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

Lucie Etienne-Mesmin, Benoit Chassaing, Mickaël Desvaux, Kim De paepe, Raphaële Gresse, Thomas Sauvaitre, Evelyne Forano, Tom Van De wiele, Stephanie Schüller, Nathalie Juge, et al.

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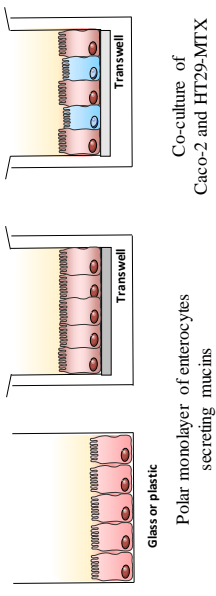
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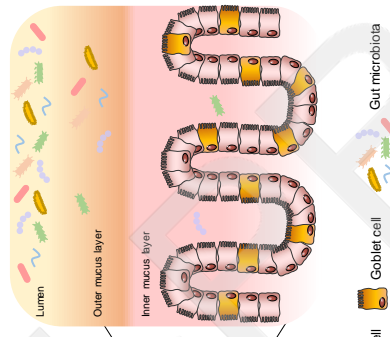
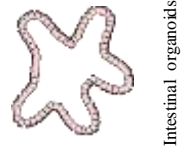
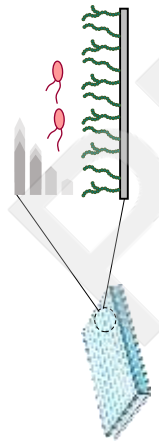
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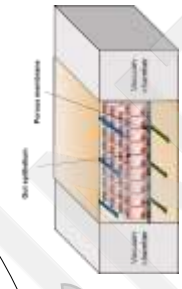
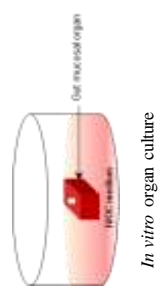


In vitro cell models

In vitro mucus/mucin binding assays



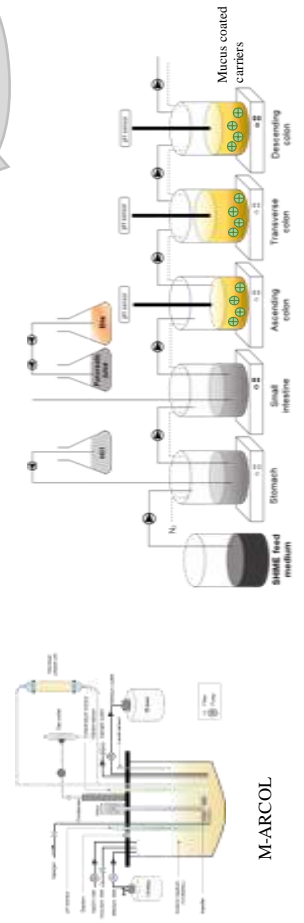
Ex-vivo organ cultures



In vivo animal models



In vivo digestive models with mucosal phase



20 **Corresponding authors:*
21 Stéphanie Blanquet-Diot (stephanie.blanquet@uca.fr)
22 28 place Henri Dunant
23 63000 Clermont-Ferrand, FRANCE
24 +33473178390
25
26 Nathalie Juge (nathalie.juge@quadram.ac.uk)
27 Gut Microbes and Health Institute Strategic Programme
28 Quadram Institute Bioscience
29 Norwich Research Park
30 NR4 7UA
31 Norwich, UK
32 +441603255068

PRE-PRINT

33 **ABSTRACT**

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

47

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

50

51 INTRODUCTION

52 The human gastrointestinal (GI) tract harbours a complex and diverse community of
53 microbes, including 10 trillion of microorganisms, collectively referred to as the gut
54 microbiota (Sender *et al.*, 2016). Several regulatory mechanisms cooperate to maintain
55 intestinal homeostasis and a disturbance of the relationship between the gut microbiota and
56 the host can result in several disorders including chronic inflammatory diseases and
57 metabolic syndromes (Rooks & Garrett, 2016). While the intestinal microbiota provides
58 important benefits to the host, such as calorie extraction and immune system maturation, it
59 also holds the power to activate various innate and adaptive immune signalling which can
60 lead to uncontrolled and deleterious intestinal inflammation (Pickard *et al.*, 2017). A key
61 component in maintaining a beneficial relationship between the commensal microbes
62 inhabiting the intestine and the host is the presence of an appropriate barrier that prevents
63 bacteria to reach and persist on the epithelial surface (Johansson & Hansson, 2016, Sicard *et*
64 *al.*, 2017, Bretin *et al.*, 2018). It is well acknowledged that intestinal epithelial cells (IECs)
65 provide a physical and biochemical barrier that prevents the translocation of commensal
66 bacteria to the underlying host tissue. In addition, there is an emerging paradigm that the
67 mucus layer is an important modulator of human health in mediating the homeostatic
68 relationship between the gut microbiota and the host. On the luminal side, the mucus layer
69 provides the first physical, chemical, and biological line of defence against large particles,
70 including commensal bacteria and invading pathogens, segregating them from IECs (Turner,
71 2009, Peterson & Artis, 2014). Furthermore, mucus provides a biological niche for a
72 microbial community, referred to as mucus-associated microbiota, which is likely to have a
73 major influence on human health (Martens *et al.*, 2018). However, advances in this field of
74 research have been hampered by the lack of suitable model systems recapitulating all the
75 interactions occurring at the mucosal interface. This review provides an overview of currently

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

79

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

81

82 1.1. Mucus structural organisation

83

84 **Mucus structure.** Mucus is a highly hydrated gel made up of more than 98% water that
85 makes it totally transparent, microscopically invisible and difficult to study. This aqueous
86 viscoelastic secretion also contains electrolytes, lipids and various proteins (Bansil & Turner,
87 2018). Mucus is found throughout the entire GI tract from the stomach to the large intestine,
88 with its thickness and structure varying depending on the location, reflecting its various
89 protective functions.

90 The mucus in the small intestine consists of one layer, while the stomach and colon
91 have a bi-layered mucus. In human stomach, the mucus is about 200–400 μm in thickness and
92 consists of an inner layer loosely attached to the epithelial surface, keeping the surface neutral
93 (pH 7) while the gastric lumen pH is acidic (pH 2), and an outer layer which is mobile on the
94 luminal side. Only few bacteria have evolved strategies to colonise the stomach, among which
95 *Helicobacter pylori* is a specialist (Atuma *et al.*, 2001, Juge, 2012). In the small intestine,
96 mucus fills up the space between the villi but is not attached to the epithelium and is
97 somewhat permeable to bacteria (Atuma *et al.*, 2001). In the colon, the two layers mediate
98 opposite interactions with the microbiota; whereas the outer layer (up to 800 μm) is densely
99 colonised by an important microbial biomass, the inner layer (>200 μm in humans) is virtually
100 devoid of bacteria leaving a space virtually free of microbes (commensals and or pathogens)
101 leaving a space virtually free of microbes above the epithelium (Johansson *et al.*, 2013).
102 However, single-cell imaging at tissue scale in mice revealed the presence of bacteria in close
103 proximity of the epithelium (Earle *et al.*, 2015). Among commensal microorganisms,
104 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.*,

130 2014). Apart from their role in secreting mucus, small intestinal goblet cells can play a role in
131 delivering luminal material to the immune system (Pelaseyed *et al.*, 2014). Interestingly, a
132 study from Gunnar Hanson's laboratory identified a subpopulation of goblet cells called
133 "sentinels" goblet cells (Birchenough *et al.*, 2016). These cells are able to sense Toll-like
134 receptor (TLR) microbial ligands at the entrance of colonic crypts and trigger the activation of
135 NLRP6 inflammasome, leading to mucus secretion from neighbouring goblet cells to defend
136 the colon against bacterial invasion (Birchenough *et al.*, 2016).

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

141
142 ***Gastro-intestinal mucins.*** The main structural components of mucus are large glycoproteins
143 called mucins. The protein sequences of mucin domains share a common core structure rich
144 in the amino acids proline (P), threonine (T) and serine (S) called the PTS domain. These
145 domains are then decorated by *O*-linked glycans made up of *N* acetylgalactosamine
146 (GalNAc), *N*-acetylglucosamine (GlcNAc), galactose (Gal) and usually terminated by sialic
147 acid and fucose (Juge, 2012, Johansson & Hansson, 2016, Sicard *et al.*, 2017). These *O*-
148 glycans render the mucin domains highly resistant to protease degradation and confer mucins
149 their high-water binding capacity.

150 Mucins are produced as transmembrane mucins or secreted gel forming mucins (Juge,
151 2012, Johansson *et al.*, 2013). In the stomach, MUC1 and MUC5AC are produced by the
152 superficial epithelium, while MUC6 are secreted by the stomach glands (Johansson *et al.*,
153 2013, Johansson & Hansson, 2016). In the small intestine and colon, mucus is structurally
154 built around the mucin-2 glycoprotein (MUC2). The folding and dimerization of MUC2 is a

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

163

164 **Mucin glycosylation.** Glycosylation is the most frequent post-translational modification of
165 proteins and can occur in *N*-linked and *O*-linked form, and *O*-glycosylation is the main
166 modification of mucins (Arike & Hansson, 2016). Mucin-type *O*-glycans are built from eight
167 core structures, with core 1, core 2, core 3 and core 4 glycans most commonly found in
168 intestinal mucins (Brockhausen *et al.*, 2009). *O*-glycosylation is initiated in the Golgi
169 apparatus by the addition of a GalNAc residue to the hydroxyl group of serine and threonine
170 of the mucin backbone. Further elongation and branching of the *O*-glycan chains is governed
171 by a large family of glycosyltransferase enzymes (Bennett *et al.*, 2012). The oligosaccharides
172 can be further modified by addition of histo-blood group antigens (ABO, Lewis), secretor (H)
173 epitopes and sialic acids and sulfate (Rossez *et al.*, 2012, Bansil & Turner, 2018). Mucin
174 glycosylation varies along the GI tract (Robbe *et al.*, 2003, Robbe *et al.*, 2004, Holmen
175 Larsson *et al.*, 2013) and is linked to microbial colonization (Juge, 2012, Bergstrom & Xia,
176 2013, Tailford *et al.*, 2015, Arike *et al.*, 2017). Mounting evidence suggests that mucin
177 glycosylation is critical to the biological and physical role played by mucus in the gut by
178 influencing the physico-chemical properties and penetrability of mucus and by modulating the
179 composition of the associated mucus-associated microbiota (see section 1.3). Not

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

184

185 **1.2. Mucus function in the gut**

186

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

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

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

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

207 The mucus layer also helps in the protection of the epithelium and, in association with
208 the immune system, plays a crucial role in intestinal homeostasis. This gel is an important
209 retention matrix for non-mucin proteins with immune regulatory molecules such as
210 antimicrobial molecules (e.g. bactericidal RegIII γ , α -defensins, secretory immunoglobulins
211 IgAs, etc), therefore limiting the number of bacteria that can reach the epithelium and the
212 underlying immune system (Peterson & Artis, 2014, Johansson & Hansson, 2016). This
213 physical and biological barrier helps to keep the tremendous amount of bacteria that reside in
214 the lumen as well as enteric pathogens at a safe distance from the epithelium (Chassaing *et*
215 *al.*, 2014, Johansson *et al.*, 2014, Chassaing *et al.*, 2015). However, this system can be
216 subverted and invading pathogens or pathobionts have evolved strategies to circumvent this
217 barrier by e.g. degrading mucins and/or influencing mucin secretion (Rolhion & Chassaing,
218 2016). In summary, mucus has a dual role in relation to the gut microbiota, it is an ecological
219 niche for bacteria by providing adhesion sites and nutrients, while protecting the underlying
220 epithelium from microbial aggressors that can breach this barrier.

221

222 **1.3. The mucus-associated microbiota**

223

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

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

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

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

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

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

268

269 **2. Mucin-bacteria interactions**

270

271 **2.1. Mechanisms of mucin binding by commensal and pathogenic microorganisms in** 272 **the gut**

273

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

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

280

281 ***Mucus binding proteins.*** Mucus-binding proteins (MUBs) containing a variable number of
282 Mub repeats are unique to gut inhabiting Lactobacilli and these proteins have been thoroughly
283 characterised in *Lactobacillus reuteri*, a gram-positive bacterial species inhabiting the GI tract
284 widely used as a probiotic (Frese *et al.*, 2011). MUB from *L. reuteri* ATCC 53608 is one of
285 the best studied examples of mucus adhesins in commensal bacteria. It is a large protein
286 consisting of six type 1 repeats (Mub1) and eight type 2 repeats (Mub2) with each repeat
287 divided into a mucin binding (MucBP) domain and an immunoglobulin binding protein
288 domain (Kuznetsova, 1990, MacKenzie *et al.*, 2009, Etzold *et al.*, 2014). The Mub repeats
289 mediate binding to mucin glycans, through interactions with terminal sialic acid (Etzold *et al.*,
290 2014, Gunning *et al.*, 2016), and Igs (MacKenzie *et al.*, 2009). MUB has the shape of a long,
291 fibre-like structure, of around 180 nm in length (Etzold *et al.*, 2014), and forms appendices
292 reminiscent to pili found in pathogenic and, more rarely, other commensal bacterial species.
293 However, in contrast to pathogenic pili which adhesin is restricted to the N-terminal tip, MUB
294 interactions with mucin glycans occur through its long and linear multi-repeat structure, as
295 shown by atomic force spectroscopy (Gunning *et al.*, 2016). This multivalent binding would
296 restrict penetration through mucus and limit access of the bacteria to the epithelium surface.
297 In addition, MUB from *L. reuteri* ATCC 53608 was recently shown to modulate
298 inflammatory responses in human monocyte-derived dendritic cells *via* interaction with DC-
299 SIGN (Bene *et al.*, 2017). The presence of mucus adhesins was also shown to mediate the
300 binding of *L. reuteri* strains to both HT-29 and mucus-producing LS174T cells. The binding
301 of *L. reuteri* to mucus led to a decreased enteropathogenic *E. coli* (EPEC) adherence to small
302 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

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

334

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

348 Although not a resident member of the gut microbiota, several *Lactococcus lactis*
349 strains have also been shown to exhibit mucus-binding properties through bacterial surface
350 proteins such as mucin-binding proteins and pili (as recently reviewed in (Mercier-Bonin &
351 Chapot-Chartier, 2017)). The mechanisms of adhesion have been extensively studied by
352 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

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

391

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

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

408

409 **2.2. Mechanisms of mucin degradation by commensal and pathogenic** 410 **microorganisms in the gut**

411

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

415

416 **Glycoside hydrolases.** Mucin glycan degradation in bacteria relies on the expression of GHs
417 such as sialidases (GH33), α -fucosidases (GH29, GH95), exo- and endo- β -N-
418 acetylglucosaminidases (GH84 and GH85), β -galactosidases (GH2, GH20, GH42), α -N-
419 acetylglucosaminidases (GH89), endo- β 1,4-galactosidases (GH98) and α -N-
420 acetylgalactosaminidases (GH101, GH129) (www.cazy.org). These enzymes have been
421 functionally characterised in resident members of the gut microbiota able to forage on mucins,
422 including *A. muciniphila*, *B. thetaiotaomicron*, *B. bifidum*, *B. fragilis*, and *R. gnavus*, as
423 recently reviewed (Tailford *et al.*, 2015, Ndeh & Gilbert, 2018). The released mono- or
424 oligosaccharides derived from mucus degradation by these commensal bacteria can be utilised
425 by the bacteria itself or scavenged by other bacteria inhabiting the mucus niche including
426 pathogenic species such as *Salmonella* species, *C. difficile*, diarrhoeagenic *E. coli*, or *Vibrio*
427 *cholerae* through cross-feeding interactions (Fabich *et al.*, 2008, Abyzov *et al.*, 2012, Ng *et*

428 *al.*, 2013). In addition, some of these pathogens have the glycolytic potential to release
429 mucus-derived sugars for their own consumption (Mondal *et al.*, 2014, Arabyan *et al.*, 2016).
430
431 **Sulfatases.** Sulfatases are being increasingly investigated for their role in modulating the gut
432 microbial ecosystem in health and disease. Some members of the gut microbiota such as *B.*
433 *thetaitaomicron*, *Bacteroides ovatus*, and Prevotella sp. strain RS2 *Bifidobacterium breve*
434 UCC2003, or *B. fragilis* possess mucin- desulfating sulfatases or glycosulfatases (Salyers *et*
435 *al.*, 1977, Berteau *et al.*, 2006, Benjdia *et al.*, 2011, Egan *et al.*, 2016, Praharaj *et al.*, 2018).
436 Mucin sulfatase activity of these species may provide them a competitive advantage in the
437 infant gut and/or the adult gut. The mucin- desulfating sulfatases that have been characterised
438 so far include sulfatases specific for the - D- galactopyranosyl 3- sulfate, -
439 Dgalactopyranosyl6- sulfate, and 2- acetamido- 2- deoxy- D- glucopyranosyl6- sulfate
440 (6- SO₃- GlcNAc) building blocks of the oligosaccharide chains. GlcNAc-6-S can be found
441 in terminal or branched positions of mucin oligosaccharide. The desulfation of mucin by
442 bacterial sulfatases may be a rate-limiting step in mucin degradation mechanism, allowing
443 glycosidases to access and act on the mucins by other members of the gut microbiota. The
444 release of sulfate from mucins may also contribute to the expansion of Sulfate-reducing
445 bacteria (SRB) in the gut (Rey *et al.*, 2013). SRB are able to produce hydrogen sulfide (H₂S)
446 which can reduce disulfide bonds present in the mucus network, leading to mucus erosion and
447 access of bacteria to the epithelium, therefore contributing to epithelial damage and
448 inflammation This mechanism has been proposed to be involved in the aetiology and/or
449 severity of IBD (Ijssennagger *et al.*, 2016). In addition, Hickey and colleagues showed that
450 sulfatases of *B. thetaitaomicron* are required for its outer membrane vesicles to transit to
451 underlying host immune cells and cause colitis (Chatzidaki-Livanis & Comstock, 2015).
452 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

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

479 Hbp (hemoglobin-binding protease), also previously known as Tsh (temperature-
480 sensitive haem-agglutinin), is capable of cleaving bovine submaxillary mucin but not hog
481 gastric mucin, which so far would appear as a feature of mucinolytic serine protease
482 autotransporter of Enterobacteriaceae (SPATE) of the peptidase S6 family (Dutta *et al.*,
483 2002). In some EHEC strains, a SPATE of the peptidase S6 family exhibiting mucinolytic
484 activity was identified on plasmid pO113, namely EpeA (EHEC plasmid-encoded
485 autotransporter) (Leyton *et al.*, 2003). In AIEC, a Vat (vacuolating autotransporter)
486 homologue belonging to the SPATE of the peptidase S6 family was demonstrated to exhibit a
487 mucinolytic activity (Gibold *et al.*, 2016). Vat-AIEC appears to significantly contribute to the
488 colonisation ability of AIEC by decreasing mucus viscosity as well as enhancing bacterial
489 penetration in mucus and access to IECs (Gibold *et al.*, 2016).

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

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

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

513

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

515

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

525

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

533 Compared to conventionally housed animals, germ-free mice have fewer goblet cells,
534 which are smaller in size (Kandori *et al.*, 1996) and harbour an impaired mucus layer,
535 indicating that the formation of the protective mucus layer depends upon the presence of
536 bacteria (Rodriguez-Pineiro & Johansson, 2015). Johansson and colleagues demonstrated that
537 the mucus of germ-free mice displayed a significant decrease in Muc2 level and was more
538 penetrable to bacterium-size fluorescent beads as compared to conventionally raised mice
539 (Johansson *et al.*, 2015). The gut microbiota composition of germ free animals is normalized
540 two weeks after colonisation in terms of microbiota composition, but up to 8 weeks are
541 needed to reach a normalized mucus phenotype (Johansson *et al.*, 2015, Hayes *et al.*, 2018).
542 In support of this, fortification of the mucus layer and increased diversity of mucin
543 glycosylation was observed within 48 hours of human intestinal organoid colonization with
544 human-derived, non-pathogenic *E. coli* (Hill *et al.*, 2017). Some bacteria, in particular
545 Anaerostipes, have been shown to display mucus-stimulating properties (Jakobsson *et al.*,
546 2015). *Lactobacillus* species can also stimulate MUC2 production and secretion by the goblet
547 cells in the human gut (Sicard *et al.*, 2017). Representative members of the two main phyla of
548 the gut microbiota, *B. thetaiotaomicron* and *Faecalibacterium prauznitzii* can modulate goblet
549 cell differentiation and thus mucus production (Wrzosek *et al.*, 2013). A recent study showed
550 that *Streptococcus thermophilus*, a transient food-borne bacterium, was able to induce mucus

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

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

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

577

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

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

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

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

621

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

624 **Fibres.** Living in symbiosis with the host, the gut microbiota depends mostly on non-
625 digestible fibres and polysaccharides as energy source. In the absence of fibres in the diet, the
626 gut microbiota shifts towards the utilisation of host glycans such as those provided by mucins,
627 resulting in a thinner protective colonic mucus (Sonnenburg & Sonnenburg, 2014, Earle *et al.*,
628 2015). Accordingly, Desai and colleagues demonstrated that a low-fibre diet promotes the
629 enrichment of mucin-degrading bacteria and the overexpression of carbohydrate-active
630 enzymes (CAZymes) that degrade the colonic mucus barrier (Desai *et al.*, 2016, Martens *et*
631 *al.*, 2018). In these mice fed with a deprived-fibre diet, infection with *C. rodentium* promotes
632 greater epithelial access and lethal colitis (Desai *et al.*, 2016).

633 A lack of fermentable fibres in the diet also leads to a reduction in epithelial cell
634 proliferation resulting in a thin mucosa with encroached bacteria (Chassaing *et al.*, 2015).
635 Further studies in mice reported that inulin supplementation increases the number of colonic
636 goblet cells, which correlates with a thicker mucus layer and an increase proportion of the
637 *Akkermansia* genus (Kleessen *et al.*, 2003, Everard *et al.*, 2013). Similarly, studies in mice
638 showed that a diet enriched in inulin fibre prevents mucus deterioration (Schroeder *et al.*,
639 2018). It was recently showed that inulin but not cellulose protects against diet-induced
640 obesity by reducing microbiota encroachment in a cytokine IL-22-dependent manner (Zou *et*
641 *al.*, 2018), demonstrating the importance of dietary factors, especially soluble fibre, in the
642 homeostasis of host-microbiota relationship. Considering the increased mucus foraging
643 activity occurring when mice are fed with a low-fibre diet, a recent study showed that
644 supplementation with probiotic bifidobacteria (*B. longum*) or prebiotic fibre (inulin) could
645 reduce such mucus defect. Notably, administration with *B. longum* was sufficient to restore
646 mucus growth, while administration with inulin could prevent the increase of mucus
647 penetrability in mice fed a western style diet (WSD) (Schroeder *et al.*, 2018).

648

649 **Western diet and food additives.** Besides fibres, other nutrients within a WSD can
650 modulate intestinal barrier function. A WSD is a rich in saturated fats and simple
651 carbohydrates but depleted in dietary fibres. As a result, a diet-induced obesity in mice leads
652 to colon mucosal barrier dysfunction with a thinner mucus layer (as described above) and
653 treatment with *A. muciniphila* appears to counteract this effect by improving mucus thickness
654 (Everard *et al.*, 2013). Similarly, mice fed a high-fat and high-sugar diet exhibit an increased
655 abundance of mucin-degrading species leading to a decrease in mucus thickness (Martinez-
656 Medina *et al.*, 2014). The diet of modern societies has dramatically changed as evidenced by a
657 steady increase in the consumption of processed foods concomitantly with an increase in the
658 use of food additives (Chassaing *et al.*, 2015). Mice treated with dietary emulsifiers
659 (polysorbate 80 or carboxymethylcellulose) show a reduced mucus thickness and increased
660 gut permeability. In these animals, some bacteria appear in close contact with the epithelium.
661 Emulsifier-treated mice have an altered microbial composition associated with increased
662 levels of mucolytic bacteria including *R. gnavus* and a marked reduction in microbial
663 diversity, with a bloom in *Verrucomicrobia* phyla, especially *A. muciniphila* (Chassaing *et al.*,
664 2015). This may further contribute to the intestinal passage of bacterial constituents such as
665 LPS and flagellin, which participates in the development of low-grade inflammation and
666 metabolic disorders in wild type mice and of colitis in susceptible host animals (Chassaing *et*
667 *al.*, 2014, Chassaing *et al.*, 2015, Chassaing *et al.*, 2017). Mice fed with diets enriched in
668 maltodextrin, a filler and thickener used in food processing, show a reduction of *Muc2*
669 expression, making the host more sensitive to low-grade inflammation but with no significant
670 change in mucosa-associated microbiota (Laudisi *et al.*, 2018).

671

672 **Effect of food contaminants on mucus.** The intestinal mucosa is increasingly appreciated as a
673 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 (TiO₂) 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.***

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

716 These changes in mucus composition were also mirrored by changes in the gut
717 microbiota composition at the mucosal surface. IBD patients exhibit a disproportion of
718 mucin-degrading (or mucinolytic) bacteria with an increased abundance of *Ruminococcus*
719 *torques* and *R. gnavus*, but a decreased abundance of *A. muciniphila*. In addition, the
720 expansion of certain pathobionts and in particular AIEC exhibiting mucinolytic activity has
721 been reported to favour gut colonisation and further induce inflammation in CD (Palmela *et*
722 *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

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

777

778 **4.1. *In vitro* mucus/mucin binding assays**

779

780 ***Microplate assays.*** Several microtiter plate assays have been developed for testing bacterial
781 adhesion to mucus and/or mucin (McNamara *et al.*, 2000, Gusils *et al.*, 2004). These
782 generally rely on the immobilisation of mucins or mucus to the wells of microtiter plates
783 following incubation overnight at 4°C or at room temperature in buffers such as such as PBS
784 (pH 7-7.5), HBSS (pH 7-7.5) or carbonate buffer (pH 9.6) (Gusils *et al.*, 2004, Dague *et al.*,
785 2010, Mackenzie *et al.*, 2010, Chagnot *et al.*, 2013). BSA (bovine serum albumin) is
786 generally used as a negative control for assessing the specificity of the binding to mucus
787 and/or mucin. Binding assays are usually performed at 37°C and the contact time with
788 bacterial cells generally ranges between 30 min to 3 hours before washing to remove non-
789 adhered bacteria. Antibiotic at growth inhibiting concentration, such as chloramphenicol, or
790 sometimes thermic treatment can be applied to prevent the growth of microorganisms in the
791 course of the adhesion assay. Binding can be determined using crystal violet staining of the
792 adhered microbial biomass (Azeredo *et al.*, 2017) or by Enzyme-linked Immunosorbent
793 Assay (ELISA) when specific antibodies against bacteria are available (Skoog *et al.*, 2012),
794 by measurement of viable counts after plating of the cells (McNamara *et al.*, 2000) or by
795 quantitative PCR (Skoog *et al.*, 2012). Alternatively, bacteria can be labelled with a
796 radioactive probe or a fluorescent dye before inoculation and the binding quantified using a
797 scintillation counter or a fluorometer, respectively (Gusils *et al.*, 2004, Mackenzie *et al.*,

798 2010). Microbial cells can also be labelled by biotinylation and further assayed using
799 streptavidin-HRP by ELISA (Sheng *et al.*, 2012). Quantification of microbial binding to
800 mucin can also be performed by flow cytometry, where microbial cells are put in contact with
801 mucin labelled with a fluorescent tag (de Repentigny *et al.*, 2000).

802

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

812

813 **Mucin microarrays.** The carbohydrate microarray technology offers a powerful platform
814 where natural or synthetic glycans are immobilized onto a solid support. (Poole *et al.*, 2018). .
815 Microarrays incorporating mucins from various sources onto different chips surfaces provide
816 a high-throughput approach to screen bacteria-mucin interactions as well as identify glycan-
817 binding proteins and glycan epitopes involved in this interaction (Clyne *et al.*, 2017). For
818 example, the use of mucin microarrays revealed that *C. jejuni* and *H. pylori* recognised
819 distinct mucin receptors despite being closely related phylogenetically (Naughton *et al.*,
820 2013). Recently, *H. pylori* was shown to interact with trefoil factor family (TFF) protein
821 TFF1 (Reeves *et al.*, 2008), and that TFF1 specifically interacts with human gastric mucin but
822 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 (Hirno *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

848 these studies, human colonic mucin (HCM) was immobilised on the sensor chip whereas
849 bacterial cells were eluted as analytes. Using sialidase or sulfatase, it was further possible to
850 discriminate some strains of lactobacilli and bifidobacteria that could specifically bind to the
851 sialic acid or sulphate residues of HCM respectively (Huang *et al.*, 2013). Single-cell force
852 spectroscopy (SFCS) has been used to quantify the adhesion forces of *L. rhamnosus* with
853 mucin at a single-cell level, pinpointing heterogeneities in the bacterial population (Sullan *et*
854 *al.*, 2014). More recently, further molecular details of mucin-bacteria interactions were
855 investigated using atomic force microscopy (AFM). Such an approach was used for the first
856 time to accurately quantify the force of adhesion of *L. lactis* cells immobilised on the AFM tip
857 to PGM at nanoscale level (Dague *et al.*, 2010). Surprisingly, it was found that PGM coating
858 strongly reduced the bacterial adhesion force compared to bare polystyrene, highlighting the
859 interplay between electrostatic, hydrophilic and steric repulsions, and that both specific and
860 non-specific interactions need to be considered (Dague *et al.*, 2010). These results were
861 consistent with a previous investigation of the muco-adhesive properties of *L. lactis* using
862 quartz crystal microbalance with dissipation monitoring (Le *et al.*, 2012). Using bacteria
863 mutant strains, AFM was also used to provide molecular insights into the respective role and
864 contribution of mucus-binding proteins and surface organelles (pili or flagella) in muco-
865 adhesion (Le *et al.*, 2013). Interactions at the protein-protein level were further investigated
866 by AFM to study the adhesive properties of *L. reuteri* Mub with mucins (Gunning *et al.*,
867 2016).

868

869 **4.2. *In vitro* mucin-secreting cell models**

870

871 **Monoculture models.** While many colon carcinoma cell lines express mRNAs encoding
872 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.c116E 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

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

928

929 **Co-culture models.** To model human intestinal epithelia, mixed cultures of enterocyte-like
930 Caco-2 cells and mucus-producing HT29-MTX cells have been widely used in drug
931 absorption and permeability studies (Hilgendorf *et al.*, 2000, Lozoya-Agullo *et al.*, 2017). Co-
932 cultures prepared with different ratios of Caco-2 and HT29-MTX cells seeded out on
933 Transwell inserts formed a continuous mucus layer similar to cultures of HT-29-MTX cells
934 grown alone (Poquet *et al.*, 2008, Beduneau *et al.*, 2014). Notably, the probiotic strains *L.*
935 *rhamnosus* GG or bifidobacteria as well as pathogenic strains of *E. coli* or *L. monocytogenes*
936 adhered better to mucus-deficient Caco-2 cells than to mucus-producing HT-29MTX cells or
937 Caco-2/HT-29 co-cultures (Laparra & Sanz, 2009). Considering the “closed” oxygen-
938 restricted environment in the human gut, Chen and colleagues developed a 3D porous silk
939 scaffolding in the shape of a hollow tube. While the inner tube wall was coated with Caco-
940 2/HT29-MTX epithelia, primary human intestinal myofibroblasts were grown in the tube
941 scaffold space underneath to support epithelial growth and differentiation. Notably, epithelia
942 grown on 3D scaffolds demonstrated increased MUC2 production compared to Transwell
943 cultures resulting in the formation of a mucus layer of 11-17 μm thickness (Chen *et al.*, 2015).

944

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

948 under cyclic strain mimicking peristaltic motion, the chambers above and below the cell
949 membrane are constantly perfused with medium, thereby generating low shear stress. This
950 environment stimulates the formation of 3D intestinal villi similar to those found in the small
951 intestine (Kim *et al.*, 2012), and the differentiation of Caco-2 cell into absorptive enterocytes,
952 and also includes enteroendocrine cells, Paneth cells and mucus-producing goblet cells (Kim
953 & Ingber, 2013). In addition, Caco-2 epithelium grown in the Gut-on-a-Chip model display
954 enhanced barrier function and mucus production as compared to static Caco-2 cell cultures
955 (Kim & Ingber, 2013). This system has recently been developed further to allow co- culture
956 with strict anaerobes (Shin *et al.*, 2019). Although the Gut-on-a-Chip devices have been
957 mostly used for long-term co-culture of IECs with commensal microbes under healthy
958 conditions, they are now being employed to model intestinal inflammation (Kim *et al.*, 2016).
959 Using a Gut-on-a-Chip model, the pathophysiological manifestation and dysregulated barrier
960 function observed during inflammation could be recapitulated which may help to gain insights
961 into disease mechanisms and assess potential therapeutic strategies (Shin & Kim, 2018).
962 Notably, probiotic VSL#3 targeted restoration of the mucosal barrier did not effectively
963 control the local inflammation nor improve mucus production (Shin & Kim, 2018). The
964 HuMix (Human Microbial Cross-talk) model is another microfluidic device enabling the co-
965 culture of Caco-2 cell monolayers with commensal bacteria under anaerobic conditions. In
966 contrast to the Gut-on-a-Chip system, the epithelial cells which do not produce mucus are
967 separated from the bacteria by a membrane coated with porcine gastric mucin (Shah *et al.*,
968 2016).

969

970

971 ***4.3. Ex vivo organ cultures***

972

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

978

979 *In vitro organ culture (IVOC)*. In 1969, Browning and Trier were the first to establish a
980 technique to culture human mucosal biopsies *ex vivo*. By using a specific culture medium and
981 incubation of the samples in 95% O₂, 5% CO₂ at 37°C, mucosal biopsies from the duodeno-
982 jejunal junction were kept alive for up to 24 hours demonstrating epithelial cell proliferation,
983 fat absorption and active mucus secretion by goblet cells (Browning & Trier, 1969). The
984 advantages of IVOC of intestinal biopsies versus cell line culture models include the presence
985 of healthy non-transformed cells including all major IEC types (enterocytes, goblet cells,
986 Paneth cells and neuroendocrine cells), underlying basement membrane and mucosal tissue,
987 and the production of mucus. While it is problematic to maintain the loose outer mucus layer
988 of colonic biopsies during sampling, the inner colonic and small intestinal mucus layers are
989 generally well preserved as evidenced by microscopy (Haque *et al.*, 2004, Walsham *et al.*,
990 2016, Hews *et al.*, 2017). IVOC of biopsy samples has been used to investigate adherence of
991 pathogenic bacteria such as EPEC (Knutton *et al.*, 1987, Schüller *et al.*, 2007), EHEC
992 (Phillips *et al.*, 2000, Fitzhenry *et al.*, 2002, Lewis *et al.*, 2015), ETEC (Knutton *et al.*, 1989,
993 Baker *et al.*, 1997) and *C. jejuni* (Grant *et al.*, 2006) to human intestinal mucosa. In addition,
994 IVOC demonstrated cytotoxic effects of bacterial toxins, such as Pet toxin from EAEC
995 (Henderson *et al.*, 1999), Shiga toxin from EHEC (Schüller *et al.*, 2004) and *C. difficile* toxin
996 A (Mahida *et al.*, 1996) on intestinal epithelium or mucosa. Interactions of enteropathogenic
997 bacteria with mucus production were observed in small intestinal and colonic biopsy tissue

998 infected with EAEC where bacteria were predominantly associated with a thick mucus layer
999 above the epithelium, which was not present in non-infected control samples (Hicks *et al.*,
1000 1996, Andrade *et al.*, 2011). This suggests that EAEC stimulates mucus secretion which
1001 agrees with the production of mucoid stools during EAEC diarrhoea (Croxen *et al.*, 2013).
1002 Similarly, stimulation of mucus secretion and bacterial binding to the mucus layer were
1003 observed in biopsy samples from the terminal ileum infected with *S. Typhimurium*. This was
1004 followed by *Salmonella* adherence and invasion of the epithelium accompanied by ruffling of
1005 the host cell membrane (Haque *et al.*, 2004). Recently, the IVOC system was used to show
1006 that the metalloprotease StcE diminishes the inner mucus layer and enhances EHEC
1007 adherence to human colonic biopsy epithelium (Hews *et al.*, 2017).

1008
1009 ***Polarised IVOC (pIVOC)***. While the traditional IVOC system allows bacterial access
1010 to the mucosal and submucosal side of the biopsy, polarised organ culture models have been
1011 developed which limit bacterial contact to the mucosal side of the tissue. This is particularly
1012 relevant when studying host responses to bacterial infections where artificial interactions with
1013 immune cells in the lamina propria might confound experimental readouts. Using a pIVOC
1014 approach by mounting colonic tissue explants between two Perspex disks in a Snapwell plate,
1015 Raffatellu and colleagues demonstrated that *Salmonella* Typhi reduced mucosal expression of
1016 the pro-inflammatory cytokine interleukin (IL)-8 by production of a capsule which masked
1017 pathogen-associated molecular patterns such as LPS and flagellin (Raffatellu *et al.*, 2005). In
1018 addition, pIVOC showed that apical exposure to EPEC or purified H6 flagellin induced IL-8
1019 expression in duodenal biopsies (Schüller *et al.*, 2009). Furthermore, infection with *C. jejuni*
1020 stimulated the production of reactive oxygen species (ROS) in duodenal and colonic mucosa
1021 (Corcionivoschi *et al.*, 2012). The pIVOC system has also been used to study the interaction
1022 of probiotic bacteria with mucosal tissue, and incubation of duodenal explants with *L. reuteri*

1023 demonstrated localisation of bacteria in the mucus layer but not in the epithelium.
1024 Nevertheless, pre-incubation with *L. reuteri* reduced EPEC adherence to the epithelium
1025 (Walsham *et al.*, 2016). A different approach to restrict bacterial access to the epithelial
1026 surface was developed by Tsilingiri and colleagues by gluing a perspex cylinder to the
1027 mucosal side of colonic resection tissue (Tsilingiri *et al.*, 2012). Surprisingly, apical
1028 incubation with probiotic *L. plantarum* resulted in degeneration of mucosal tissue from
1029 healthy donors, whilst all three strains studied (*L. paracasei*, *L. rhamnosus*, *L. plantarum*)
1030 caused tissue damage in resections from patients with IBD. In contrast, supernatants from *L.*
1031 *paracasei* reduced inflammation in *Salmonella*-infected and IBD tissue. As the maintenance
1032 of larger tissue samples requires incubation in high levels of oxygen (95-99%), the use of
1033 IVOC to study interactions of oxygen-sensitive bacteria with human intestinal mucosa
1034 remains problematic. However, a novel murine 3D-intestinal organ culture system was
1035 recently developed whereby an intact intestinal fragment was lumenally perfused with de-
1036 gassed medium containing anaerobic bacteria while the serosal side of the tissue was
1037 maintained under humidified oxygenated conditions. Whilst preserving gut tissue
1038 architecture, the system also supported the growth of commensal microbes (*Clostridium*
1039 *ramosum* and SFB) and allowed assessment of their impact on the immune and nervous
1040 system (Yissachar *et al.*, 2017).

1041
1042 ***Human enteroids/colonoids and intestinal organoids.*** New technologies have been
1043 developed which enable the generation of self-propagating spheres of primary intestinal
1044 epithelial cells (“mini-guts”). Enteroids or colonoids are derived from adult stem cells isolated
1045 from the crypts of human small intestinal or colonic tissue, respectively (Jung *et al.*, 2011,
1046 Sato *et al.*, 2011). In contrast, human intestinal organoids (HIOs) are established by
1047 differentiation of embryonic or, more often, induced pluripotent stem cells (genetically

1048 reprogrammed adult stem cells) (Spence *et al.*, 2011). In comparison to enteroids, HIOs lack
1049 maturation and more closely resemble foetal than adult intestine. In addition, they are devoid
1050 of functional intestinal stem cells and surrounded by a mesenchyme which is absent in
1051 enteroids (Sinagoga & Wells, 2015, Leslie & Young, 2016). As the apical side of the
1052 epithelium is facing inwards, infection of spheroid enteroids/HIOs with bacteria requires
1053 microinjection. Studies on the anaerobic pathogen *C. difficile* showed that injected bacteria
1054 remained alive in HIOs for up to 12 hours and caused disruption of epithelial barrier function
1055 *via* secretion of the toxin TcdA. Interestingly, oxygen measurements indicated reduced
1056 oxygen levels in the lumen of HIOs (5 to 15%). Furthermore, infection with *C. difficile*
1057 resulted in reduced MUC2 and mucus production in HIOs (Engevik *et al.*, 2015). HIOs also
1058 supported growth of EHEC and commensal *E. coli*. Infection with EHEC induced ROS
1059 production and an inflammatory response associated with recruitment of external neutrophils
1060 into HIO spheres (Karve *et al.*, 2017). Interestingly, colonisation of HIOs with commensal *E.*
1061 *coli* (ECOR2) stimulated enterocyte maturation, antimicrobial peptide secretion, production
1062 of a MUC2-containing mucus layer, and increased epithelial barrier function, thereby
1063 indicating the establishment of stable host-microbe symbiosis (Hill *et al.*, 2017).

1064 To facilitate incubations with bacteria, 2D enteroid systems have now been
1065 successfully developed where primary intestinal cells are grown as monolayers on permeable
1066 membrane supports. Previous studies showed that differentiated human enteroid and colonoid
1067 monolayers contained MUC2-producing goblet cells and formed a mucus layer of more than
1068 25 μm thickness (VanDussen *et al.*, 2015, In *et al.*, 2016). Two-dimensional enteroids and
1069 colonoids supported binding of EAEC, EHEC and EPEC (VanDussen *et al.*, 2015). More
1070 specifically, apical EHEC infection of colonoids resulted in the formation of characteristic
1071 attaching and effacing lesions, mucus degradation and reduced expression of the microvillar
1072 protein protocadherin 24, which was mediated by the secreted serine protease EspP (In *et al.*,

1073 2016). The 2D enteroid model was further refined by adding primary human macrophages to
1074 the basolateral side of the membrane support. Intriguingly, enteroid monolayers grown in the
1075 presence of macrophages exhibited increased cell height and barrier function. In addition,
1076 underlying macrophages were able to capture and kill EPEC and ETEC by extending
1077 projections across the epithelial monolayer (Noel *et al.*, 2017).

1078 In another approach to mimic the gut environment more closely, cells from human
1079 small intestinal enteroids were seeded on tubular silk sponge scaffolds and supported by
1080 primary human intestinal myofibroblasts as described for Caco-2/HT29-MTX (Section 4.2).
1081 The resulting intestinal model epithelium contained all four major epithelial cell types and
1082 exhibited tight junction formation, microvillus polarisation, digestive enzyme secretion, and
1083 low oxygen tension in the lumen. Moreover, infection with a laboratory strain of *E. coli*
1084 resulted in a significant innate immune response (Chen *et al.*, 2017). Recently, a Gut-on-a-
1085 Chip model based on primary intestinal epithelial cells has been developed which also
1086 includes co-culture of an underlying endothelium. Human enteroids are cultured on a side of a
1087 porous membrane within a microfluidic device whereas the intestinal microvascular
1088 endothelium is established on the other side of the filter.. This device reproduces the epithelial
1089 cells proliferation and host defenses more accurately (Kasendra *et al.*, 2018). Kim and
1090 colleagues showed that a human Gut-on-a-Chip micro device colonized by non-pathogenic
1091 bacteria (commensal and probiotic bacteria) was able to induce production of a key set of
1092 pro-inflammatory cytokines. This device enabled high level of mucus production on micro
1093 engineered intestinal villi, therefore providing a protective barrier to maintain long-term
1094 stable host–microbe coexistence (Kim *et al.*, 2016).

1095

1096 **4.4. *In vitro* human fermentation models involving a mucosal phase**

1097

1098 As aforementioned, the spatial positioning of gut microorganisms in the mucus layer is
1099 important with respect to their functional role in the human gut ecosystem. The microbial
1100 community residing in the mucus layer across the length of the GI tract is, however, hard to
1101 study given the difficulty to sample this region *in vivo*, especially in human (Macfarlane *et al.*,
1102 1997, Flint *et al.*, 2012, Donaldson *et al.*, 2016). *In vitro* colonic models involving a mucosal
1103 phase are a valuable alternative to study the fine-scale spatial organisation of the gut
1104 microbial ecosystem.

1105 Multiple colon *in vitro* models have been developed over the years, ranging from
1106 simple, single stage batch incubations to more complex and representative three stage
1107 continuous and semi-continuous reactor models (Miller & Wolin, 1981, Gibson *et al.*, 1988,
1108 Allison *et al.*, 1989, Blanquet-Diot *et al.*, 2012, McDonald *et al.*, 2013, Van den Abbeele *et*
1109 *al.*, 2013). These continuous fermentation models, inoculated with faecal samples of donors,
1110 recapitulate the main biotic and abiotic parameters of the human colon, such as temperature,
1111 pH, residence time, supply of nutritive medium reproducing the composition of ileal effluents,
1112 therefore enabling the study of a complex and metabolically active gut microbiota under
1113 anaerobiosis conditions. In these fermentation models, the bioreactors can be inoculated with
1114 fresh or frozen stools provided by individual or pool of different donors being healthy human
1115 volunteers with no history of antibiotic treatment 2 to 6 months before the beginning of the
1116 study.

1117
1118 ***Introduction of mucus carriers in human in vitro colonic models.*** Mucins in solution have
1119 been frequently included in these colonic models to provide a nutrient source to the gut
1120 bacteria, but the study of mucus colonisation by gut bacteria in these systems has been
1121 revolutionized by the ability to simulate the viscoelastic gel-like nature of the mucus layer
1122 through the incorporation of mucus carriers (Gibson *et al.*, 1988, Macfarlane *et al.*, 1989,

1123 Macfarlane *et al.*, 2005, Van Herreweghen *et al.*, 2017). MacFarlane first demonstrated a
1124 rapid colonisation of an agar-mucus layer during a 48-hour incubation in a two-stage
1125 continuous fermentor system by a mixture of *Bacteroides*, enterobacteria and facultative
1126 anaerobes (Macfarlane *et al.*, 2005). However, the use of glass tubes, containing this agar-
1127 mucus layer, in this set-up did not permit a practical long-term implementation (Van den
1128 Abbeele *et al.*, 2009). Mucus-coated beads (mixture of 5% porcine mucin type II and 1%
1129 agar) have since been identified as crucial platforms in sustaining microbial diversity by
1130 selectively enriching species, which are not thriving in the luminal environment. This mucus
1131 interphase was introduced in the Simulator of the Human Intestinal Microbial Ecosystem
1132 (SHIME) resulting in the M-SHIME (Mucus SHIME) configuration (Van den Abbeele *et al.*,
1133 2012). This system was first used to assess the fitness of potential probiotic lactobacilli,
1134 revealing a pronounced enrichment of *Lactobacillus mucosae* and *L. rhamnosus* GG in the
1135 mucus beads compared to the luminal environment, contributing to their long-term
1136 persistence in M-SHIME (Van den Abbeele *et al.*, 2012). The use of next-generation 16S
1137 rRNA gene amplicon sequencing methods and the incorporation of mucus beads in both
1138 proximal and distal colon conditions in the M-SHIME, further resulted in the detection of
1139 several additional mucus-associated species (De Paepe *et al.*, 2018). Besides *Roseburia*
1140 *faecis/Enterococcus rectale*, a strong enrichment of *Ruminococcus inulinivorans*,
1141 *Clostridium*, *Bilophila*, *Anaeroglobus* and *Veillonellaceae* species was observed in the
1142 proximal mucus layer compared to the lumen (De Paepe *et al.*, 2018). *A. muciniphila*,
1143 *Cloacibacillus evryensis*, *Pyramidobacter piscolens*, *Eubacterium contortum* and species
1144 belonging to *Odoribacter*, *Enterobacteriaceae* and *Desulfovibrio* were predominantly
1145 residing in the distal mucus layer (De Paepe *et al.*, 2018). It can be expected that the list of
1146 species inhabiting the mucus layer will continue to expand in the future M-SHIME
1147 experiments, by continuing to explore the inter-individual variability of faecal samples.

1148 Another major advantage of *in vitro* fermentation models is the possibility to capture
1149 dynamics by time-resolved analyses. To minimize disturbance of the system during such
1150 analyses, the M-SHIME system was adapted to facilitate a rapid, anaerobic, frequent
1151 sampling by mounting sampling ports with an airlock system on top of the SHIME lids. These
1152 sampling ports moreover enable the anaerobic addition, transfer and sampling of any
1153 insoluble dietary substrate. The adapted model was correspondingly termed Dietary Particle-
1154 Mucosal-Simulator of the Human Intestinal Microbial Ecosystem (DP-M-SHIME). The DP-
1155 M-SHIME offers a closer proxy for the diversity of *in vivo* GI microenvironments, as aside
1156 from the mucus layer, insoluble dietary particles present an interesting additional niche for
1157 microorganisms to physically interact with and colonise (De Paepe *et al.* 2018).

1158 In addition, these colonic *in vitro* fermentation models enable the study of severe gut
1159 microbiome perturbations such as antibiotic therapy or pathogen invasion, which cannot be
1160 performed in humans for obvious ethical reasons. Using M-SHIME, the mucus bead carriers
1161 were shown to confer resilience to lactobacilli species against a combined treatment of
1162 antibiotics including tetracycline, amoxicillin and ciprofloxacin (Van den Abbeele *et al.*,
1163 2012). In addition, the M-SHIME model has been used to demonstrate the antagonistic effects
1164 of probiotics and prebiotics such as *L. reuteri*, long-chain arabinoxylans or inulin towards
1165 AIEC colonisation in the mucosal environment (Van den Abbeele *et al.*, 2016). Another study
1166 reported the importance of mucus for providing a protective environment to beneficial gut
1167 microbes, such as *L. reuteri*, to help them escape from stress induced by high loads of linoleic
1168 acid, the most common polyunsaturated fatty acid in a WSD (De Weirdt *et al.*, 2013). The M-
1169 SHIME model has also been shown to preserve disease signatures, as illustrated by a reduced
1170 luminal and mucosal *Clostridium* cluster XIVa colonisation in colon vessels inoculated with
1171 the faecal material of ulcerative colitis patients compared to healthy donors (Vermeiren *et al.*,
1172 2012). The mucin-beads technology has recently been transferred into the Artificial Colon

1173 (ARCOL), with adaptations to avoid flushing the bioreactor with nitrogen when mucin-coated
1174 beads are being renewed (Cordonnier *et al.*, 2015, Thevenot *et al.*, 2015) .

1175
1176 ***Combining colon fermentation models with in vitro cell culture.*** Recent advancements in the
1177 field of *in vitro* fermentation involve the combination of gut microbiota models with a host
1178 compartment to assess host-microbiota interactions (Bahrami *et al.*, 2011, Marzorati *et al.*,
1179 2014, Defois *et al.*, 2018). The three-way interactions between host, microbiome and dietary
1180 interventions can be examined by applying the supernatants of colonic samples onto human
1181 cell lines or combinations thereof, such as co-cultures of enterocytes and immune cells, in
1182 Transwell systems (Marzorati *et al.*, 2014). Cytokine and TNF- α production are followed up
1183 as markers of intestinal inflammation, whereas the trans-epithelial electrical resistance
1184 (TEER) and Lucifer yellow translocation give an indication of epithelial barrier function
1185 (Geirnaert *et al.*, 2017). While typically, in human cell line experiments, the short-term effects
1186 of a single treatment application onto differentiated cells with a disrupted barrier are being
1187 evaluated, recently, a method was developed to test the effects of a probiotic treatment on the
1188 development of epithelial barrier integrity during cell differentiation, which is more
1189 representative of the *in vivo* situation (Geirnaert *et al.*, 2017). In order to further improve the
1190 *in vivo* relevance, a host module, such as the Host Microbiota Interaction (HMI) Module
1191 (Marzorati *et al.*, 2014), can be coupled to the colonic *in vitro* fermentation systems described
1192 above to directly and continuously recirculate the colonic microbial suspension over a mucus
1193 layer that is in indirect contact with enterocytes and/or immune cells.

1194

1195 ***4.5. In vivo animal models***

1196

1197 As described above, *in vitro* mucin-secreting cell cultures, *ex-vivo* organ cultures as
1198 well as *in vitro* fermentation models have yielded fundamental insights into the role of mucins
1199 and mucus in bacterial interactions with the host. However, the use of *in vivo* models is
1200 necessary to study the biological roles of mucins under physiological or pathological
1201 conditions at the level of entire organism. Genetically modified mouse models with an
1202 impaired mucin production or glycosylation have been developed to assess the role of mucus
1203 in the interaction between gut bacteria and the host *in vivo*.

1204
1205 ***Muc2^{-/-} mouse model.*** Many *in vivo* animal studies investigating the role of mucus in gut
1206 homeostasis have relied on the use of *Muc2^{-/-}* mice, lacking the major intestinal mucin Muc2.
1207 The first studies based on *Muc2^{-/-}* mice showed that these animals displayed an impaired
1208 epithelial barrier function characterised by aberrant intestinal crypt morphology and altered
1209 cell maturation and migration, and that the mice frequently developed adenomas in the small
1210 intestine, as well as rectal tumours (Velcich *et al.*, 2002). The microscopic analysis of the
1211 colon indicated mucosal thickening, increased proliferation and superficial erosions (Van der
1212 Sluis *et al.*, 2006). The development of spontaneous colitis in Muc2 deficient mice indicated
1213 that Muc2 is critical for colonic protection (Van der Sluis *et al.*, 2006). A gut microbiota
1214 dysbiosis was also observed in the *Muc2^{-/-}* mice which harbored a pro-inflammatory-like
1215 microbiota profile, characterized by an increase in *Clostridiales* and a decrease in
1216 *Lactobacillaceae* (Huang *et al.*, 2015). Furthermore, it was shown that the spatial
1217 compartmentalization of bacteria in the intestine of *Muc2^{-/-}* mice was compromised and
1218 transcriptomic analysis revealed a downregulation of TLR, immune and chemokine signaling
1219 pathways compared to wild type mice (Sovran *et al.*, 2015) Also, the expression of the
1220 network of IL-22-regulated defense genes was increased in *Muc2^{-/-}* mice (Sovran *et al.*, 2015).
1221 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

1247 significantly higher barrier disruption and higher mortality rates than wild type mice
1248 (Zarepour *et al.*, 2013). Colonisation of *Muc2*^{-/-} mice by enterotoxigenic *B. fragilis*, a
1249 causative agent of acute diarrhoea in humans, led to lethal disease (Hecht *et al.*, 2017). The
1250 protective function of Muc2 was also demonstrated in models of *T. muris* parasitic infection
1251 (Hasnain *et al.*, 2010). *T. muris* is a murine infecting nematode which is used as model of *T.*
1252 *trichiura* infection in humans, a threat in developing countries. After infection, *Muc2*^{-/-} mice
1253 showed a delayed expulsion of the worms from the intestine compared to wild type mice. In
1254 addition, an increase in Muc2 production, observed exclusively in resistant mice, correlated
1255 with worm expulsion. The nematodes demonstrated a decrease in their energy status in wild
1256 type mice compared to the susceptible *Muc2*^{-/-} mice (Hasnain *et al.*, 2010). *E. histolytica* is a
1257 human parasite infecting the colon and responsible of amoebic dysentery and/or liver
1258 abscesses. *E. histolytica* specifically colonises the mucus layer by adhering to galactose and
1259 GalNAc residues present in Muc2 (Kissoon-Singh *et al.*, 2013). The parasite also induces
1260 potent hypersecretion from goblet cells. Kissoon-Singh and colleagues showed that *E.*
1261 *histolytica* induced a pronounced time-dependent secretory exudate with increased gross
1262 pathology scores and serum albumin leakage in *Muc2*^{-/-} mice. Colonic pathology, secretory
1263 responses, and increased pro-inflammatory cytokine secretions were also correlated with
1264 altered expression of tight junction proteins (Kissoon-Singh *et al.*, 2013). These results
1265 demonstrate that colonic mucins confer both luminal and epithelial barrier functions and that,
1266 in the absence of Muc2, mice are more susceptible to *E. histolytica*-induced secretory and
1267 pro-inflammatory responses. A recent study using antibiotic treated *Muc2*^{-/-} and *Muc2*^{+/+}
1268 littermates showed that *E. histolytica* elicited robust mucus and water secretions, enhanced
1269 pro-inflammatory cytokines and chemokine expression and higher pathology scores as
1270 compared to the modest response observed in non-antibiotic treated littermates. Host
1271 responses were microbiota specific as mucus secretion and pro-inflammatory responses were

1272 attenuated following homologous faecal microbial transplants in antibiotic-treated *Muc2*^{+/+}
1273 quantified by secretion of ³H-glucosamine newly synthesized mucin, Muc2 mucin
1274 immunostaining and immunohistochemistry (Leon-Coria *et al.*, 2018). The mechanism
1275 controlling mucus release in the presence of *E. histolytica* was further studied by Cornick and
1276 colleagues who identified vesicle-associated membrane protein 8 (VAMP8) present on mucin
1277 granules as orchestrating regulated exocytosis in human goblet cells in response to the
1278 presence of *E. histolytica* (Cornick *et al.*, 2017). In *Vamp8*^{-/-} mice, *E. histolytica* induced
1279 enhanced killing of epithelial cells and aggressive proinflammatory response with elevated
1280 levels of IL-1 α , IL-1 β , and TNF- α secretion, highlighting the downstream consequences of
1281 improper mucin secretion in mucosal barrier defence. Taken together, these results
1282 demonstrate the critical involvement of Muc2 in host protection from nematode infection, by
1283 constituting an effective physical and biological barrier against pathogenic infection.

1284
1285 ***Muc1*^{-/-} mouse model.** Mice impaired in the production of cell surface mucins have also been
1286 engineered. The *Muc1*^{-/-} mouse model revealed the role played by Muc1 in *H. pylori*
1287 infection, a pathogen involved in gastric ulcers and adenocarcinoma (McGuckin *et al.*, 2007,
1288 Linden *et al.*, 2009). *Muc1*^{-/-} mice displayed a 5-fold increase in *H. pylori* colonisation as
1289 compared to wild type mice (McGuckin *et al.*, 2007). This study further demonstrated the
1290 ability of *H. pylori* to bind to purified Muc1 *in vitro*, suggesting that Muc1 limits the access of
1291 *H. pylori* to the epithelial surface thereby providing protection from infection and
1292 proinflammatory bacterial products. Muc1 deficiency also resulted in increased epithelial cell
1293 apoptosis in *H. pylori* infected mice (Linden *et al.*, 2009). More recently, the long-term
1294 consequence of Muc1 deficiency on *H. pylori* pathogenesis was investigated in *Muc1*^{-/-} mice
1295 (Ng *et al.*, 2016). *Muc1*^{-/-} mice began to die 6 months after *H. pylori* challenge, indicating that
1296 a deficiency in Muc1 leads to lethal infection. This study also revealed that *Muc1* was an

1297 important, previously unidentified negative regulator of the NLRP3 inflammasome, and loss
1298 of this regulation resulted in the development of severe pathology (Ng *et al.*, 2016).
1299 Consistent with these studies, *Muc1^{-/-}* mice have a higher rate of systemic infection in a
1300 murine *C. jejuni* model of gastroenteritis (McAuley *et al.*, 2007).

1301

1302 ***Muc13^{-/-} mouse model.*** The MUC13 transmembrane mucin is highly and constitutively
1303 expressed in the small and large intestines and MUC13 polymorphisms have been associated
1304 with human IBD and susceptibility to *E. coli* infection in pigs. While *Muc13*-deficient mice
1305 did not show intestinal pathology, they developed more severe acute colitis than wild type
1306 mice after DSS challenge, as reflected by increased weight loss, rectal bleeding, diarrhoea and
1307 histological colitis scores (Sheng *et al.*, 2011).

1308

1309 ***Mouse models with an altered mucin glycosylation.*** Loss of *O*-glycans impairs the
1310 expression and function of several intestinal mucins, thereafter causing more profound defects
1311 in the function of the intestinal barrier than a flaw caused by the deficiency of an individual
1312 mucin. A number of transgenic mouse models have been developed to decipher the
1313 mechanisms underpinning the role of mucin glycosylation in gut homeostasis. Mice lacking
1314 core 3–derived *O*-glycans (also known as *C3GnT^{-/-}* mice) display a substantial reduction of
1315 *Muc2* protein and an increased susceptibility to DSS-induced colitis and accelerated
1316 colorectal tumorigenesis (An *et al.*, 2007). In addition, core 3 *O*-glycosylation was shown to
1317 play a major role in controlling *Salmonella* intestinal burdens in *C3GnT^{-/-}* mice (Zarepour *et*
1318 *al.*, 2013). Similarly, *C2GnT2^{-/-}* mice (mice lacking core 2-derived *O*-glycans) displayed an
1319 increased susceptibility to DSS-induced colitis but with no change in *Muc2* expression (Stone
1320 *et al.*, 2009). Mice with intestinal epithelial cell–specific deficiency of core 1–derived *O*-
1321 glycans (IEC *C1galt1^{-/-}*) develop spontaneous colitis (Fu *et al.*, 2011). Mice lacking both core

1322 1- and core 3–derived *O*-glycans (DKO mice) have an impaired mucus barrier function and
1323 develop colitis-associated colon cancer in which the dysbiotic microbiota promote
1324 inflammation and cancer (Bergstrom *et al.*, 2016). In a water avoidance model in rats,
1325 psychological stress lead to less-protective mucus layer. In particular, *O*-glycosylation of
1326 mucins was strongly affected and these changes were associated with flattening and loss of
1327 the mucus layer cohesive properties (Da Silva *et al.*, 2014). Altogether, these data suggest that
1328 the lack of a proper *O*-glycosylation impairs Muc2 expression or secretion and alters gut
1329 barrier function of the mucus layer.

1330 In addition to modifications of mucin core glycans, mouse models have been
1331 developed targeting epitope modifications of the mucin glycans chains. Dawson and
1332 colleagues reported that deletion of the sulfate transporter NaS1 in mice (*Nas1^{-/-}* mice)
1333 resulting in a decrease in mucin sulfation, enhanced susceptibility to experimental DSS colitis
1334 and systemic infection by *C. jejuni* (Dawson *et al.*, 2009). In addition, mice with a deletion of
1335 the sulfo-transferase GlcNAc6ST2 enzyme adding sulfate to GlcNAC residues on *O*-mucin
1336 glycan chains exhibited an increased susceptibility to DSS-induced colitis (Tobisawa *et al.*,
1337 2010). Mice deleted for Sat-1 (sulphate anion transporter-1) were more susceptible to chronic
1338 infection by parasite *T. muris* (Hasnain *et al.*, 2017). Collectively, these findings indicate that
1339 mucin abnormalities can initiate the onset of inflammatory related diseases in the gut.

1340 In addition to mice harbouring a deletion in genes encoding proteins directly involved
1341 in mucin expression or glycosylation, several transgenic mouse models have been shown to
1342 display alterations in mucus properties. These include the Winnie and Eeyore mice which
1343 carry single missense mutations in two different D-domains of Muc2 (Heazlewood *et al.*,
1344 2008). These mice display fewer goblet cells and a reduction in secreted mucus with *O*-
1345 glycosylated and non-*O*-glycosylated Muc2. The misfolding results in endoplasmic reticulum
1346 stress, goblet cell apoptosis, depletion of the secreted mucus layer and development of

1347 chronic intestinal inflammation (Heazlewood *et al.*, 2008). In combination with mucus, a
1348 large population of intraepithelial lymphocytes (IELs) bearing the $\gamma\delta$ T cell receptor is
1349 mediating immune protection against invading bacteria. In $\gamma\delta$ T-cell-deficient ($TCR\delta^{-/-}$) mice,
1350 mucin expression and glycosylation is altered, mucus-secreting goblet cells are significantly
1351 reduced in number and those animals are more prone to DSS-induced colitis (Kober *et al.*,
1352 2014). Mouse models deficient in TLR5, IL-10 and Sodium hydrogen antiporter 3 (Slc9a3 or
1353 Nhe3) revealed bacteria in contact with the epithelium. Additional analysis of the less
1354 inflamed $IL-10^{-/-}$ mice revealed a thicker mucus layer but a more penetrable inner mucus
1355 allowing bacteria to penetrate and reach the epithelium (Johansson *et al.*, 2014).

1356

1357 ***Non-rodent models.*** Differences in mucus thickness and composition have been observed
1358 between rats, pigs and rabbits, and suggests that the pig mucus pattern resembles more closely
1359 that of humans (Varum *et al.*, 2012). The zebrafish larva is an emerging model system for
1360 investigating components of the innate immune system, including mucus physiology. It has
1361 been shown that five gel-forming secreted mucin genes are found in zebrafish with a high
1362 degree of homology to other vertebrate mucins regarding their genomic and protein domain
1363 organisation, as well as their tissue specific expression (Jevtov *et al.*, 2014).

1364

1365 **5. Limitations of current experimental models involving mucus and future challenges**

1366

1367 Given the importance of the gut microbiota as a modulator of health and disease,
1368 increasing attention has been devoted to the role played by mucus in the interaction with gut
1369 bacteria (Juge, 2019). As described above (section 4), various experimental models increasing
1370 in complexity from simple *in vitro* assays to cell lines, organ-on-chips, *in vitro* colon
1371 fermentation systems or animal models have been developed and successfully applied to the

1372 study of gut microbe-mucus interactions. However, one of the limitations common to most *in*
1373 *vitro* models is the origin of mucins used to assess the interactions with bacteria doubled by
1374 the inability to reproduce a colonic mucus gel recapitulating the *in vivo* situation. This is
1375 important as the nature and origin of purified mucins used in these assays greatly influence
1376 the outcome of binding as demonstrated using microtitre plate (Owen *et al.*, 2017), dot-blot
1377 (Ringot-Destrez *et al.*, 2018) or mucin microarrays (Clyne *et al.*, 2017). Mucin glycosylation
1378 plays a critical role in the interaction between gut bacteria and mucus and significant
1379 glycosylation differences occur between purified mucins used in *in vitro* assays from different
1380 sites of the murine GI tract or from goblet cells (e.g. LS174T), as analysed by mass
1381 spectrometry (Leclaire *et al.*, 2018, Ringot-Destrez *et al.*, 2018). In addition, the purification
1382 steps alter the properties of native glycoproteins and purified mucins used in these assays lack
1383 the ability to form viscoelastic hydrogels (Kocevar-Nared *et al.*, 1997). A similar situation
1384 occurs with mucus secreting cell lines where the type of mucins and structure of mucus differ
1385 from the colonic environment. For example, the HT29 cell line secretes mostly MUC5AC
1386 whereas MUC2 is the main mucin secreted in the small and large intestines. These differences
1387 are due to the use of cancer cells which show an alteration in the expression and glycosylation
1388 of mucins. In addition, the production of mucus by epithelial cell lines can be influenced by
1389 culture conditions. For example, growing cells on Transwell filters with a small amount of
1390 apical medium (semi-wet interface culture) in combination with mechanical stimulation (on a
1391 rocking platform) and addition of the Notch γ -secretase inhibitor DAPT resulted in
1392 polarisation and secretion of MUC2 and MUC5AC by HT29 MTX-P8, HT29 MTX-E12 and
1393 LS513 cells (Navabi *et al.*, 2013). Additionally, the mucus produced by goblet cells in *in vitro*
1394 co-culture cell models is not continuous nor homogenate which is not fully representative of
1395 the *in vivo* situation. Lastly, the formation of the bi-layered mucus found in the colon remains
1396 a challenge in these models. Novel strategies such as multiple cell layers, 2D-organoid

1397 techniques or Organ-on-a-Chip devices are currently being developed to better mimic the
1398 human intestinal epithelial microenvironment. Such multiple cell models exhibit intestinal
1399 villus morphogenesis associated with mucus production. These models are also needed to
1400 recapitulate antimicrobial defense and inflammatory reactions normally occurring in mucosal
1401 tissues. Another advantage of these systems is that, unlike cell lines, organoids can be used to
1402 evaluate long-term interactions between mucus and gut microbes. However, these more
1403 advanced biopsy- based models remain low throughput and expensive as compared to *in vitro*
1404 assays and are limited by the availability and variability of clinical specimens.

1405 In addition, to the host side, several microbial factors must be taken into consideration
1406 when assaying the interactions between the gut bacteria and mucus. These include the
1407 handling and labelling microbial cells which may affect the surface molecular determinants
1408 potentially involved in mucus/mucin interactions (e.g. cell-surface adhesins, pili or flagella)
1409 (Chagnot *et al.*, 2014). The growth conditions (e.g. growth media with different nutrient
1410 compositions; temperature, pH, osmolarity or redox potential) can also influence the
1411 expression of the bacterial receptors mediating the interactions with mucins. To date, most
1412 studies have focused on the interactions between mucus and probiotic or pathogenic strains
1413 and assessing strictly anaerobe gut symbionts or complex microbial communities remain a
1414 challenge in this field of research.

1415 An alternative to the systems described above is the use of dynamic *in vitro*
1416 fermentation models of the human gut, such as the M-SHIME (Marzorati *et al.*, 2014) or DP-
1417 M-SHIME (De Paepe *et al.*, 2018) models. In these *in vitro* colonic models, the introduction
1418 of mucin-covered beads allows to study the long-term *in vitro* microbial colonisation of
1419 mucin, in the presence of a complex anaerobic intestinal microbiota (Marzorati *et al.*, 2014,
1420 Shah *et al.*, 2016). These models provide a mean to study gut microbiota functionality and
1421 niche differentiation, during treatments with xenobiotics (for example antibiotics, synthetic

1422 chemicals such as food additives, environmental pollutants like persistent organic pollutants
1423 (POPs)), pathogens or functional foods. Future developments in this field will be the
1424 introduction of mucus secretion and/or a mucus surface layer in dynamic *in vitro* models of
1425 the upper GI tract, such as in the gastric and small intestinal TNO Gastro-Intestinal model
1426 (TIM) (Guerra *et al.*, 2012). This is important so to take into account the successive stressful
1427 events (e.g. acidic gastric pH, bile salts) that commensal or pathogenic microbes undergo in
1428 the human GI tract before reaching the intestinal epithelium and that may greatly influence
1429 their physiological stage, virulence and/or activity.

1430 However, as mentioned above, a limitation of these *in vitro* GI models is that they rely
1431 on commercially available mucins used for the mucin bead technology. These secretory
1432 mucins, usually MUC5AC and MUC6 porcine gut gastric mucin, differ in terms of structure
1433 and glycosylation from intestinal MUC2 and cannot form a bi-layered mucus gel. It has been
1434 proposed that in the future, *in vitro* engineered mucus may be used to mimic human-derived
1435 mucus in a more reproducible manner. The colonic *in vitro* models could also be improved by
1436 including immunoglobulins, specific antimicrobial peptides, or secreted phosphatidylcholine,
1437 which have been shown to modulate mucus surface properties, thereby influencing bacterial
1438 adhesion (Martens *et al.*, 2018). Future *in vitro* colon models should also better mimic the *in*
1439 *vivo* transit, and particularly retrograde movements (Hiroz *et al.*, 2009), as back-flow was
1440 recently suggested to be crucial for the persistence of gut microbes in the GI tract (Cremer *et*
1441 *al.*, 2016). Current technological advances include the coupling of these fermentation models
1442 to intestinal epithelial cells or more complex units such as the HMI module. A next step will
1443 be to couple the digestive/fermentation models with enteroids/colonoids or HIO. However,
1444 despite their increased complexity, most of these approaches remain limited by the absence of
1445 important host functions, such as variable peristalsis-like motions. This is a critical limitation
1446 because mechanical deformations resulting from peristalsis both influence normal epithelial

1447 cell differentiation and control microbial overgrowth in the living intestine (Gayer & Basson,
1448 2009, Benam *et al.*, 2015). The development of microfluidic systems and organ-on-chips is
1449 currently addressing this important technological gap (Kim & Ingber, 2013, Kim *et al.*, 2016).

1450 The development of these advanced *in vitro* systems is essential to help reduce
1451 dependence on animal studies. Due to the invasive nature of the experiments, the mechanisms
1452 underpinning microbe-mucus interactions *in vivo* have mainly been investigated in animal
1453 models, mostly rodents. Genetically engineered mice impaired in mucin secretion or
1454 glycosylation have been instrumental to decipher the role of mucins and mucus in the
1455 protection of the intestinal epithelium and the interactions between pathogenic bacteria,
1456 commensal microbiota and the mucus barrier. However, although the domain organisation
1457 and expression pattern of mucins appear largely conserved between human and mouse (Joshi
1458 *et al.*, 2015), mucin glycosylation (Thomsson *et al.*, 2012) and gut microbiota (Nguyen *et al.*,
1459 2015) differ between these two species. It has been speculated that differences in mucin
1460 glycosylation between mammalian species may underlie some of the differences in infectivity
1461 and/or pathogenicity for individual microbial pathogens (Linden *et al.*, 2008) or the different
1462 commensal microbiota (Thomsson *et al.*, 2012). Therefore, caution should be applied when
1463 translating data obtained in mouse models to humans. Lastly, unlike *in vitro* assays, *in vivo*
1464 studies are restricted to end-point measurements.

1465 Recent years have witnessed unprecedented technological advances in the
1466 development of *in vitro* GI models that more closely resemble the gut mucosal interface. Our
1467 next challenge will be to simulate these models at different stages of development or disease
1468 conditions (e.g. IBD, obesity or CF). Special attention should be paid to inter-individual
1469 differences and intra-individual variability in gut microbiota composition and intestinal
1470 biopsies from different donors or patients. This is important to better understand the role of
1471 gut microbe-mucus interactions in the aetiology of a particular disease or condition and

1472 determine the microbial and biochemical signature that could differentiate between diseased
1473 or healthy status. In particular, more research is warranted to determine how the
1474 physicochemical properties and/or thickness of the mucus layer and mucin glycosylation are
1475 altered during a specific disease. In the future, these pre-clinical models will help screen novel
1476 therapeutic strategies aimed at restoring gut barrier function and tailored to the individual
1477 patient as a step towards personalised medicine.

1478

1479 **Conflicts of interest.** None declared.

1480

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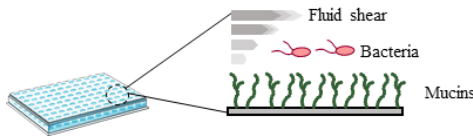
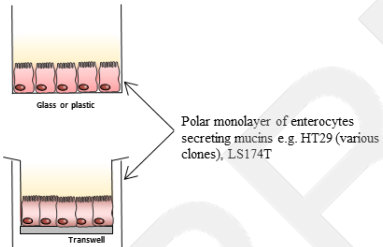
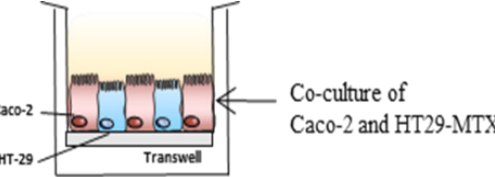
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PRE-PRINT

| Types of models | Description | Applications | Advantages | Limitations | References |
|--|--|---|--|--|---|
| <p data-bbox="11 79 515 111"><i>In vitro</i> mucus/mucin binding assays</p> <p data-bbox="11 119 515 151"><i>Microplates - Flow chambers</i></p>  | <ul style="list-style-type: none"> * Immobilization of mucus/mucin on the microtiter plate * Microtiter plate : adhesion in static conditions * Flow chambers : adhesion under dynamic conditions (fluid shear) | <ul style="list-style-type: none"> * Evaluation of bacterial adhesion (commensals and pathogens) to mucins and molecular mechanisms associated | <ul style="list-style-type: none"> * Fast, quantitative and high throughput method to study mucus-microbe interactions independently from other <i>in vivo</i> conditions * Identification of molecular determinants involved in adhesion of microbes * Coupling with biophysical techniques (Surface Plasmon Resonance, Atomic Force Microscopy) | <ul style="list-style-type: none"> * 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 | <p>McNamara <i>et al.</i>, 2000, Gusils <i>et al.</i>, 2004 ; Ringot-Clyne <i>et al.</i>, 2017 Dunne <i>et al.</i> 2018</p> |
| <p data-bbox="11 566 515 598"><i>In vitro</i> cell models</p> <p data-bbox="11 606 515 638"><i>Monoculture models</i></p>  | <ul style="list-style-type: none"> * Gut-derived epithelial cells resembling intestinal tissue consisting mainly of mature goblet cells that secrete an adherent | <ul style="list-style-type: none"> * 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 | <ul style="list-style-type: none"> * 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 | <ul style="list-style-type: none"> * Derived from cancer cells, different from healthy tissue * Not representative of various cell types recovered in mucosal epithelial tissues * Not representative of appropriate <i>MUC</i> 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) | <p>Linden <i>et al.</i>, 2007, Navabi <i>et al.</i>, 2013, Hews <i>et al.</i>, 2017</p> |
| <p data-bbox="11 1220 515 1252"><i>Co-culture models</i></p>  | <ul style="list-style-type: none"> * Mixed culture of enterocytes and mucin secreting cells | <ul style="list-style-type: none"> * Adherence of commensal and pathogenic bacteria to host cells * Effect of commensals/pathogens on host cell mucin synthesis and / or composition of the | <ul style="list-style-type: none"> * Better representation of cell-type ratio recovered in mucosal epithelial tissues * Simple model, well described in literature | <ul style="list-style-type: none"> * 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 | <p>Hilgendorf <i>et al.</i>, 2000, Lozoya-Agullo <i>et al.</i>, 2017</p> |

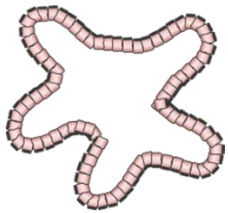
mucus layer

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

PRE-PRINT

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

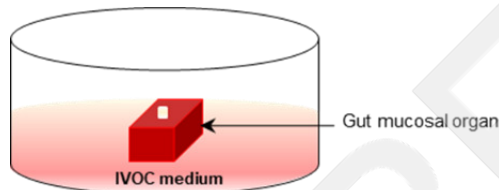
- * Study of advanced aspects of mucus development in a more complex scenario
- * Study of host-commensals and pathogens interactions

- * 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*

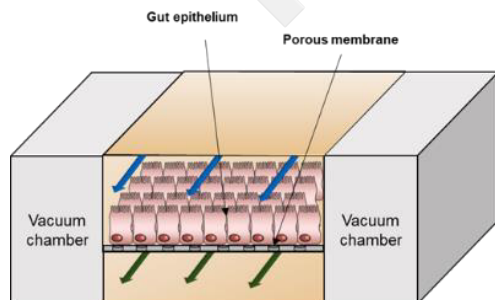
- * Study of host-commensals and pathogens interactions

- * 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

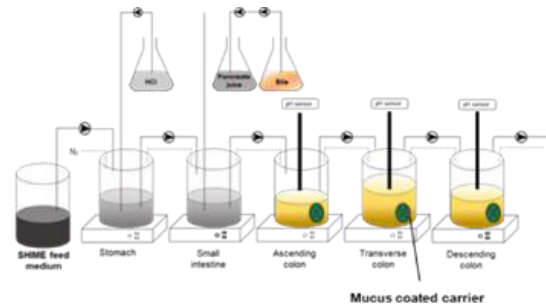
- * Presence of non-transformed cells including all major cell types (enterocytes, goblet cells, Paneth cells and endocrine cells)
- * Reproduction of peristalsis like motions
- * Possible use of biopsies from disease patients (e.g. IBD)

- * 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

***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

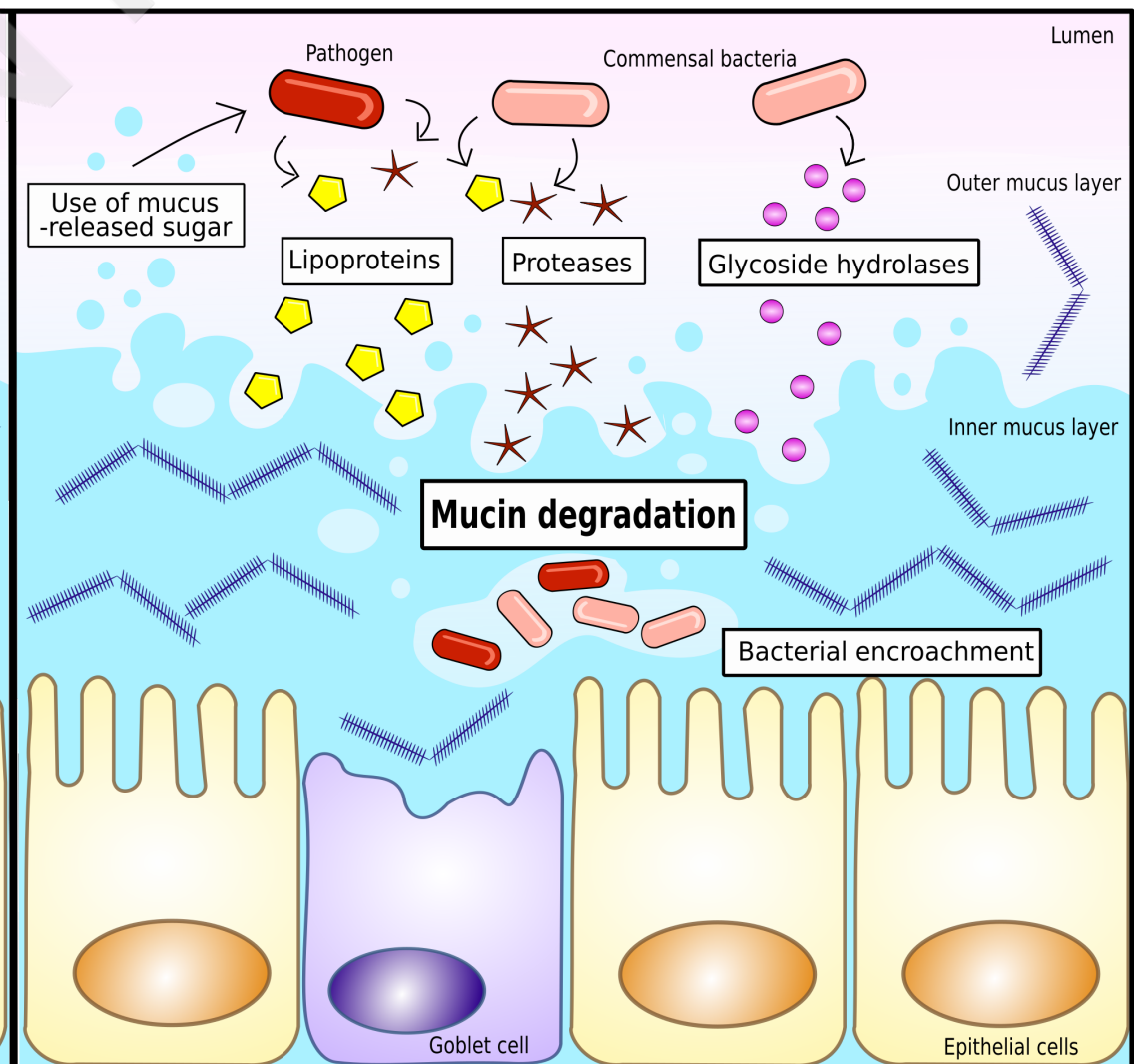
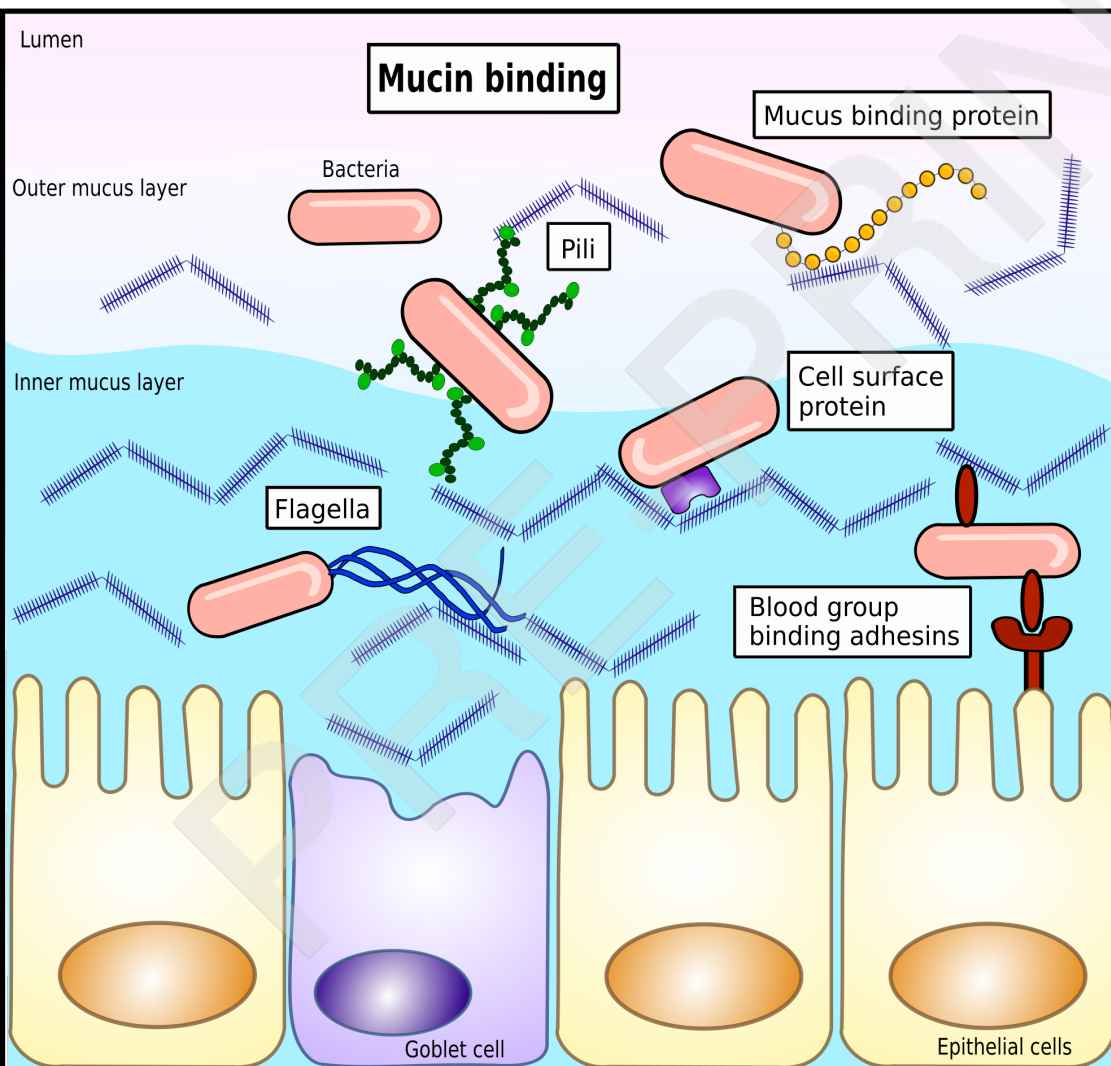
- * Requires housing facility and adequate agreements
- * Expensive to maintain colonies
- * Housing husbandries and diets can modulate mouse microbiota
- * Murine gut microbiota different from the human gut microbiota
- * Mucin glycosylation profile of mice different from human intestinal mucins
- * 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

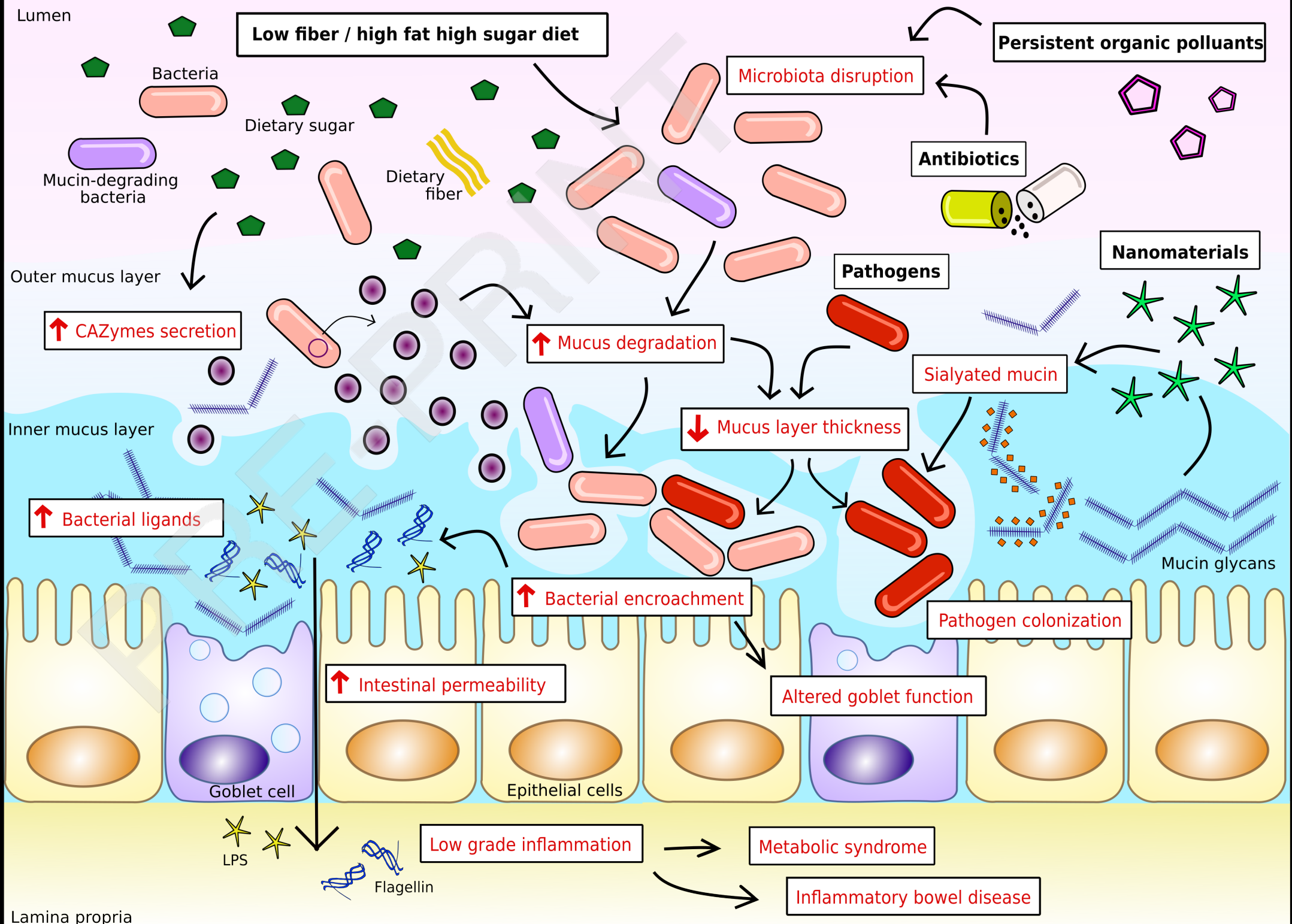
Van der Sluis *et al.*, 2006
 Velcich *et al.*, 2002

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

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

PRE-PRINT





Low fiber / high fat high sugar diet

Persistent organic pollutants

Microbiota disruption

Antibiotics

Nanomaterials

Pathogens

Bacteria

Dietary sugar

Dietary fiber

Mucin-degrading bacteria

Outer mucus layer

↑ CAZymes secretion

↑ Mucus degradation

↓ Mucus layer thickness

Sialyated mucin

Inner mucus layer

↑ Bacterial ligands

↑ Bacterial encroachment

Pathogen colonization

Mucin glycans

↑ Intestinal permeability

Altered goblet function

Goblet cell

Epithelial cells

Lamina propria

LPS

Flagellin

Low grade inflammation

Metabolic syndrome

Inflammatory bowel disease

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.