degrading (or mucinolytic) bacteria with an increased abundance of *Ruminococcus torques* and *R. gnavus*, but a decreased abundance of *A. muciniphila*. In addition, the expansion of certain pathobionts and in particular AIEC exhibiting mucinolytic activity has been reported to favour gut colonisation and further induce inflammation in CD (Palmela *et al.*, 2018). Taken together, these data suggest that mucus-bacteria interactions contribute to

723 the intestinal barrier dysfunction in IBD patients and future work is needed to better
724 understand the influence or consequence of these interactions on the disease.
725
726 ***Obesity and metabolic-related disorders.*** A correlation between adiposity,
727 dysglycemia and microbiota encroachment has been reported in a number of animal studies.
728 *Muc2*$^{-/-}$ mice fed a High Fat Diet (HFD) are protected from diet-induced weight gain, fatty
729 liver, and insulin resistance as they displayed less inflammation and increased systemic levels
730 of IL-22 (Hartmann *et al.*, 2016). This study supports a role of Muc2 during obesity and
731 highlights the importance of the crosstalk between microbiota, mucus and immune mediators.
732 In mice fed a HFD, mucus secretion is altered in the ileum but not in the duodenum and
733 jejunum, largely in response to an alteration of PPAR-γ signalling. In these mice, Muc2
734 accumulates at the apical side of goblet cells, leading to a reduction in the expansion capacity
735 of the mucins, thus strongly altering the phenotype of the mucus layer (Tomas *et al.*, 2016).
736 Studies by Chassaing and colleagues in different mouse models of metabolic syndrome and in
737 humans demonstrated that bacteria have the ability to infiltrate the mucus layer and reach the
738 epithelium (Chassaing *et al.*, 2014, Chassaing *et al.*, 2015, Chassaing *et al.*, 2017). Further,
739 measurement of bacterial-epithelial distance reveals that microbiota encroachment is a feature
740 of insulin resistance-associated dysglycemia in humans that may promote inflammation
741 (Chassaing *et al.*, 2017). Several studies demonstrated that *A. muciniphila* is less abundant in
742 the intestinal microbiota of both genetic and diet-induced obese and diabetic mice, as well as
743 in individuals with obesity, when compared to the faecal microbial population of healthy
744 individuals (Everard *et al.*, 2013, Shin *et al.*, 2014). *A. muciniphila* treatment has been shown
745 to reverse fat gain, serum LPS levels, gut barrier function, and insulin resistance. In addition,
746 oral administration of an outer-membrane protein from *A. muciniphila* led to reduced fat mass
747 and metabolic syndrome in mice fed an obesity induced diet (Plovier *et al.*, 2017).

748 Conversely, anti-diabetic treatments such as metformin administration led to an increase in
749 the *Akkermansia* spp. population (Shin *et al.*, 2014).

750 Human studies have shown that alcohol abuse induced alcoholic liver diseases (ALD)
751 are associated with an increase in intestinal mucus thickness in patients, using wheat germ
752 agglutinin staining on duodenal biopsies (Hartmann *et al.*, 2013). Animal studies
753 demonstrated that when compared to wild type animals, *Muc-2*$^{-/-}$ mice are protected from
754 alcoholic steatohepatitis in an experimental alcohol-induced liver disease model (Hartmann *et*
755 *al.*, 2013). In addition, *Muc-2*$^{-/-}$ mice are protected from Non Alcoholic Fatty Liver Disease
756 (NAFLD) when fed a high-fat diet inducing liver steatosis (Hartmann *et al.*, 2016).
757 Altogether, these data highlight the role of mucus and mucins in the gut-liver axis.

758

759 ***Cancer.*** The role of mucins in cancer progression has been extensively reviewed
760 (Hollingsworth & Swanson, 2004, Kufe, 2009). *Muc2*$^{-/-}$ mice displayed spontaneous
761 development of adenomas in the small intestine that progressed to invasive adenocarcinoma,
762 as well as rectal tumours (Velcich *et al.*, 2002). In humans, high levels of expression of
763 MUC2 by pancreatic and biliary tumours has been associated with a low degree of
764 invasiveness, malignancy and a better prognosis as compared to tumours not expressing
765 MUC2 (Hollingsworth & Swanson, 2004). An abnormal mucin *O*-glycosylation has been
766 associated with an increased inflammation that could contribute to the development of colitis-
767 associated colon cancer in mice (Bergstrom & Xia, 2013, Bergstrom *et al.*, 2016). Together
768 these studies support the role of MUC2 as a tumour suppressor.

769

770 **4. Potential of experimental models to study mucus/mucin interactions with gut**
771 **microbes**

772

As mounting evidences highlight the importance of mucus in the cross-talk between the gut microbiota and the host, a wide range of experimental models has been developed to study mucus-bacteria interactions (Table 1). These include the use of purified mucins, mucin-secreting cells or tissues, or mucin-containing fermentation models, as described below.

4.1. *In vitro* mucus/mucin binding assays

Microplate assays. Several microtiter plate assays have been developed for testing bacterial adhesion to mucus and/or mucin (McNamara *et al.*, 2000, Gusils *et al.*, 2004). These generally rely on the immobilisation of mucins or mucus to the wells of microtiter plates following incubation overnight at 4°C or at room temperature in buffers such as such as PBS (pH 7-7.5), HBSS (pH 7-7.5) or carbonate buffer (pH 9.6) (Gusils *et al.*, 2004, Dague *et al.*, 2010, Mackenzie *et al.*, 2010, Chagnot *et al.*, 2013). BSA (bovine serum albumin) is generally used as a negative control for assessing the specificity of the binding to mucus and/or mucin. Binding assays are usually performed at 37°C and the contact time with bacterial cells generally ranges between 30 min to 3 hours before washing to remove non-adhered bacteria. Antibiotic at growth inhibiting concentration, such as chloramphenicol, or sometimes thermic treatment can be applied to prevent the growth of microorganisms in the course of the adhesion assay. Binding can be determined using crystal violet staining of the adhered microbial biomass (Azeredo *et al.*, 2017) or by Enzyme-linked Immunosorbent Assay (ELISA) when specific antibodies against bacteria are available (Skoog *et al.*, 2012), by measurement of viable counts after plating of the cells (McNamara *et al.*, 2000) or by quantitative PCR (Skoog *et al.*, 2012). Alternatively, bacteria can be labelled with a radioactive probe or a fluorescent dye before inoculation and the binding quantified using a scintillation counter or a fluorometer, respectively (Gusils *et al.*, 2004, Mackenzie *et al.*,

2010). Microbial cells can also be labelled by biotinylation and further assayed using streptavidin-HRP by ELISA (Sheng *et al.*, 2012). Quantification of microbial binding to mucin can also be performed by flow cytometry, where microbial cells are put in contact with mucin labelled with a fluorescent tag (de Repentigny *et al.*, 2000).

Dot blot assay. More recently, a dot-blot method was developed for the sensitive and rapid detection of microorganisms able to bind to mucins (Ringot-Destrez *et al.*, 2018). In brief, purified mucins were spotted on a nitrocellulose membrane, whereas the bacterial cells were labelled using a fluorescent dye, such as 4',6-diamidino-2-phenylindole (DAPI), Syto9 or Fluorescein isothiocyanate (FITC), before being overlaid (Ringot-Destrez *et al.*, 2018). The adhesion capacities of the microorganisms tested differed depending on the nature of the mucins including purified GI tract mucins, PGM and mucins from the mucus-secreting cell line such as HT29-MTX (see detailed description of this cell line in Section 4.2) (Ringot-Destrez *et al.*, 2018).

Mucin microarrays. The carbohydrate microarray technology offers a powerful platform where natural or synthetic glycans are immobilized onto a solid support. (Poole *et al.*, 2018). . Microarrays incorporating mucins from various sources onto different chips surfaces provide a high-throughput approach to screen bacteria-mucin interactions as well as identify glycan-binding proteins and glycan epitopes involved in this interaction (Clyne *et al.*, 2017). For example, the use of mucin microarrays revealed that *C. jejuni* and *H. pylori* recognised distinct mucin receptors despite being closely related phylogenetically (Naughton *et al.*, 2013). Recently, *H. pylori* was shown to interact with trefoil factor family (TFF) protein TFF1 (Reeves *et al.*, 2008), and that TFF1 specifically interacts with human gastric mucin but not with human colonic mucins nor mucins from other animal sources as shown using mucin

823 microarrays (Dunne *et al.*, 2018). This indicates that TFF1 may play an important role in the
824 development of gastric cancer in *H. pylori* infections (Reeves *et al.*, 2008, Dunne *et al.*,
825 2018). Mucin microarrays were also used to identify the interactions of commensal strains
826 with mucus (*Lactobacillus salivarius* AH102 and *Bifidobacteria longum* AH1205),
827 highlighting the importance of mucin glycans in the preference of the two bacteria to mucins
828 (Naughton *et al.*, 2013, Flannery *et al.*, 2015).

829

830 ***Binding assays in flow chamber.*** As a consequence of fluid shear gradient in the gut, the
831 bacteria located in the outer mucus layer are exposed to a more turbulent flow compared to
832 those that reside between the microvilli of the epithelial cells and therefore less exposed to
833 physical perturbation (De Weirdt & Van de Wiele, 2015). While the assays described above
834 correspond to adhesion under static conditions, experiments can also be performed in
835 dynamic conditions using flow chamber, where the shear force can be controlled (Le *et al.*,
836 2013). Low-fluid shear environments and high shear rates are known to provide laminar
837 pattern. Over time and with different laminar flow rates, the surface coverage of microbial
838 cells to coupons coated with mucin provides an estimate detachment profile as a function of
839 the shear stress.

840

841 ***Biophysical assays.*** In order to gain further molecular insights into the interactions of
842 microbial cells with mucin, various biophysical techniques have been developed and applied
843 over the years. Optical biosensors based on resonant mirrors have been used to determine the
844 binding kinetics of *H. pylori* cells to mucin (Hirmo *et al.*, 1999). Following competition
845 binding assays, the recognition of sialylated and sulphated moieties of mucin by *H. pylori* was
846 demonstrated. Surface plasmon resonance (SPR) has been used to evaluate the adhesion
847 abilities of a range of *Lactobacillus* species (Uchida *et al.*, 2004, Kinoshita *et al.*, 2007). In

these studies, human colonic mucin (HCM) was immobilised on the sensor chip whereas bacterial cells were eluted as analytes. Using sialidase or sulfatase, it was further possible to discriminate some strains of lactobacilli and bifidobacteria that could specifically bind to the sialic acid or sulphate residues of HCM respectively (Huang *et al.*, 2013). Single-cell force spectroscopy (SFCS) has been used to quantify the adhesion forces of *L. rhamnosus* with mucin at a single-cell level, pinpointing heterogeneities in the bacterial population (Sullan *et al.*, 2014). More recently, further molecular details of mucin-bacteria interactions were investigated using atomic force microscopy (AFM). Such an approach was used for the first time to accurately quantify the force of adhesion of *L. lactis* cells immobilised on the AFM tip to PGM at nanoscale level (Dague *et al.*, 2010). Surprisingly, it was found that PGM coating strongly reduced the bacterial adhesion force compared to bare polystyrene, highlighting the interplay between electrostatic, hydrophilic and steric repulsions, and that both specific and non-specific interactions need to be considered (Dague *et al.*, 2010). These results were consistent with a previous investigation of the muco-adhesive properties of *L. lactis* using quartz crystal microbalance with dissipation monitoring (Le *et al.*, 2012). Using bacteria mutant strains, AFM was also used to provide molecular insights into the respective role and contribution of mucus-binding proteins and surface organelles (pili or flagella) in muco-adhesion (Le *et al.*, 2013). Interactions at the protein-protein level were further investigated by AFM to study the adhesive properties of *L. reuteri* Mub with mucins (Gunning *et al.*, 2016).

4.2. *In vitro* mucin-secreting cell models

Monoculture models. While many colon carcinoma cell lines express mRNAs encoding surface-associated and/or secreted intestinal mucins (Deplancke & Gaskins, 2001), few of

873 them secrete MUC2 or form a mucus layer (Linden *et al.*, 2007, Navabi *et al.*, 2013, Hews *et*
874 *al.*, 2017). Most mucus-secreting cell lines are derived from the heterogeneous
875 adenocarcinoma cell line HT-29 which can be differentiated into a mucus-secreting phenotype
876 by growth under metabolic stress conditions. After an initial phase of cell mortality, adapted
877 subpopulations of highly differentiated cells emerge (Lievin-Le Moal & Servin, 2013). HT29-
878 18N2 cells are often used as a model system for goblet cell differentiation and mucin
879 secretion; these cells have been established by growth under glucose deprivation in galactose-
880 containing culture medium (Phillips *et al.*, 1988). In contrast, HT29-MTX cells and their
881 clonal derivatives have been obtained by sequential adaptation to increasing concentrations of
882 methotrexate (Lesuffleur *et al.*, 1990). When grown on Transwell filter supports, some HT-29
883 MTX clones (e.g. MTX-D1 and MTX-E12) form polarised monolayers mostly constituted of
884 mature goblet cells secreting an adherent mucus layer of 50-150 µm thickness as revealed by
885 Alcian Blue staining (Behrens *et al.*, 2001). In addition, the mucin-secreting clonal cell line
886 HT-29.cl16E emerged from parental HT-29 cells after subculture in sodium butyrate whilst
887 HT29-FU cells were established by treatment with 5-fluorouracil (Lesuffleur *et al.*, 1991).
888 These mucus-producing HT-29 derivatives have been widely used to investigate the
889 adherence of commensal and pathogenic bacteria to host cells (Coconnier *et al.*, 1992, Bernet
890 *et al.*, 1993, Eveillard *et al.*, 1993, Bernet *et al.*, 1994, Kerneis *et al.*, 1994, Favre-Bonte *et al.*,
891 1999, Gopal *et al.*, 2001, Schild *et al.*, 2005, Barketi-Klai *et al.*, 2011, Dolan *et al.*, 2012,
892 Gagnon *et al.*, 2013, Naughton *et al.*, 2013, Martins *et al.*, 2015, Martins *et al.*, 2016) and/or
893 evaluate the effect of commensal bacteria on infection with enteropathogens (Bernet *et al.*,
894 1993, Bernet *et al.*, 1994, Coconnier *et al.*, 1998, Gopal *et al.*, 2001, Alemka *et al.*, 2010,
895 Zihler *et al.*, 2011, Zivkovic *et al.*, 2015, Vazquez-Gutierrez *et al.*, 2016). Some studies
896 investigated the direct effect of commensal or pathogenic bacteria on host cell mucin
897 synthesis and/or composition of the mucus layer. Infection with atypical EPEC increased

898 expression of secreted MUC2 and MUC5AC as well as membrane-bound MUC3 and MUC4
899 in HT29-MTX cells, thereby enhancing bacterial growth by providing nutrients for adherent
900 bacteria (Vieira *et al.*, 2010). Another study showed that apical infection with *Listeria*
901 *monocytogenes* stimulated mucus secretion by polarised HT29-MTX cells. This effect was
902 mediated by binding of the toxin listeriolysin O to a receptor on the epithelial brush border
903 (Coconnier *et al.*, 1998) and reduced bacterial invasion and colonisation of the host
904 epithelium (Lievin-Le Moal *et al.*, 2005). Interestingly, probiotic *Lactobacillus* strains which
905 adhering to mucus-producing HT-29 cells upregulated the transcription and secretion of
906 MUC3 which reduced adherence of EPEC in co-incubation experiments (Mack *et al.*, 2003).
907 Modulation of mucus production and mucin glycosylation by commensal bacteria can also
908 occur independently of adhesion. For example, a small soluble peptide of the gut commensal
909 *R. gnavus* E1 strain has been shown to increase HT-29 MTX cell glycosylation via enhanced
910 transcription of glycosyltransferases and MUC2-encoding genes (Graziani *et al.*, 2016).
911 Similarly, a soluble low molecular weight compound from *B. thetaiotaomicron* has been
912 reported to enhance galactosylation in HT29-MTX cells. While no change in transcription
913 was detected, galactosyltransferase activity was increased in HT29-MTX cells treated with
914 soluble bacterial extract suggesting post-translational mechanisms of regulation (Miguel *et*
915 *al.*, 2001).

916 In addition to HT-29 cell derivatives, mucus-producing LS174T colon carcinoma cells
917 have been used to study host-bacteria interactions. LS174T cells secrete mature MUC2,
918 MUC5AC and human gallbladder mucin (van Klinken *et al.*, 1996) but do not produce an
919 organised adherent mucus layer (Navabi *et al.*, 2013). Recent studies using this cell line
920 showed that the secreted metalloprotease StcE reduced MUC2 levels during infection with
921 EHEC and thereby facilitated bacterial adherence to the intestinal epithelium (Hews *et al.*,
922 2017). In addition, the soluble protein p40 from *L. rhamnosus* GG stimulated MUC2 mRNA

and protein expression in LS174T cells, and this effect was dependent on the epidermal growth factor receptor (Wang *et al.*, 2014). Furthermore, treatment with butyrate, a product of bacteria fermentation, increased mucin production in LS174T cells (Burger-van Paassen *et al.*, 2009, Jung *et al.*, 2015). Recently, LS174T cells were used to decipher *E. histolytica*-elicited suppressed goblet cell transcription (Leon-Coria *et al.*, 2018).

Co-culture models. To model human intestinal epithelia, mixed cultures of enterocyte-like Caco-2 cells and mucus-producing HT29-MTX cells have been widely used in drug absorption and permeability studies (Hilgendorf *et al.*, 2000, Lozoya-Agullo *et al.*, 2017). Co-cultures prepared with different ratios of Caco-2 and HT29-MTX cells seeded out on Transwell inserts formed a continuous mucus layer similar to cultures of HT-29-MTX cells grown alone (Poquet *et al.*, 2008, Beduneau *et al.*, 2014). Notably, the probiotic strains *L. rhamnosus* GG or bifidobacteria as well as pathogenic strains of *E. coli* or *L. monocytogenes* adhered better to mucus-deficient Caco-2 cells than to mucus-producing HT-29MTX cells or Caco-2/HT-29 co-cultures (Laparra & Sanz, 2009). Considering the "closed" oxygen-restricted environment in the human gut, Chen and colleagues developed a 3D porous silk scaffolding in the shape of a hollow tube. While the inner tube wall was coated with Caco-2/HT29-MTX epithelia, primary human intestinal myofibroblasts were grown in the tube scaffold space underneath to support epithelial growth and differentiation. Notably, epithelia grown on 3D scaffolds demonstrated increased MUC2 production compared to Transwell cultures resulting in the formation of a mucus layer of 11-17 μm thickness (Chen *et al.*, 2015).

Organ-on-a-chip. Another approach to simulate a mucin-producing human intestinal epithelium is the 'Gut-on-a-Chip' system, where Caco-2 cells are grown on a porous membrane support in a microfluidic device. While the cell membrane support is maintained

under cyclic strain mimicking peristaltic motion, the chambers above and below the cell membrane are constantly perfused with medium, thereby generating low shear stress. This environment stimulates the formation of 3D intestinal villi similar to those found in the small intestine (Kim *et al.*, 2012), and the differentiation of Caco-2 cell into absorptive enterocytes, and also includes enteroendocrine cells, Paneth cells and mucus-producing goblet cells (Kim & Ingber, 2013). In addition, Caco-2 epithelium grown in the Gut-on-a-Chip model display enhanced barrier function and mucus production as compared to static Caco-2 cell cultures (Kim & Ingber, 2013). This system has recently been developed further to allow co- culture with strict anaerobes (Shin *et al.*, 2019). Although the Gut-on-a-Chip devices have been mostly used for long-term co-culture of IECs with commensal microbes under healthy conditions, they are now being employed to model intestinal inflammation (Kim *et al.*, 2016). Using a Gut-on-a-Chip model, the pathophysiological manifestation and dysregulated barrier function observed during inflammation could be recapitulated which may help to gain insights into disease mechanisms and assess potential therapeutic strategies (Shin & Kim, 2018). Notably, probiotic VSL#3 targeted restoration of the mucosal barrier did not effectively control the local inflammation nor improve mucus production (Shin & Kim, 2018). The HuMix (Human Microbial Cross-talk) model is another microfluidic device enabling the co-culture of Caco-2 cell monolayers with commensal bacteria under anaerobic conditions. In contrast to the Gut-on-a-Chip system, the epithelial cells which do not produce mucus are separated from the bacteria by a membrane coated with porcine gastric mucin (Shah *et al.*, 2016).

4.3. Ex vivo organ cultures

As described above, traditional culture of human cells represents a valuable predictor of human physiology, pathology, and therapeutic responses but is limited by the absence of the tissue microenvironment. Culture approaches using human intestinal biopsy samples therefore represent an upscale platform to investigate the involvement of the mucus layer in healthy conditions or in the onset of various diseases.

In vitro organ culture (IVOC). In 1969, Browning and Trier were the first to establish a technique to culture human mucosal biopsies *ex vivo*. By using a specific culture medium and incubation of the samples in 95% O_2, 5% CO_2 at 37°C, mucosal biopsies from the duodeno-jejunal junction were kept alive for up to 24 hours demonstrating epithelial cell proliferation, fat absorption and active mucus secretion by goblet cells (Browning & Trier, 1969). The advantages of IVOC of intestinal biopsies versus cell line culture models include the presence of healthy non-transformed cells including all major IEC types (enterocytes, goblet cells, Paneth cells and neuroendocrine cells), underlying basement membrane and mucosal tissue, and the production of mucus. While it is problematic to maintain the loose outer mucus layer of colonic biopsies during sampling, the inner colonic and small intestinal mucus layers are generally well preserved as evidenced by microscopy (Haque *et al.*, 2004, Walsham *et al.*, 2016, Hews *et al.*, 2017). IVOC of biopsy samples has been used to investigate adherence of pathogenic bacteria such as EPEC (Knutton *et al.*, 1987, Schüller *et al.*, 2007), EHEC (Phillips *et al.*, 2000, Fitzhenry *et al.*, 2002, Lewis *et al.*, 2015), ETEC (Knutton *et al.*, 1989, Baker *et al.*, 1997) and *C. jejuni* (Grant *et al.*, 2006) to human intestinal mucosa. In addition, IVOC demonstrated cytotoxic effects of bacterial toxins, such as Pet toxin from EAEC (Henderson *et al.*, 1999), Shiga toxin from EHEC (Schüller *et al.*, 2004) and *C. difficile* toxin A (Mahida *et al.*, 1996) on intestinal epithelium or mucosa. Interactions of enteropathogenic bacteria with mucus production were observed in small intestinal and colonic biopsy tissue

infected with EAEC where bacteria were predominantly associated with a thick mucus layer above the epithelium, which was not present in non-infected control samples (Hicks *et al.*, 1996, Andrade *et al.*, 2011). This suggests that EAEC stimulates mucus secretion which agrees with the production of mucoid stools during EAEC diarrhoea (Croxen *et al.*, 2013). Similarly, stimulation of mucus secretion and bacterial binding to the mucus layer were observed in biopsy samples from the terminal ileum infected with *S.* Typhimurium. This was followed by *Salmonella* adherence and invasion of the epithelium accompanied by ruffling of the host cell membrane (Haque *et al.*, 2004). Recently, the IVOC system was used to show that the metalloprotease StcE diminishes the inner mucus layer and enhances EHEC adherence to human colonic biopsy epithelium (Hews *et al.*, 2017).

Polarised IVOC (pIVOC). While the traditional IVOC system allows bacterial access to the mucosal and submucosal side of the biopsy, polarised organ culture models have been developed which limit bacterial contact to the mucosal side of the tissue. This is particularly relevant when studying host responses to bacterial infections where artificial interactions with immune cells in the lamina propria might confound experimental readouts. Using a pIVOC approach by mounting colonic tissue explants between two Perspex disks in a Snapwell plate, Raffatellu and colleagues demonstrated that *Salmonella* Typhi reduced mucosal expression of the pro-inflammatory cytokine interleukin (IL)-8 by production of a capsule which masked pathogen-associated molecular patterns such as LPS and flagellin (Raffatellu *et al.*, 2005). In addition, pIVOC showed that apical exposure to EPEC or purified H6 flagellin induced IL-8 expression in duodenal biopsies (Schüller *et al.*, 2009). Furthermore, infection with *C. jejuni* stimulated the production of reactive oxygen species (ROS) in duodenal and colonic mucosa (Corcionivoschi *et al.*, 2012). The pIVOC system has also been used to study the interaction of probiotic bacteria with mucosal tissue, and incubation of duodenal explants with *L. reuteri*

demonstrated localisation of bacteria in the mucus layer but not in the epithelium. Nevertheless, pre-incubation with *L. reuteri* reduced EPEC adherence to the epithelium (Walsham *et al.*, 2016). A different approach to restrict bacterial access to the epithelial surface was developed by Tsilingiri and colleagues by gluing a perspex cylinder to the mucosal side of colonic resection tissue (Tsilingiri *et al.*, 2012). Surprisingly, apical incubation with probiotic *L. plantarum* resulted in degeneration of mucosal tissue from healthy donors, whilst all three strains studied (*L. paracasei, L. rhamnosus, L. plantarum*) caused tissue damage in resections from patients with IBD. In contrast, supernatants from *L. paracasei* reduced inflammation in *Salmonella*-infected and IBD tissue. As the maintenance of larger tissue samples requires incubation in high levels of oxygen (95-99%), the use of IVOC to study interactions of oxygen-sensitive bacteria with human intestinal mucosa remains problematic. However, a novel murine 3D-intestinal organ culture system was recently developed whereby an intact intestinal fragment was luminally perfused with de-gassed medium containing anaerobic bacteria while the serosal side of the tissue was maintained under humidified oxygenated conditions. Whilst preserving gut tissue architecture, the system also supported the growth of commensal microbes (*Clostridium ramosum* and SFB) and allowed assessment of their impact on the immune and nervous system (Yissachar *et al.*, 2017).

Human enteroids/colonoids and intestinal organoids. New technologies have been developed which enable the generation of self-propagating spheres of primary intestinal epithelial cells ("mini-guts"). Enteroids or colonoids are derived from adult stem cells isolated from the crypts of human small intestinal or colonic tissue, respectively (Jung *et al.*, 2011, Sato *et al.*, 2011). In contrast, human intestinal organoids (HIOs) are established by differentiation of embryonic or, more often, induced pluripotent stem cells (genetically

reprogrammed adult stem cells) (Spence *et al.*, 2011). In comparison to enteroids, HIOs lack maturation and more closely resemble foetal than adult intestine. In addition, they are devoid of functional intestinal stem cells and surrounded by a mesenchyme which is absent in enteroids (Sinagoga & Wells, 2015, Leslie & Young, 2016). As the apical side of the epithelium is facing inwards, infection of spheroid enteroids/HIOs with bacteria requires microinjection. Studies on the anaerobic pathogen *C. difficile* showed that injected bacteria remained alive in HIOs for up to 12 hours and caused disruption of epithelial barrier function *via* secretion of the toxin TcdA. Interestingly, oxygen measurements indicated reduced oxygen levels in the lumen of HIOs (5 to 15%). Furthermore, infection with *C. difficile* resulted in reduced MUC2 and mucus production in HIOs (Engevik *et al.*, 2015). HIOs also supported growth of EHEC and commensal *E. coli*. Infection with EHEC induced ROS production and an inflammatory response associated with recruitment of external neutrophils into HIO spheres (Karve *et al.*, 2017). Interestingly, colonisation of HIOs with commensal *E. coli* (ECOR2) stimulated enterocyte maturation, antimicrobial peptide secretion, production of a MUC2-containing mucus layer, and increased epithelial barrier function, thereby indicating the establishment of stable host-microbe symbiosis (Hill *et al.*, 2017).

To facilitate incubations with bacteria, 2D enteroid systems have now been successfully developed where primary intestinal cells are grown as monolayers on permeable membrane supports. Previous studies showed that differentiated human enteroid and colonoid monolayers contained MUC2-producing goblet cells and formed a mucus layer of more than 25 μm thickness (VanDussen *et al.*, 2015, In *et al.*, 2016). Two-dimensional enteroids and colonoids supported binding of EAEC, EHEC and EPEC (VanDussen *et al.*, 2015). More specifically, apical EHEC infection of colonoids resulted in the formation of characteristic attaching and effacing lesions, mucus degradation and reduced expression of the microvillar protein protocadherin 24, which was mediated by the secreted serine protease EspP (In *et al.*,

2016). The 2D enteroid model was further refined by adding primary human macrophages to the basolateral side of the membrane support. Intriguingly, enteroid monolayers grown in the presence of macrophages exhibited increased cell height and barrier function. In addition, underlying macrophages were able to capture and kill EPEC and ETEC by extending projections across the epithelial monolayer (Noel *et al.*, 2017).

In another approach to mimic the gut environment more closely, cells from human small intestinal enteroids were seeded on tubular silk sponge scaffolds and supported by primary human intestinal myofibroblasts as described for Caco-2/HT29-MTX (Section 4.2). The resulting intestinal model epithelium contained all four major epithelial cell types and exhibited tight junction formation, microvillus polarisation, digestive enzyme secretion, and low oxygen tension in the lumen. Moreover, infection with a laboratory strain of *E. coli* resulted in a significant innate immune response (Chen *et al.*, 2017). Recently, a Gut-on-a-Chip model based on primary intestinal epithelial cells has been developed which also includes co-culture of an underlying endothelium. Human enteroids are cultured on a side of a porous membrane within a microfluidic device whereas the intestinal microvascular endothelium is established on the other side of the filter.. This device reproduces the epithelial cells proliferation and host defenses more accurately (Kasendra *et al.*, 2018). Kim and colleagues showed that a human Gut-on-a-Chip micro device colonized by non-pathogenic bacteria (commensal and probiotic bacteria) was able to induce production of a key set of pro-inflammatory cytokines. This device enabled high level of mucus production on micro engineered intestinal villi, therefore providing a protective barrier to maintain long-term stable host–microbe coexistence (Kim *et al.*, 2016).

4.4. *In vitro human fermentation models involving a mucosal phase*

As aforementioned, the spatial positioning of gut microorganisms in the mucus layer is important with respect to their functional role in the human gut ecosystem. The microbial community residing in the mucus layer across the length of the GI tract is, however, hard to study given the difficulty to sample this region *in vivo*, especially in human (Macfarlane *et al.*, 1997, Flint *et al.*, 2012, Donaldson *et al.*, 2016). *In vitro* colonic models involving a mucosal phase are a valuable alternative to study the fine-scale spatial organisation of the gut microbial ecosystem.

Multiple colon *in vitro* models have been developed over the years, ranging from simple, single stage batch incubations to more complex and representative three stage continuous and semi-continuous reactor models (Miller & Wolin, 1981, Gibson *et al.*, 1988, Allison *et al.*, 1989, Blanquet-Diot *et al.*, 2012, McDonald *et al.*, 2013, Van den Abbeele *et al.*, 2013). These continuous fermentation models, inoculated with faecal samples of donors, recapitulate the main biotic and abiotic parameters of the human colon, such as temperature, pH, residence time, supply of nutritive medium reproducing the composition of ileal effluents, therefore enabling the study of a complex and metabolically active gut microbiota under anaerobiosis conditions. In these fermentation models, the bioreactors can be inoculated with fresh or frozen stools provided by individual or pool of different donors being healthy human volunteers with no history of antibiotic treatment 2 to 6 months before the beginning of the study.

Introduction of mucus carriers in human in vitro colonic models. Mucins in solution have been frequently included in these colonic models to provide a nutrient source to the gut bacteria, but the study of mucus colonisation by gut bacteria in these systems has been revolutionized by the ability to simulate the viscoelastic gel-like nature of the mucus layer through the incorporation of mucus carriers (Gibson *et al.*, 1988, Macfarlane *et al.*, 1989,

Macfarlane *et al.*, 2005, Van Herreweghen *et al.*, 2017). MacFarlane first demonstrated a rapid colonisation of an agar-mucus layer during a 48-hour incubation in a two-stage continuous fermentor system by a mixture of *Bacteroides*, enterobacteria and facultative anaerobes (Macfarlane *et al.*, 2005). However, the use of glass tubes, containing this agar-mucus layer, in this set-up did not permit a practical long-term implementation (Van den Abbeele *et al.*, 2009). Mucus-coated beads (mixture of 5% porcine mucin type II and 1% agar) have since been identified as crucial platforms in sustaining microbial diversity by selectively enriching species, which are not thriving in the luminal environment. This mucus interphase was introduced in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) resulting in the M-SHIME (Mucus SHIME) configuration (Van den Abbeele *et al.*, 2012). This system was first used to assess the fitness of potential probiotic lactobacilli, revealing a pronounced enrichment of *Lactobacillus mucosae* and *L. rhamnosus* GG in the mucus beads compared to the luminal environment, contributing to their long-term persistence in M-SHIME (Van den Abbeele *et al.*, 2012). The use of next-generation 16S rRNA gene amplicon sequencing methods and the incorporation of mucus beads in both proximal and distal colon conditions in the M-SHIME, further resulted in the detection of several additional mucus-associated species (De Paepe *et al.*, 2018). Besides *Roseburia faecis/Enterococcus rectale,* a strong enrichment of *Ruminococcus inulinivorans*, *Clostridium*, *Bilophila*, *Anaeroglobus* and *Veillonellaceae* species was observed in the proximal mucus layer compared to the lumen (De Paepe *et al.*, 2018). *A. muciniphila, Cloacibacillus evryensis, Pyramidobacter piscolens, Eubacterium contortum* and species belonging to *Odoribacter, Enterobacteriaceae* and *Desulfovibrio* were predominantly residing in the distal mucus layer (De Paepe *et al.*, 2018). It can be expected that the list of species inhabiting the mucus layer will continue to expand in the future M-SHIME experiments, by continuing to explore the inter-individual variability of faecal samples.

Another major advantage of *in vitro* fermentation models is the possibility to capture dynamics by time-resolved analyses. To minimize disturbance of the system during such analyses, the M-SHIME system was adapted to facilitate a rapid, anaerobic, frequent sampling by mounting sampling ports with an airlock system on top of the SHIME lids. These sampling ports moreover enable the anaerobic addition, transfer and sampling of any insoluble dietary substrate. The adapted model was correspondingly termed Dietary Particle-Mucosal-Simulator of the Human Intestinal Microbial Ecosystem (DP-M-SHIME). The DP-M-SHIME offers a closer proxy for the diversity of *in vivo* GI microenvironments, as aside from the mucus layer, insoluble dietary particles present an interesting additional niche for microorganisms to physically interact with and colonise (De Paepe *et al.* 2018).

In addition, these colonic *in vitro* fermentation models enable the study of severe gut microbiome perturbations such as antibiotic therapy or pathogen invasion, which cannot be performed in humans for obvious ethical reasons. Using M-SHIME, the mucus bead carriers were shown to confer resilience to lactobacilli species against a combined treatment of antibiotics including tetracycline, amoxicillin and ciprofloxacin (Van den Abbeele *et al.*, 2012). In addition, the M-SHIME model has been used to demonstrate the antagonistic effects of probiotics and prebiotics such as *L. reuteri*, long-chain arabinoxylans or inulin towards AIEC colonisation in the mucosal environment (Van den Abbeele *et al.*, 2016). Another study reported the importance of mucus for providing a protective environment to beneficial gut microbes, such as *L. reuteri*, to help them escape from stress induced by high loads of linoleic acid, the most common polyunsaturated fatty acid in a WSD (De Weirdt *et al.*, 2013). The M-SHIME model has also been shown to preserve disease signatures, as illustrated by a reduced luminal and mucosal *Clostridium* cluster XIVa colonisation in colon vessels inoculated with the faecal material of ulcerative colitis patients compared to healthy donors (Vermeiren *et al.*, 2012). The mucin-beads technology has recently been transferred into the Artificial Colon

(ARCOL), with adaptations to avoid flushing the bioreactor with nitrogen when mucin-coated beads are being renewed (Cordonnier *et al.*, 2015, Thevenot *et al.*, 2015) .

Combining colon fermentation models with in vitro cell culture. Recent advancements in the field of *in vitro* fermentation involve the combination of gut microbiota models with a host compartment to assess host-microbiota interactions (Bahrami *et al.*, 2011, Marzorati *et al.*, 2014, Defois *et al.*, 2018). The three-way interactions between host, microbiome and dietary interventions can be examined by applying the supernatants of colonic samples onto human cell lines or combinations thereof, such as co-cultures of enterocytes and immune cells, in Transwell systems (Marzorati *et al.*, 2014). Cytokine and TNF-α production are followed up as markers of intestinal inflammation, whereas the trans-epithelial electrical resistance (TEER) and Lucifer yellow translocation give an indication of epithelial barrier function (Geirnaert *et al.*, 2017). While typically, in human cell line experiments, the short-term effects of a single treatment application onto differentiated cells with a disrupted barrier are being evaluated, recently, a method was developed to test the effects of a probiotic treatment on the development of epithelial barrier integrity during cell differentiation, which is more representative of the *in vivo* situation (Geirnaert *et al.*, 2017). In order to further improve the *in vivo* relevance, a host module, such as the Host Microbiota Interaction (HMI) Module (Marzorati *et al.*, 2014), can be coupled to the colonic *in vitro* fermentation systems described above to directly and continuously recirculate the colonic microbial suspension over a mucus layer that is in indirect contact with enterocytes and/or immune cells.

4.5. In vivo animal models

As described above, *in vitro* mucin-secreting cell cultures, *ex-vivo* organ cultures as well as *in vitro* fermentation models have yielded fundamental insights into the role of mucins and mucus in bacterial interactions with the host. However, the use of *in vivo* models is necessary to study the biological roles of mucins under physiological or pathological conditions at the level of entire organism. Genetically modified mouse models with an impaired mucin production or glycosylation have been developed to assess the role of mucus in the interaction between gut bacteria and the host *in vivo*.

Muc2$^{-/-}$ mouse model. Many *in vivo* animal studies investigating the role of mucus in gut homeostasis have relied on the use of *Muc2*$^{-/-}$ mice, lacking the major intestinal mucin Muc2. The first studies based on *Muc2*$^{-/-}$ mice showed that these animals displayed an impaired epithelial barrier function characterised by aberrant intestinal crypt morphology and altered cell maturation and migration, and that the mice frequently developed adenomas in the small intestine, as well as rectal tumours (Velcich *et al.*, 2002). The microscopic analysis of the colon indicated mucosal thickening, increased proliferation and superficial erosions (Van der Sluis *et al.*, 2006). The development of spontaneous colitis in Muc2 deficient mice indicated that Muc2 is critical for colonic protection (Van der Sluis *et al.*, 2006). A gut microbiota dysbiosis was also observed in the *Muc2*$^{-/-}$ mice which harbored a pro-inflammatory-like microbiota profile, characterized by an increase in *Clostridiales* and a decrease in *Lactobacillaceae* (Huang *et al.*, 2015). Furthermore, it was shown that the spatial compartmentalization of bacteria in the intestine of *Muc2*$^{-/-}$ mice was compromised and transcriptomic analysis revealed a downregulation of TLR, immune and chemokine signaling pathways compared to wild type mice (Sovran *et al.*, 2015) Also, the expression of the network of IL-22-regulated defense genes was increased in *Muc2*$^{-/-}$ mice (Sovran *et al.*, 2015). Recent work also confirmed a clear shift in the microbiota composition of *Muc2*$^{-/-}$ mice, with

1222 the Firmicutes phylum enriched and the Bacteroidetes phylum decreased, as well as an
1223 increase in genera considered as potential pathogens also (Wu *et al.*, 2018).

1224 *Muc2*$^{-/-}$ mice have been used to test the effect of the probiotic mixture VSL#3 on
1225 colonic inflammation and intestinal barrier function (Kumar *et al.*, 2017). This probiotic
1226 mixture contains eight strains belonging to *Lactobacillus*, *Bifidobacterium* and *Streptococcus*
1227 genera which are usually found in the human intestinal microbiota. In *Muc2*$^{-/-}$ mice, VSL#3
1228 reduced basal colonic proinflammatory cytokine levels and improved epithelial barrier
1229 function. In addition, VSL#3 reduced the level of proinflammatory chemokines and
1230 upregulated tissue regeneration growth factors leading to a faster resolution of colitis
1231 symptoms in *Muc2*$^{-/-}$ mice with DSS-induced colitis. This was associated with the restoration
1232 of antimicrobial peptide gene expression in the small intestine, and an increased abundance of
1233 commensal bacteria in the gut. The authors proposed that these beneficial effects were
1234 mediated by acetate, produced by the gut bacteria (Kumar *et al.*, 2017). Treatment of *Muc2*$^{-/-}$
1235 mice with *Lactobacillus* spp. could ameliorate spontaneous colitis and led to an increased
1236 production of SCFA (Morampudi *et al.*, 2016).

1237 *Muc2*$^{-/-}$ mice have also been used to investigate the role of this mucin to prevent
1238 bacterial and parasite infection. Upon infection with *C. rodentium*, a murine pathogen related
1239 to diarrhoeagenic attaching-effacing *E. coli, Muc2*$^{-/-}$ mice exhibited a rapid weight loss and up
1240 to 90% mortality (Bergstrom *et al.*, 2010). Mucin secretion was increased in wild type mice
1241 during infection as compared to the uninfected controls, suggesting that mucin production is
1242 critical to clear the mucosal surface from pathogenic bacteria. In *Muc2*$^{-/-}$ mice, commensal
1243 bacteria were also found to interact with *C. rodentium* and host tissues, indicating that Muc2
1244 regulates all forms of intestinal microbiota at the gut surface (Bergstrom *et al.*, 2010). When
1245 *Muc2*$^{-/-}$ mice were infected with *Salmonella*, they showed a dramatic susceptibility to
1246 infection, carrying significantly higher caecal and liver pathogen burdens, and developing

significantly higher barrier disruption and higher mortality rates than wild type mice (Zarepour *et al.*, 2013). Colonisation of *Muc2*$^{-/-}$ mice by enterotoxigenic *B. fragilis,* a causative agent of acute diarrhoea in humans, led to lethal disease (Hecht *et al.*, 2017). The protective function of Muc2 was also demonstrated in models of *T. muris* parasitic infection (Hasnain *et al.*, 2010). *T. muris* is a murine infecting nematode which is used as model of *T. trichiura* infection in humans, a threat in developing countries. After infection, *Muc2*$^{-/-}$ mice showed a delayed expulsion of the worms from the intestine compared to wild type mice. In addition, an increase in Muc2 production, observed exclusively in resistant mice, correlated with worm expulsion. The nematodes demonstrated a decrease in their energy status in wild type mice compared to the susceptible *Muc2*$^{-/-}$ mice (Hasnain *et al.*, 2010). *E. histolytica* is a human parasite infecting the colon and responsible of amoebic dysentery and/or liver abscesses. *E. histolytica* specifically colonises the mucus layer by adhering to galactose and GalNAc residues present in Muc2 (Kissoon-Singh *et al.*, 2013). The parasite also induces potent hypersecretion from goblet cells. Kissoon-Singh and colleagues showed that *E. histolytica* induced a pronounced time-dependent secretory exudate with increased gross pathology scores and serum albumin leakage in *Muc2*$^{-/-}$ mice. Colonic pathology, secretory responses, and increased pro-inflammatory cytokine secretions were also correlated with altered expression of tight junction proteins (Kissoon-Singh *et al.*, 2013). These results demonstrate that colonic mucins confer both luminal and epithelial barrier functions and that, in the absence of Muc2, mice are more susceptible to *E. histolytica*-induced secretory and pro-inflammatory responses. A recent study using antibiotic treated *Muc2*$^{-/-}$ and *Muc2*$^{+/+}$ littermates showed that *E. histolytica* elicited robust mucus and water secretions, enhanced pro-inflammatory cytokines and chemokine expression and higher pathology scores as compared to the modest response observed in non-antibiotic treated littermates. Host responses were microbiota specific as mucus secretion and pro-inflammatory responses were

attenuated following homologous faecal microbial transplants in antibiotic-treated *Muc2*$^{+/+}$ quantified by secretion of ^{3}H-glucosamine newly synthesized mucin, Muc2 mucin immunostaining and immunohistochemistry (Leon-Coria *et al.*, 2018). The mechanism controlling mucus release in the presence of *E. histolytica* was further studied by Cornick and colleagues who identified vesicle-associated membrane protein 8 (VAMP8) present on mucin granules as orchestrating regulated exocytosis in human goblet cells in response to the presence of *E. histolytica* (Cornick *et al.*, 2017). In *Vamp8*$^{-/-}$ mice, *E. histolytica* induced enhanced killing of epithelial cells and aggressive proinflammatory response with elevated levels of IL-1α, IL-1β, and TNF-α secretion, highlighting the downstream consequences of improper mucin secretion in mucosal barrier defence. Taken together, these results demonstrate the critical involvement of Muc2 in host protection from nematode infection, by constituting an effective physical and biological barrier against pathogenic infection.

Muc1*$^{-/-}$ *mouse model. Mice impaired in the production of cell surface mucins have also been engineered. The *Muc1*$^{-/-}$ mouse model revealed the role played by Muc1 in *H. pylori* infection, a pathogen involved in gastric ulcers and adenocarcinoma (McGuckin *et al.*, 2007, Linden *et al.*, 2009). *Muc1*$^{-/-}$ mice displayed a 5-fold increase in *H. pylori* colonisation as compared to wild type mice (McGuckin *et al.*, 2007). This study further demonstrated the ability of *H. pylori* to bind to purified Muc1 *in vitro*, suggesting that Muc1 limits the access of *H. pylori* to the epithelial surface thereby providing protection from infection and proinflammatory bacterial products. Muc1 deficiency also resulted in increased epithelial cell apoptosis in *H. pylori* infected mice (Linden *et al.*, 2009). More recently, the long-term consequence of Muc1 deficiency on *H. pylori* pathogenesis was investigated in *Muc1*$^{-/-}$ mice (Ng *et al.*, 2016). *Muc1*$^{-/-}$ mice began to die 6 months after *H. pylori* challenge, indicating that a deficiency in Muc1 leads to lethal infection. This study also revealed that *Muc1* was an

important, previously unidentified negative regulator of the NLRP3 inflammasome, and loss of this regulation resulted in the development of severe pathology (Ng *et al.*, 2016). Consistent with these studies, *Muc1*$^{-/-}$ mice have a higher rate of systemic infection in a murine *C. jejuni* model of gastroenteritis (McAuley *et al.*, 2007).

Muc13*$^{-/-}$ *mouse model. The MUC13 transmembrane mucin is highly and constitutively expressed in the small and large intestines and MUC13 polymorphisms have been associated with human IBD and susceptibility to *E. coli* infection in pigs. While Muc13-deficient mice did not show intestinal pathology, they developed more severe acute colitis than wild type mice after DSS challenge, as reflected by increased weight loss, rectal bleeding, diarrhoea and histological colitis scores (Sheng *et al.*, 2011).

Mouse models with an altered mucin glycosylation. Loss of *O*-glycans impairs the expression and function of several intestinal mucins, thereafter causing more profound defects in the function of the intestinal barrier than a flaw caused by the deficiency of an individual mucin. A number of transgenic mouse models have been developed to decipher the mechanisms underpinning the role of mucin glycosylation in gut homeostasis. Mice lacking core 3–derived *O*-glycans (also known as *C3GnT*$^{-/-}$ mice) display a substantial reduction of Muc2 protein and an increased susceptibility to DSS-induced colitis and accelerated colorectal tumorigenesis (An et al., 2007). In addition, core 3 *O*-glycosylation was shown to play a major role in controlling Salmonella intestinal burdens in *C3GnT*$^{-/-}$ mice (Zarepour *et al.*, 2013). Similarly, *C2GnT2*$^{-/-}$ mice (mice lacking core 2-derived *O*-glycans) displayed an increased susceptibility to DSS-induced colitis but with no change in Muc2 expression (Stone *et al.*, 2009). Mice with intestinal epithelial cell–specific deficiency of core 1–derived *O*-glycans (IEC *C1galt1*$^{-/-}$) develop spontaneous colitis (Fu *et al.*, 2011). Mice lacking both core

1- and core 3–derived *O*-glycans (DKO mice) have an impaired mucus barrier function and develop colitis-associated colon cancer in which the dysbiotic microbiota promote inflammation and cancer (Bergstrom *et al.*, 2016). In a water avoidance model in rats, psychological stress lead to less-protective mucus layer. In particular, *O*-glycosylation of mucins was strongly affected and these changes were associated with flattening and loss of the mucus layer cohesive properties (Da Silva *et al.*, 2014). Altogether, these data suggest that the lack of a proper *O*-glycosylation impairs Muc2 expression or secretion and alters gut barrier function of the mucus layer.

In addition to modifications of mucin core glycans, mouse models have been developed targeting epitope modifications of the mucin glycans chains. Dawson and colleagues reported that deletion of the sulfate transporter NaS1 in mice (*Nas1*$^{-/-}$ mice) resulting in a decrease in mucin sulfation, enhanced susceptibility to experimental DSS colitis and systemic infection by *C. jejuni* (Dawson et al., 2009). In addition, mice with a deletion of the sulfo-transferase GlcNAc6ST2 enzyme adding sulfate to GlcNAC residues on *O*-mucin glycan chains exhibited an increased susceptibility to DSS-induced colitis (Tobisawa et al., 2010). Mice deleted for Sat-1 (sulphate anion transporter-1) were more susceptible to chronic infection by parasite *T. muris* (Hasnain et al., 2017). Collectively, these findings indicate that mucin abnormalities can initiate the onset of inflammatory related diseases in the gut.

In addition to mice harbouring a deletion in genes encoding proteins directly involved in mucin expression or glycosylation, several transgenic mouse models have been shown to display alterations in mucus properties. These include the Winnie and Eeyore mice which carry single missense mutations in two different D-domains of Muc2 (Heazlewood *et al.*, 2008). These mice display fewer goblet cells and a reduction in secreted mucus with O-glycosylated and non-O-glycosylated Muc2. The misfolding results in endoplasmic reticulum stress, goblet cell apoptosis, depletion of the secreted mucus layer and development of

chronic intestinal inflammation (Heazlewood *et al.*, 2008). In combination with mucus, a large population of intraepithelial lymphocytes (IELs) bearing the γδ T cell receptor is mediating immune protection against invading bacteria. In γδ T-cell-deficient (*TCRδ*$^{-/-}$) mice, mucin expression and glycosylation is altered, mucus-secreting goblet cells are significantly reduced in number and those animals are more prone to DSS-induced colitis (Kober *et al.*, 2014). Mouse models deficient in TLR5, IL-10 and Sodium hydrogen antiporter 3 (Slc9a3 or Nhe3) revealed bacteria in contact with the epithelium. Additional analysis of the less inflamed *IL-10*$^{-/-}$ mice revealed a thicker mucus layer but a more penetrable inner mucus allowing bacteria to penetrate and reach the epithelium (Johansson *et al.*, 2014).

Non-rodent models. Differences in mucus thickness and composition have been observed between rats, pigs and rabbits, and suggests that the pig mucus pattern resembles more closely that of humans (Varum *et al.*, 2012). The zebrafish larva is an emerging model system for investigating components of the innate immune system, including mucus physiology. It has been shown that five gel-forming secreted mucin genes are found in zebrafish with a high degree of homology to other vertebrate mucins regarding their genomic and protein domain organisation, as well as their tissue specific expression (Jevtov *et al.*, 2014).

5. Limitations of current experimental models involving mucus and future challenges

Given the importance of the gut microbiota as a modulator of health and disease, increasing attention has been devoted to the role played by mucus in the interaction with gut bacteria (Juge, 2019). As described above (section 4), various experimental models increasing in complexity from simple *in vitro* assays to cell lines, organ-on-chips, *in vitro* colon fermentation systems or animal models have been developed and successfully applied to the

study of gut microbe-mucus interactions. However, one of the limitations common to most *in vitro* models is the origin of mucins used to assess the interactions with bacteria doubled by the inability to reproduce a colonic mucus gel recapitulating the *in vivo* situation. This is important as the nature and origin of purified mucins used in these assays greatly influence the outcome of binding as demonstrated using microtitre plate (Owen *et al.*, 2017), dot-blot (Ringot-Destrez *et al.*, 2018) or mucin microarrays (Clyne *et al.*, 2017). Mucin glycosylation plays a critical role in the interaction between gut bacteria and mucus and significant glycosylation differences occur between purified mucins used in *in vitro* assays from different sites of the murine GI tract or from goblet cells (e.g. LS174T), as analysed by mass spectrometry (Leclaire *et al.*, 2018, Ringot-Destrez *et al.*, 2018). In addition, the purification steps alter the properties of native glycoproteins and purified mucins used in these assays lack the ability to form viscoelastic hydrogels (Kocevar-Nared *et al.*, 1997). A similar situation occurs with mucus secreting cell lines where the type of mucins and structure of mucus differ from the colonic environment. For example, the HT29 cell line secretes mostly MUC5AC whereas MUC2 is the main mucin secreted in the small and large intestines. These differences are due to the use of cancer cells which show an alteration in the expression and glycosylation of mucins. In addition, the production of mucus by epithelial cell lines can be influenced by culture conditions. For example, growing cells on Transwell filters with a small amount of apical medium (semi-wet interface culture) in combination with mechanical stimulation (on a rocking platform) and addition of the Notch γ-secretase inhibitor DAPT resulted in polarisation and secretion of MUC2 and MUC5AC by HT29 MTX-P8, HT29 MTX-E12 and LS513 cells (Navabi *et al.*, 2013). Additionally, the mucus produced by goblet cells in *in vitro* co-culture cell models is not continuous nor homogenate which is not fully representative of the *in vivo* situation. Lastly, the formation of the bi-layered mucus found in the colon remains a challenge in these models. Novel strategies such as multiple cell layers, 2D-organoid

techniques or Organ-on-a-Chip devices are currently being developed to better mimic the human intestinal epithelial microenvironment. Such multiple cell models exhibit intestinal villus morphogenesis associated with mucus production. These models are also needed to recapitulate antimicrobial defense and inflammatory reactions normally occurring in mucosal tissues. Another advantage of these systems is that, unlike cell lines, organoids can be used to evaluate long-term interactions between mucus and gut microbes. However, these more advanced biopsy- based models remain low throughput and expensive as compared to *in vitro* assays and are limited by the availability and variability of clinical specimens.

In addition, to the host side, several microbial factors must be taken into consideration when assaying the interactions between the gut bacteria and mucus. These include the handling and labelling microbial cells which may affect the surface molecular determinants potentially involved in mucus/mucin interactions (e.g. cell-surface adhesins, pili or flagella) (Chagnot *et al.*, 2014). The growth conditions (e.g. growth media with different nutrient compositions; temperature, pH, osmolarity or redox potential) can also influence the expression of the bacterial receptors mediating the interactions with mucins. To date, most studies have focused on the interactions between mucus and probiotic or pathogenic strains and assessing strictly anaerobe gut symbionts or complex microbial communities remain a challenge in this field of research.

An alternative to the systems described above is the use of dynamic *in vitro* fermentation models of the human gut, such as the M-SHIME (Marzorati *et al.*, 2014) or DP-M-SHIME (De Paepe *et al.*, 2018) models. In these *in vitro* colonic models, the introduction of mucin-covered beads allows to study the long-term *in vitro* microbial colonisation of mucin, in the presence of a complex anaerobic intestinal microbiota (Marzorati *et al.*, 2014, Shah *et al.*, 2016). These models provide a mean to study gut microbiota functionality and niche differentiation, during treatments with xenobiotics (for example antibiotics, synthetic

chemicals such as food additives, environmental pollutants like persistent organic pollutants (POPs)), pathogens or functional foods. Future developments in this field will be the introduction of mucus secretion and/or a mucus surface layer in dynamic *in vitro* models of the upper GI tract, such as in the gastric and small intestinal TNO Gastro-Intestinal model (TIM) (Guerra *et al.*, 2012). This is important so to take into account the successive stressful events (e.g. acidic gastric pH, bile salts) that commensal or pathogenic microbes undergo in the human GI tract before reaching the intestinal epithelium and that may greatly influence their physiological stage, virulence and/or activity.

However, as mentioned above, a limitation of these *in vitro* GI models is that they rely on commercially available mucins used for the mucin bead technology. These secretory mucins, usually MUC5AC and MUC6 porcine gut gastric mucin, differ in terms of structure and glycosylation from intestinal MUC2 and cannot form a bi-layered mucus gel. It has been proposed that in the future, *in vitro* engineered mucus may be used to mimic human-derived mucus in a more reproducible manner. The colonic *in vitro* models could also be improved by including immunoglobulins, specific antimicrobial peptides, or secreted phosphatidylcholine, which have been shown to modulate mucus surface properties, thereby influencing bacterial adhesion (Martens *et al.*, 2018). Future *in vitro* colon models should also better mimic the *in vivo* transit, and particularly retrograde movements (Hiroz *et al.*, 2009), as back-flow was recently suggested to be crucial for the persistence of gut microbes in the GI tract (Cremer *et al.*, 2016). Current technological advances include the coupling of these fermentation models to intestinal epithelial cells or more complex units such as the HMI module. A next step will be to couple the digestive/fermentation models with enteroids/colonoids or HIO. However, despite their increased complexity, most of these approaches remain limited by the absence of important host functions, such as variable peristalsis-like motions. This is a critical limitation because mechanical deformations resulting from peristalsis both influence normal epithelial

cell differentiation and control microbial overgrowth in the living intestine (Gayer & Basson, 2009, Benam *et al.*, 2015). The development of microfluidic systems and organ-on-chips is currently addressing this important technological gap (Kim & Ingber, 2013, Kim *et al.*, 2016).

The development of these advanced *in vitro* systems is essential to help reduce dependence on animal studies. Due to the invasive nature of the experiments, the mechanisms underpinning microbe-mucus interactions *in vivo* have mainly been investigated in animal models, mostly rodents. Genetically engineered mice impaired in mucin secretion or glycosylation have been instrumental to decipher the role of mucins and mucus in the protection of the intestinal epithelium and the interactions between pathogenic bacteria, commensal microbiota and the mucus barrier. However, although the domain organisation and expression pattern of mucins appear largely conserved between human and mouse (Joshi et al., 2015), mucin glycosylation (Thomsson *et al.*, 2012) and gut microbiota (Nguyen *et al.*, 2015) differ between these two species. It has been speculated that differences in mucin glycosylation between mammalian species may underlie some of the differences in infectivity and/or pathogenicity for individual microbial pathogens (Linden *et al.*, 2008) or the different commensal microbiota (Thomsson *et al.*, 2012). Therefore, caution should be applied when translating data obtained in mouse models to humans. Lastly, unlike *in vitro* assays, *in vivo* studies are restricted to end-point measurements.

Recent years have witnessed unprecedented technological advances in the development of *in vitro* GI models that more closely resemble the gut mucosal interface. Our next challenge will be to simulate these models at different stages of development or disease conditions (e.g. IBD, obesity or CF). Special attention should be paid to inter-individual differences and intra-individual variability in gut microbiota composition and intestinal biopsies from different donors or patients. This is important to better understand the role of gut microbe-mucus interactions in the aetiology of a particular disease or condition and

determine the microbial and biochemical signature that could differentiate between diseased or healthy status. In particular, more research is warranted to determine how the physicochemical properties and/or thickness of the mucus layer and mucin glycosylation are altered during a specific disease. In the future, these pre-clinical models will help screen novel therapeutic strategies aimed at restoring gut barrier function and tailored to the individual patient as a step towards personalised medicine.

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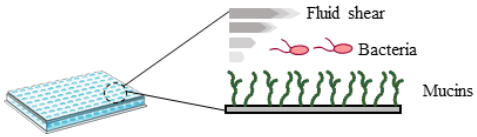
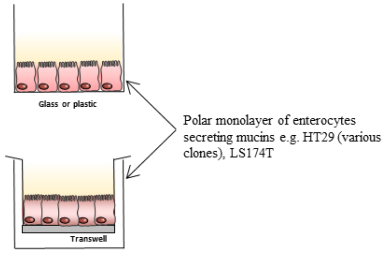
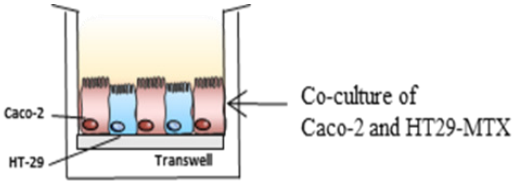
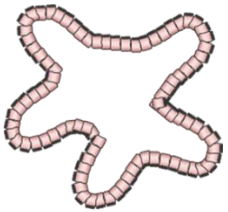
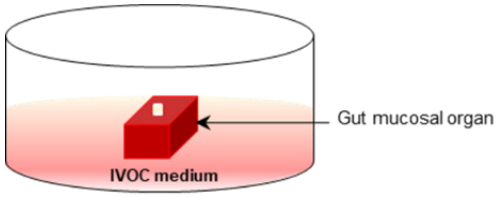
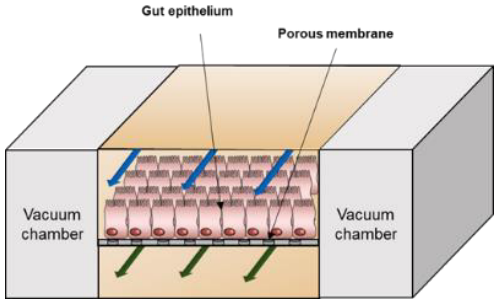
Types of models	Description	Applications	Advantages	Limitations	References
***In vitro* mucus/mucin binding assays**					
Microplates - Flow chambers					
	* Immobilization of mucus/mucin on the microtiter plate * Microtiter plate : adhesion in static conditions * Flow chambers : adhesion under dynamic conditions (fluid shear)	* Evaluation of bacterial adhesion (commensals and pathogens) to mucins and molecular mechanisms associated	* Fast, quantitative and high throughput method to study mucus-microbe interactions independently from other *in vivo* conditions * Identification of molecular determinants involved in adhesion of microbes * Coupling with biophysical techniques (Surface Plasmon Resonance, Atomic Force Microscopy)	* Influence of experimental conditions (antibiotics, mechanical treatments, growth conditions, hydrophobic interactions) * Limited availability of purified mucins (mainly use of pig gastric mucin) * Absence of gut microbiota	McNamara *et al.*, 2000, Gusils *et al.*, 2004 ; Ringot-Clyne et al.,2017 Dunne et al. 2018
***In vitro* cell models**					
Monoculture models					
	* Gut–derived epithelial cells resembling intestinal tissue consisting mainly of mature goblet cells that secrete an adherent	* Adherence of commensal and pathogenic bacteria to host cells * Effect of commensals/pathogens on host cell mucin synthesis and/or composition of the mucus layer	* Reproducible and easily handled in laboratories * Identification of molecular determinants involved in adhesion of microbes and host cell mucin synthesis * Good platform for screening and characterizing probiotic activity	* Derived from cancer cells, different from healthy tissue * Not representative of various cell types recovered in mucosal epithelial tissues * Not representative of appropriate *MUC* gene expression * Modulation of mucus production by culture conditions * Absence of gut microbiota * Difficulty to maintain for long-term experiments (>1 month) * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Linden *et al.*, 2007, Navabi *et al.*, 2013, Hews *et al.*, 2017
Co-culture models					
	* Mixed culture of enterocytes and mucin secreting cells	* Adherence of commensal and pathogenic bacteria to host cells * Effect of commensals/pathogens on host cell mucin synthesis and / or composition of the	* Better representation of cell-type ratio recovered in mucosal epithelial tissues * Simple model, well described in literature	* Absence of M-cells (development of triple co-culture Caco-2/HT29-MTX/Raji B) * Variations in seeding ratios of HT29 MTX/Caco-2 can impede results interpretation * Modulation of mucus production by culture conditions * Absence of gut microbiota * Difficulty to maintain for	Hilgendorf *et al.*, 2000, Lozoya-Agullo *et al.*, 2017

		mucus layer	long-term experiments (>1 month) * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)

***Ex-vivo* organ cultures**					
Intestinal organoids					
	* Generation of self-propagating spheres of primary intestinal epithelial cells * Enteroids = derived from adult stem cells isolated from the crypts of human small intestinal * Colonoids = derived from adult stem cells isolated from the crypts of human colonic tissue	* Study of advanced aspects of mucus development in a more complex scenario * Study of host–commensals and pathogens interactions	* Often collected from mice tissues, possible use of patient-derived tissues * Assay that more accurately mimics *in vivo* conditions * Amenable to long-term culture	* Highly expensive and requires specialized expertise * Requires access to biopsies/tissues * Donor-to-donor variability * Requirement of injection to infect organoids with bacteria * Absence of gut microbiota * No reproduction of peristalsis motions and GI stressful events * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Jung *et al.*, 2011, Sato *et al.*, 2011
In vitro organ culture *(IVOC)*					
	* Whole organs maintained *in vitro*	* Study of host–commensals and pathogens interactions	* Better maintenance of tissue architecture * Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) * Often collected from animal tissues, possible use of patient-derived tissues * Possible use of biopsies from disease patients (e.g. IBD)	* Requires access to biopsies/tissues * Expensive and requires expertise * Donor-to-donor variability * Difficulty to maintain for long-term experiments * No reproduction of peristalsis motions and GI stressful events * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes)	Browning & Trier, 1969, Schüller *et al.*, 2007
Gut- on-a chip					
	* Reproduction of the multicellular structures, cell–cell and tissue–tissue interactions, and the native microenvironment * Closely reproduction of the *in vivo* situation	* Study of the complex physiological and pathophysiological responses of tissues at an organ level * Study of host–commensals and pathogens interactions	* Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells) * Reproduction of peristalsis like motions * Possible use of biopsies from disease patients (e.g. IBD)	* Expensive and requires dedicated expertise and instrumentation * Stem cell differentiation is difficult to achieve * Flow rate of the medium can influence cell metabolism * Absence of gut microbiota * No input from immune and nervous system * Requirement of high oxygen levels (difficulty to study oxygen-sensitive microbes) * No reproduction of the full	Kasendra *et al.*, 2018

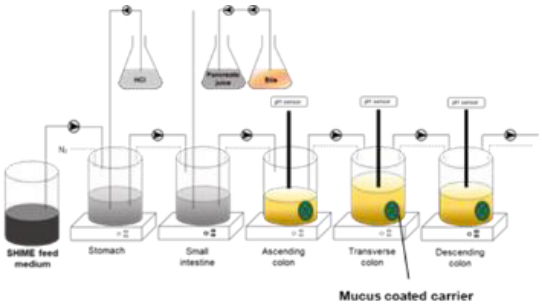
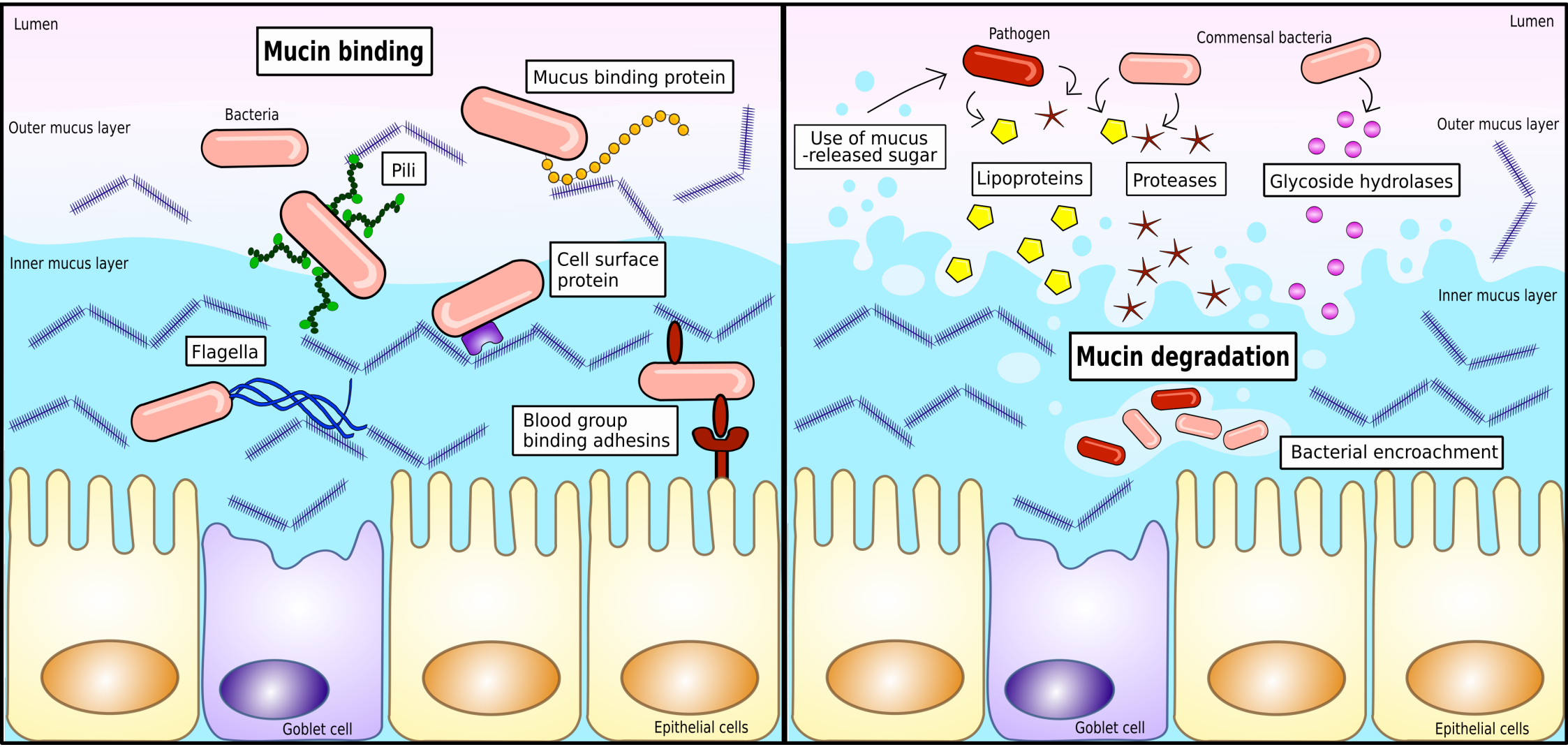
				complexity of the human gut microbiota	
***In vitro* human colonic models involving a mucosal phase**					
M-SHIME					
	* Series of bioreactor modeling the different parts of the human gut * Introduction of mucus-coated carriers (Mucus SHIME)	* Study the fine-scale spatial organization of the gut microbial ecosystem * Investigation of the interactions between commensals, pathogens, probiotics and luminal/mucosal gut microbiota	* Integration of human GI - related parameters and possibility to modulate them depending on diet, age and diseases (e.g. ulcerative colitis) * Capture dynamics by time-resolved analyses * Capture inter-individual variability of human gut microbiota * Possible long term experiments * Possible coupling with cell culture models and Host-Microbe Interactions (HMI) module	* Expensive and requires expertise and specialized instrumentation * Use of pig gastric mucin * No reproduction of immune and nervous system * No reproduction of the full complexity of the human gut microbiota * Donor-to-donor variability	Van den Abbeele *et al.*, 2012 Van den Abbeele *et al.*, 2013 De Paepe *et al.*, 2018
***In vivo* animal models**					
	* Whole organism models * Development of genetically modified mice with impaired mucin production (comparison with wild type animals)	* Study of the functional roles of mucin and mucus under physiological or pathological conditions at the level of entire organism * Investigation of downstream consequences of mucin modulation in mucosal barrier defense * Investigation of the interactions between commensals, pathogens, probiotics and luminal/mucosal gut microbiota	* Physiological model * Allow targeting of a specific gene/pathway in the complex gut-microbiota – host interactions * Amenable to diet or microbiome-based interventions * Possible long-term experiments	* Requires housing facility and adequate agreements * Expensive to maintain colonies *Housing husbandries and diets can modulate mouse microbiota * Murine gut microbiota different from the human gut microbiota * Mucin glycosylation profile of mice different from human intestinal mucins * No reproduction of the full complexity of the human gut microbiota * Limited translational capacity to human situation * Mice generally inbred so no reproduction of the genetic variations found in the human population	Van der Sluis *et al.*, 2006 Velcich *et al.*, 2002

Table 1. Experimental models available to study mucus-bacteria interactions.

GI : Gastro-Intestinal, HMI : Host-Microbe Interactions, IBD : Inflammatory Bowel Ddiseases, IVOC : *In vitro* organ culture, M-SHIME : Mucus Simulator of the Human Intestinal Microbial Ecosystem.


Lumen
Mucin binding
Mucus binding protein
Bacteria
Outer mucus layer
Pili
Inner mucus layer
Cell surface protein
Flagella
Blood group binding adhesins
Goblet cell
Epithelial cells
Lumen
Pathogen
Commensal bacteria
Use of mucus -released sugar
Outer mucus layer
Lipoproteins
Proteases
Glycoside hydrolases
Inner mucus layer
Mucin degradation
Bacterial encroachment
Goblet cell
Epithelial cells

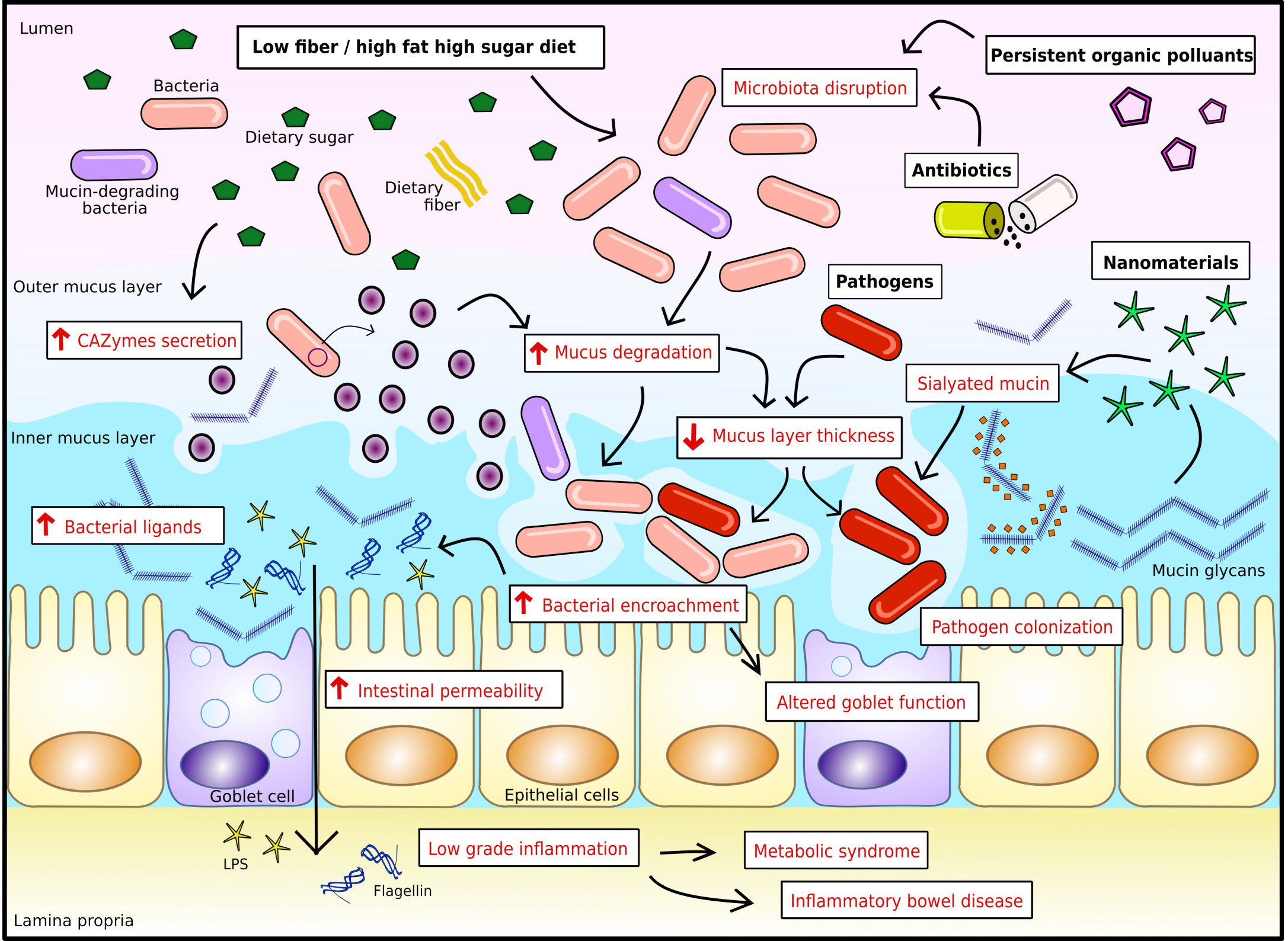

Lumen
Low fiber / high fat high sugar diet
Persistent organic polluants
Bacteria
Microbiota disruption
Dietary sugar
Antibiotics
Mucin-degrading bacteria
Dietary fiber
Nanomaterials
Outer mucus layer
Pathogens
CAZymes secretion
Mucus degradation
Sialyated mucin
Mucus layer thickness
Inner mucus layer
Bacterial ligands
Mucin glycans
Bacterial encroachment
Pathogen colonization
Intestinal permeability
Altered goblet function
Goblet cell
Epithelial cells
LPS
Low grade inflammation
Metabolic syndrome
Flagellin
Inflammatory bowel disease
Lamina propria

FIGURE LEGENDS

Fig. 1. Mucin-bacterial interactions in the digestive tract

Left panel: Mucins display various and diverse oligosaccharide structures representing potential binding sites for microbial adhesion. Commensal and pathogenic microbes can use cell-surface appendages, such as pili, flagella or fimbriae or adhesins to bind to mucus.

Right panel: Mucin glycans are an important energy source for microbes inhabiting the mucus niche that further confer them with an ecological advantage over other members of the gut microbiota. Commensal and pathogenic microorganisms can degrade mucin glycan chains leading to the release of mono- or oligosaccharides from that can be subsequently metabolized by other gut microbes in the mucosal environment.

Fig.2. Perturbations of the mucus barrier in response to environmental and microbial stimuli.

This figure represents an overview of the various factors (diets, nanomaterials, pollutants, antibiotics or invading pathogens) affecting the gut microbiota composition and/or the thickness, structure and composition of the mucus barrier. Disruption of the mucus layer promotes bacterial encroachment leading to the subsequent development of low-grade inflammation, associated with inflammatory bowel diseases and metabolic disorders.