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**Running title:** Intestinal mucus and microbiota: new targets for mycotoxins?

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## Abstract

There is an increasing awareness of the deleterious effects attributed to mycotoxins during their fate within the gut, particularly for deoxynivalenol (DON), zearalenone (ZEN), ochratoxin A (OTA), fumonisin B1 (FB1), aflatoxin B1 (AFB1) and patulin (PAT). Disruption of the epithelial barrier is well-established. However, intestinal barrier function on its luminal side involves two other partners, mucus and microbiota which have rarely been considered in the context of mycotoxin exposure. The current review aimed at providing a summary of DON, ZEN, OTA, FB1, AFB1 and PAT effects on intestinal barrier function, with special focus on mucus and microbiota. DON, ZEN, OTA, FB1, AFB1 and PAT are known to markedly affect epithelial cell integrity and functions. Regarding mucus, DON is undoubtedly the most documentated mycotoxin. *In vivo*, toxicological impact of DON generally has only been assessed through goblet cell number. Evaluation of the mycotoxins/mucus interplay considering other indicators such as composition, thickness and penetrability of mucus, mucin O-glycosylation thus warrants further attention. With respect to microbiota, few short-term studies to date have been reported indicating deleterious effects. However, long-term exposure to mycotoxins may also produce significant changes in microbiota composition and metabolic activity, which requires further experimentation. In conclusion, mucus and microbiota are key targets for dietary mycotoxins although assessment of induced effects is preliminary. A significant research effort is now underway to determine the adverse consequences of mycotoxins on mucus and microbiota considered as individual but also as tightly-connected gut players.

**Keywords:** dietary mycotoxins, gut, epithelium, mucus, microbiota

## Introduction

Mycotoxins are secondary metabolites produced by fungal genera mainly *Aspergillus, Fusarium* and *Penicillium*. These metabolites represent the most frequently occurring natural food contaminants in human and animal diets and occur worldwide in foods such as cereals and animal forages (Bondy and Pestka 2000; Ok et al. 2009), leading to acute and chronic exposures in humans and animals (Akbari et al. 2017). Despite the improvement of good agricultural and manufacturing practices, mycotoxin contamination especially occupational cannot be avoided (Viegas et al. 2012; 2013; Pinto et al. 2015; Follmann et al. 2016) and recent surveys indicate that 70% of the raw materials are contaminated with these toxins (Bryden 2012; Streit et al. 2013). Mycotoxins are (1) produced before, during and after harvest; (2) variably resistant to technological treatments and (3) sometimes difficult to eliminate. Therefore, these metabolites are present both in human food and animal feed. The aim of this review is to focus on major mycotoxins: deoxynivalenol (DON), zearalenone (ZEN), ochratoxin A (OTA), fumonisin B1 (FB1), aflatoxin B1 (AFB1) and patulin (PAT). Generalities, regarding chemical structure, major producing fungi, predominant contaminated crops, adverse effects, mode of action and health-based guidance values are given in Table 1. As noted in this Table, the clinical toxicological syndromes initiated by chronic or sub-chronic ingestion of mycotoxins on animal health have been described leading to regulations for human food in many countries including USA and Europe.

The adverse effects of exposure to several mycotoxins on various indicators of growth performance have also been demonstrated. Indeed, both animal experiments and human epidemiological studies have shown an impaired growth upon exposure to aflatoxin, fumonisin or deoxynivalenol (Andretta et al. 2011; Khlangwiset et al. 2011; Andretta et al. 2012; Wu et al. 2014; Smith et al. 2015). The intestine might play a role in growth retardation induced by mycotoxins; however, except for DON, the underlying mechanism is not identified. From studies mainly carried out in mice, Pestka and his colleagues have demonstrated that DON acts at different levels to induce impaired growth and weight gain, including on neuroendocrine signaling, immune responses, growth hormone and on central nervous system (Wu et al. 2014; Lebrun et al. 2015; Zhou and Pestka 2015; Wu et al. 2017).

There is an increasing awareness of the deleterious effects of mycotoxins during their fate within the gastrointestinal tract (GIT). Mycotoxins induce intestinal pathologies in humans and animals, including necrosis of intestinal epithelium (Pinton & Oswald, 2014), disturb intestinal barrier function, modulate immune responses and indirectly affect feed consumption by impairing growth hormone signaling (Payros et al. 2016). Some mycotoxins cause DNA modifications and mutations in enterocytes (Maresca & Fantini, 2010). Due to the diversity in their bioavailability, differences between animal species and entero-hepatic recycling, mycotoxins may compromise intestinal homeostasis before absorption in the upper compartments and/or throughout the entire intestine by a non-adsorbed or reabsorbed fraction (Grenier & Applegate 2013). These characteristics i indicate that the GIT is repeatedly exposed to a high proportion of ingested mycotoxins in its upper and lower segments. Indeed, the primary function of the GIT is digestion accompanied by water and nutrient absorption from the lumen to the blood through the single-layered epithelium which acts a selective filter against luminal pathogens and dietary antigens (Turner 2009). In addition, the ability of intestinal epithelial cells to act as immune cells and to crosstalk with mucosal immunity (Shao et al. 2005; Wells et al. 2011; Gourbeyre et al. 2015) will not be discussed in this review.

Numerous studies demonstrated the mycotoxin-mediated disruption of the intestinal barrier integrity through an impairment of the epithelium barrier, as evidenced by increased

permeability and translocation of harmful stressors such as pathogens ( Grenier & Applegate 2013; Pinton & Oswald 2014; Ghareeb et al. 2015; Akbari et al. 2017). However, intestinal barrier function on its luminal side also involves two other partners, mucus and microbiota, which, together with epithelium, provide a physical, chemical and biological line of defense for the host, probably in an orchestrated manner (Burger-Van Paassen et al. 2009; Lu et al. 2011; Tomas et al. 2013; Wrzosek et al. 2013; Da Silva et al. 2014). Although mucus and microbiota are pivotal players in digestive health, these components have been frequently neglected in food toxicology and rarely considered in the context of mycotoxins. Based upon this background, the current review aimed to provide a summary of the available evidence regarding direct effects of individual mycotoxins introduced above on intestinal barrier function, with special focus on mucus and microbiota.

## The constituents of the intestinal barrier

### *Intestinal epithelium*

The intestinal epithelium is the largest of the body's mucosal surfaces, covering approximately 400 $m^2$ surface area with a single layer of columnar cells, organized into crypts and villi and surrounded by the glycocalyx. There are many similarities in the histologic structure of the mucosa in the small and large intestine. The most apparent difference is that the large intestine is devoid of villi, which are "finger-like" structures conferring an increase in small intestinal absorptive surface. One absorptive (enterocytes/colonocytes) and 4 main differentiated (goblet cells, Paneth cells, enteroendocrine cells and tuft cells) cell types have to date been described in the intestinal epithelium. The most abundant are the enterocytes/colonocytes, which enable nutrients and water to be transported along the small and large intestine respectively into the internal environment. In the secretory cell lineage, the largest number is represented by goblet cells characterized by mucin-granule containing distended theca. Their number increases from the proximal to distal intestine, constituting 4, 6, 12 and 16% in the duodenum, jejunum, ileum, and distal colon, respectively. Paneth cells specifically located in the small intestine provide antibacterial peptides and proteins. The other secretory cells types such as enteroendocrine and tuft cells are less abundant (1 and 0.4%, respectively) and distributed all along the epithelium. The intestinal epithelial surface is continually renewed by pluripotent intestinal epithelial stem cells that reside in the base of crypts, where proliferation, differentiation and functional potential of epithelial cell progenitors are regulated by local stem cell microenvironment (Crosnier et al. 2006; van der Flier & Clevers 2009).

Intestinal epithelial cells display two crucial albeit opposite functions. Intestinal epithelial cells regulate nutrients and water absorption while restricting access for luminal antigens to permeate internal organs. Thus, these cells constitute a selective - and dynamic – barrier, mediating transport of molecules through the transcellular pathway (across cells) and/or the paracellular pathway (between cells). The major functional elements involved in the separation between apical (luminal) and basolateral compartments are the tight junction (TJ) proteins, sealing the intercellular space between adherent epithelial cells. TJ surround the apical ends of the lateral membranes of epithelial cells and determine the selective paracellular permeability to solutes (Tsukita et al. 2001). TJ are multiple protein complexes composed of transmembrane proteins, and a wide spectrum of cytosolic proteins. Four integral transmembrane proteins, including occludin, claudins, junctional adhesion molecule (JAM) and tricellulin have been identified with the claudin family consisting of at least 24 members. The intracellular domains of these transmembrane proteins interact with cytosolic scaffold proteins, such as zonula occludens (ZO) proteins, which subsequently anchor transmembrane proteins to the perijunctional actomyosin ring (Suzuki 2013). TJ ensure three main functions to: (i) provide

a barrier to adverse luminal agents, (ii) allow the permeation of ions, solutes, and water and (iii) maintain cell polarity by blocking free diffusion of proteins and lipids between apical and basolateral domains of the plasma membrane (Turner 2009). The modification of TJ barrier function and paracellular permeability is dynamically regulated by various extracellular stimuli and closely associated with health and susceptibility to disease (Turner 2009). Experimentally, the integrity of intestinal epithelial layer integrity may be assessed by measuring transepithelial electrical resistance (TEER). In addition, the intestinal permeability might be more specifically determined using markers of different sizes to evaluate the paracellular pathway such as Lucifer yellow, fluorescein isothiocyanate FITC, FITC-dextran, or chromium-labeled ethylenediamine-tetraacetic acid $^{51}$Cr-EDTA and/or the transcellular pathway with larger molecules > 4kDa, such as horseradish peroxidase.

***Intestinal mucus***

*Mucus and mucins*

Few investigations have focused on gastrointestinal mucus since this substance is transparent and may collapse upon any manipulation. Indeed, mucus is mostly water (>95%) and shrinks to a thin structure when dehydrated. That is why mucus is not normally observed on formaldehyde-fixed tissue sections and is best preserved using Carnoy fixative based on dry methanol, dry chloroform and glacial acetic acid (Puchtler et al. 1970). Atuma et al. (2001) took a big step forward in showing that the mucus luminal surface was visualized in rats by allowing charcoal particles to sediment down on top of the mucus. Following this procedure, a relatively thick mucus layer was observed along the whole intestine. This pioneer work then fuelled numerous studies in mucus science for unravelling why this intestinal component functions as a key interface and its necessity for maintaining gut health in humans and animals. Mucus is a viscoelastic gel that lines and protects the intestinal epithelium, separating it from the contents of the lumen. Mucus functions as a dynamic barrier that is permeable to gases, water and nutrients, but impermeable to most microorganisms. This substance was long considered to act as a "simple" physical barrier, but is now known to exert other key functions essential for maintaining intestinal homeostasis, such as (i) lubrication of the epithelium, facilitating the progress of material along the digestive tract, (ii) maintenance of a stable microenvironment at the epithelial surface, (iii) protection of the epithelium through the presence of immune system molecules and (iv) provision of an ecological niche for the intestinal microbiota (Johansson et al. 2011; Juge 2012; Ouwerkerk et al. 2013).

The main constituents of mucus are mucins, which are produced, stored and released by goblet cells. Mucins are large glycoproteins in which the glycans make up more than 80% of the molecular mass. The central parts have a protein backbone containing sequences rich in proline, threonine, and serine, the so-called PTS-sequences, which are highly O-glycosylated and constitute mucin domains. The O-linked glycan chains contain 1-20 sugar residues, which might be attached in a linear and branched manner. The O-glycans are initially attached to a serine or a threonine of the protein backbone with N-acetylgalactosamine (GalNac). The chain is elongated with core structures and two potential backbone regions containing N-acetylglucosamine (GlcNac) and galactose (Gal). Termination usually occurs with fucose (Fuc), Gal, GalNac or sialic acid residues that form the different histo-blood-group antigens. Both Gal and GlcNac residues may be sulphated, thus offering a high degree of diversification (Linden et al. 2008). MUC2 is the secreted gel-forming mucin present in the intestine (Johansson et al. 2011). MUC2 oligomerizes into large net-like polymers when the C- and N-termini form disulfide bond-stabilized di- and trimers (Ambort et al. 2012). In humans, MUC2 is coated with more than 100 different O-linked glycan structures (Larsson et al. 2009), which notably differ from those in rodents (Thomsson et al. 2012). The difference in the glycan

"preferences" of bacterial species was suggested to explain host-specific selection of a characteristic microbiome profile (Donaldson et al. 2015).

*Mucus in the small intestine and colon*

Mucus covers the intestinal epithelium to a different extent along the GIT both in humans and animals. The stomach and colon have a two-layered system with an inner and an outer mucus layers whereas the small intestine has only a single layer (Ermund et al. 2013). In this region, the mucus fills up the space between the villi and covers them, but is not attached to the epithelium and is penetrable to particles with the same size as bacteria (Ermund et al. 2013). As mucus is not anchored to the epithelial surface, this substance moves with peristaltic waves in a distal direction. Fresh mucus is constantly secreted from the goblet cells, especially from the crypt openings.

In contrast to the small intestine, the colon exhibits a two-layered mucus system organization. The inner mucus layer is continuously formed by secretion from the surface goblet cells (Johansson 2012). Upon secretion, the MUC2 mucin unfolds and forms large net-like structures that are arranged by interacting with previously secreted inner mucus layer (Ambort et al. 2012). The inner mucus layer is firmly attached to the epithelium with a thickness of 50 μm and 200–300 μm in mice and humans, respectively (Atuma et al. 2001; Johansson et al. 2008; Hansson & Johansson 2010). An estimate of the turnover of the inner mucus layer in live murine distal colonic tissue suggests that it is renewed every 1–2 hr (Johansson 2012). This turnover kinetics is important for maintaining this layer free from bacteria (Johansson et al. 2008). Another important factor for the host protection is the staggered layers of MUC2 that act as a size-exclusion filter (Johansson et al. 2008; Round et al. 2012) and are thus not penetrable to bacteria or beads with sizes down to 0.5 μm (Johansson et al. 2014). The inner mucus layer is then converted into the outer layer. This conversion, also observed in the mucus of germ-free animals, probably depends upon host protease activities (Johansson et al. 2008). However, the nature of the protease(s) involved and the activation trigger(s) remain to date unknown.

***Intestinal microbiota***

Gut microbiota of monogastric mammals contains members of the three domains of life (Archae, Bacteria and Eukarya). Results presented here only focus on the bacterial microbiota composition and functions.

*Composition and functions of the gut microbiota*

Adult mammalian intestinal microbiota are dominated by two phyla, *Bacteroidetes* and *Firmicutes*, whereas *Actinobacteria* (including *Bifidobacterium* spp.), *Proteobacteria* (including *Escherichia coli* and *Enterobacteriaceae* family), *Verrucomicrobia* (including *Akkermansia muciniphila*) phyla are only present in minor proportions (Bäckhed et al. 2005; Eckburg et al. 2005; Ley et al. 2008). Along the GIT, microbiota are not homogeneous and displays both longitudinal from stomach to distal colon (Gu et al. 2013; Donaldson et al. 2015) and latitudinal from crypts to lumen heterogeneities (Nava et al. 2011; Pédron et al. 2012). Although the microbiota composition under healthy conditions is relatively stable at the phylum level among individuals, a high variable bacterial composition at the species level was detected. However, the functional composition including metabolism of indigestible carbohydrates, proteins and bile acids remains relatively stable (Rajilić-Stojanović et al. 2012; Shafquat et al. 2014). Some external host factors, such as diet (Davenport et al. 2014; Salonen & De Vos 2014), stressor exposure (Bailey et al. 2010), antibiotic treatment (Maurice et al. 2013), are known to exert a marked influence on bacterial gut microbiota. These changes may be transient for

moderate or non-permanent perturbations as gut microbiota is considered to be relatively resilient (Lozupone et al. 2012).

The concentration and microbial diversity of the gut microbiota increase from duodenum to distal colon segments. In fact, in humans, the bacterial concentration commonly rises by approximately 8 $Log_{10}$ orders of magnitude from the beginning of small intestine ($10^{3-4}$ CFU/g) to distal colon ($10^{11-12}$ CFU/g) (Sekirov et al. 2010). For healthy subjects, such high variations depend upon local gut physical parameters including pH, redox potential, oxygen concentration (Albenberg et al. 2014) and/or the presence of antimicrobial molecules (α– and β–defensins, lysozyme, RegIIIγ), bile salts, reactive oxygen species (ROS) and digestive enzymes secreted by the host in the lumen.

*Small intestine microbiota*

The bacterial population inhabiting the small intestine possesses common traits to cope with a harsh physico-chemical environment, characterized by (1) high concentration of secreted bile acids, (2) rapid luminal flow and (3) presence of a small amount of oxygen. In humans, small intestine microbiota consist of facultative anaerobes (*Lactobacilliaceae*, *Streptococcaceae*, *Enterobacteriaceae*) and strict anaerobes (*Veillonella* spp. and *Clostridium* cluster XIVa), belonging to *Proteobacteria* and *Firmicutes* phyla.

*Large intestine microbiota*

In the large intestine, non-digestible carbohydrates such as cellulose, hemicellulose and resistant starch as well as host mucins are hydrolyzed. Degradation of polysaccharides including cellulose or hemicellulose, is performed by members of *Ruminococcus* and *Roseburia* genera belonging to the *Lachnospiraceae* family (*Clostridium* cluster XIVa) and *Firmicutes* phylum. Some members of *Prevotella* and *Bacteroides* genera (*Bacteroidetes* phylum) are also active in this degradation process (Rajilić-Stojanović 2013; Louis et al. 2014). Fermentation of monosaccharides then leads to the production of short chain fatty acids, in particular acetate, propionate and butyrate. The latter is the primary energy source for colonocytes. This substance plays a key role in colon epithelial protection, against colorectal cancer by inhibiting HDACs (histone deacetylases) in cancerous cells and activating HATs (histone acetyltransferases) in normal cells. Further, butyrate makes immune system hyporesponsive to beneficial commensals (Hamer et al. 2008; Donohoe et al. 2011; Koh et al. 2016). The main butyrate producers identified to date belong to the *Lachnospiraceae* or the *Ruminococcaceae* family, notably *Faecalibacterium prausnitzii* (Louis et al. 2010), which also displays anti-inflammatory properties (Sokol et al. 2008). Non-digested proteins or amino acids may be fermented by some proteolytic bacteria, mainly *Bacteroides* spp. However, some end-products of protein fermentation such as p-cresol or hydrogen sulfide ($H_2S$) are considered as toxic metabolites for the host. Further, bile acids that escape from absorption in terminal ileum are deconjugated to a less toxic form by members of *Bacteroides*, *Lactobacillus* and *Bifidobacterium* genera.

Gut microbiota determination is generally based on fecal material but in recent years, an increasing attention has been paid to bacteria living in close proximity to mucus, mucosa and/or crypts, and depicted as spatially organized biofilms at the mucosal surface (Nava et al. 2011; Pédron et al. 2012; De Vos 2015).

**Impact of mycotoxins on the gut: focus on epithelium, mucus and microbiota**

Data dealing with the impact of major mycotoxins, DON, ZEN, OTA, FB1, AFB1 and PAT on intestinal epithelium, mucus and microbiota are summarized below.

***Deoxynivalenol (DON)***

Among the mycotoxins produced by the *Fusarium* genus, the broad family of trichothecenes is prevalent; in particular, deoxynivalenol (DON) is present in cereal-based products (Table 1). DON is toxic to humans and many animal species, with pig being the most sensitive species to this mycotoxin and chicken the most resistant one (Pestka & Smolinski 2005; Pinton & Oswald 2014). Ingestion of DON typically results in a variety of adverse symptoms in animals, such as irritation or necrosis of intestinal mucosa, diarrhea, vomiting, reduction of feed intake, low body weight gain and alteration in immunological functions (Bondy and Pestka, 2000; Pestka 2010; Maresca 2013; Wang et al. 2014). In humans, food poisoning with nausea, diarrhea, and vomiting was associated with Fusarium-infested or DON contaminated cereals. For example, in a gastroenteritis outbreak in the Anhui province of China affecting more than 130,000 people, wheat samples collected were found to contain 2 to 50 mg DON/kg food (Wu et al. 2014). At the molecular level, DON binds to the 60S ribosomal subunit, induces a ribotoxic stress leading to the activation of MAP Kinases, cellular cell cycle arrest and apoptosis (Pestka 2010; Payros et al. 2016).

*DON and intestinal epithelium*

DON was shown to induce intestinal lesions in the jejunum and ileum in pigs, further confirmed in *ex vivo* studies using piglet jejunal explants (Pinton et al. 2012; Lucioli et al. 2013; Pierron et al. 2016a). In particular, shortening of villi height was found suggesting an effect of DON in the balance between epithelial cell proliferation and apoptosis. In rodents, sub-chronic exposure to DON-contaminated diet induced significant jejunal lesions associated with a lower height of villi (Payros et al. 2017). Such changes, also observed in broilers, lead to an altered absorption by intestinal epithelial cells, thus adversely impacting animal growth (Ghareeb et al. 2015). In pigs with a low-dose exposure and treatment duration between 1 and 6 weeks, no significant differences in morphometric parameters characterizing the architecture of the mucosa that is thickness of the mucosa, height of the villi and villus-to-crypt length ratio were noted in the duodenum of DON-exposed vs. control animals (Lewczuk et al. 2016).

Regarding the effect of DON on the intestinal epithelial barrier, DON was demonstrated to affect TEER in porcine and human cells lines (Pinton & Oswald 2014; Ghareeb et al. 2015; Pierron et al. 2016a). Kasuga et al. (1998) reported that DON reduced TEER and increased permeability of Lucifer yellow in human Caco-2 and T84 cells (). Similarly, DON enhanced paracellular permeability of porcine IPEC-1 cells and human Caco-2 cells to FITC-dextran in a time and dose-dependent manner (Pinton et al. 2009). In piglet jejunal explants, a 2-fold elevation in paracellular passage of FITC-dextran was observed in comparison with untreated jejunal tissue (Pinton et al. 2009). *In vivo*, a reduction of TEER was associated with an increase in paracellular passage of FITC-dextran in the intestine of chickens, pigs and mice exposed to DON (Akbari et al. 2014; Pinton & Oswald 2014; Ghareeb et al. 2015). In porcine epithelial cell monolayers, the rise in intestinal permeability was related to a specific reduction in expression of TJ proteins and more specifically claudins (Diesing et al. 2011; Akbari et al. 2014; Pinton & Oswald 2014). In particular, the diminished expression of claudin 4 was shown to be mediated by activation of the MAP kinase pathway (Pinton et al. 2010; Lucioli et al. 2013). The fall in protein expression was accompanied by an up-regulation, at least to some extent, of the *TJ* mRNAs levels both *in vitro* and *in vivo* (Osselaere et al. 2013; Akbari et al. 2014). Further, through activation of the MAP kinase pathway, DON induced a direct pro-inflammatory response increasing production of cytokines or chemokines, such as IL8, by intestinal epithelial cells (Maresca et al. 2008; Cano et al. 2013; Alassane-Kpembi et al. 2017b; Pierron et al. 2016a). Recently, Payros et al. (2017) demonstrated for the first time the implication of DON in DNA alteration of intestinal tissue. A significant increase in DNA

damage was observed both *in vitro* and *in vivo* in intestinal epithelial cells of rodents colonized since birth by a commensal *E. coli* strain producing a genotoxin, the colibactin, and co-exposed to DON, compared to animals colonized only with *E. coli* strain and fed a control diet.

Alterations in the intestinal epithelium integrity are subsequently responsible for the entry of commensal and/or pathogenic bacteria which normally are restricted to the gut lumen. Pinton et al. (2009) found that DON induced a concentration-dependent translocation of a pathogenic strain of *E. coli* across porcine IPEC-1 cells. Similarly, a significant elevation in passage of *Salmonella* Typhimurium was observed in porcine IPEC-J2 cells after DON exposure (Vandenbroucke et al. 2011). In human DON-exposed Caco-2 cells, Maresca et al. (2008) confirmed the facilitated transepithelial passage of non-invasive commensal *E. coli*. In mice, ingestion of DON-contaminated feed potentiated *Salmonella* Enteritidis infection (Hara-Kudo et al. 1996).

*DON and intestinal mucus*

Based on *in vivo* experiments, results suggest that DON may compromise mucus barrier integrity. In piglets fed DON-contaminated feed for 5 weeks, Bracarense et al. (2012) reported a decrease in number of goblet cells in the jejunum, in comparison with controls. In a study on piglet jejunal explants exposed to DON, Basso et al. (2013) noted the deleterious effect exerted by this mycotoxin on goblet cells. However, in pigs fed DON-contaminated feed for 1-6 weeks, the % goblet cells in the epithelium covering the villi and forming the crypts was similar in duodenum of treated and control animals (Lewczuk et al. 2016). Interestingly, Pinton et al. (2015) used an *in vitro* human muco-secreting cell line (HT29-16E), as well as porcine jejunal explants, to determine the influence of DON on intestinal mucus. Sub-toxic doses of DON were found to reduce intestinal mucin production through a specific decrease in levels of mRNA encoding for the membrane-associated mucin MUC1 and the secreted mucins MUC2 and MUC3. Wan et al. (2014) also examined the DON-induced impact on mucus by considering human muco-secreting HT29-MTX cells alone or in co-culture with Caco-2 cells. *MUC5AC* and *MUC5B* mRNA and protein, and total mucin-like glycoprotein secretion were significantly modulated. In particular, in the HT29-MTX monoculture, a significant downregulation of *MUC5AC* mRNA level was observed. In the Caco-2/HT29-MTX (70/30) co-culture, a significant downregulation of *MUC5AC* mRNA expression was found while the *MUC5B* mRNA level was upregulated. When the HT29-MTX monoculture was used, a significant increase in the MUC5AC and MUC5B protein levels was observed in cell lysate. In the 90/10 co-culture, a significant increase in the MUC5B protein supernatant level was observed. In the 70/30 co-culture, a decrease in the MUC5AC protein level was also found in cell supernatant. Finally, when the HT29-MTX monoculture was examined, DON significantly increased the total mucin in cell lysate whereas, in the 70/30 co-culture, it significantly decreased the total mucin level in cell lysate and in cell supernatant. To date, the only *in vivo* apparent study reported on the properties of the mucus layer, referred here as an "extrinsic component" of the intestinal barrier, has been conducted by in broiler chickens fed a DON-contaminated diet for 2 weeks (Antonissen et al. 2015b). In the mycotoxin-exposed group, mRNA expression levels of *MUC2* were diminished in the duodenum but not jejunum and ileum. Significant differences were observed in the mucin monosaccharide composition between control and mycotoxin-challenged animals as evidenced by increase in GalNac and NeuAc proportions, and decrease in Gal proportion in the duodenum (Antonissen et al. 2015b). Antonissen et al. (2015b) suggested that such changes in mucin O-linked oligosaccharide composition might affect the mucus layer integrity (Da Silva et al. 2014) and modulate intestinal microbiota composition, even though no experimental evidence was provided.

*DON and intestinal microbiota*

One of the first studies in pigs examined the possible interaction between DON and gut microbiota based upon the use of the Capillary Electrophoresis-Single Strand Conformation Polymorphism (CE-SSCP) method to determine an effect on microbial populations through increase in total number of cultivable aerobic bacteria in feces (Waché et al. 2009). Further, after a 4-week DON administration in human microbiota-associated rats which are gnotobiotic rats harboring human intestinal microbiota, Saint-Cyr et al. (2013) employed a qPCR approach to demonstrate a significant elevation of 0.5 $\log_{10}$ in the *Bacteroides*/*Prevotella* group and a decrease in *E. coli* in the fecal population. Recently, a 16S rRNA microbiota analysis showed that ingestion of a DON-contaminated diet in rodents did not significantly alter composition and diversity of gut microbiota (Payros et al. 2017).

It appears that DON may or may not exert a global effect on microbiota composition. Reciprocally, gut microbiota of various animals including rat, pig, cow and chicken metabolizes DON (Swanson et al. 1987; Hedman & Pettersson 1997; Payros et al. 2016). Young et al. (2007) demonstrated de-epoxidation of DON to DOM-1 (deepoxy-deoxynivalenol), a less toxic metabolite by chicken intestinal microbiota (Pierron et al. 2016b). Interestingly, a member of the *Eubacterium* genus, the strain BBSH 797 isolated from bovine rumen fluid, is also able to de-epoxidize DON and used commercially (Fuchs et al. 2002; He & Zhou 2010). Similarly, in humans, the capacity of fecal microbiota to metabolize DON to DOM-1 was noted by Gratz et al. (2013) albeit this process is variable among individuals depending on microbiota composition. A summary of the effects of DON on intestinal epithelium, mucus and microbiota is provided in Table 2.

***Zearalenone (ZEN)***

Zearalenone (ZEN), which is produced by *Fusarium* species, is a natural contaminant of the food chain in countries with temperate climate (Bennett & Klich 2003; Richard 2007) (Table 1). Toxicity of ZEN on the reproductive tract is well characterized (Kowalska et al. 2016); ZEN possesses an estrogen-like activity competing with endogenous hormones for binding to the estrogen receptors leading to hyper-estrogenism, precocious puberty and reproductive disorders (Afriyie-Gyawu et al. 2005; Zhao et al. 2013).

*ZEN and intestinal epithelium*

ZEN decreased the survival of intestinal epithelial cells without altering TEER *in vitro*. This mycotoxin also up-regulated expression of some genes implicated in cellular differentiation (*TFF2*, *BMP4*) and down-regulated the expression of tumor suppressor genes *DKK1*, *PCDH11X* or *TC5313860* (Wan et al. 2013a; Marin et al. 2015; Taranu et al. 2015). *In vivo*, in pigs fed ZEN-contaminated feed for 1-6 weeks, the thickness of the mucosa, height of villi and villus-to-crypt length ratio were similar in duodenum of treated vs. controls (Lewczuk et al. 2016).

*ZEN and intestinal mucus*

Few investigations to date examined the role of ZEN on intestinal mucus. The influence of ZEN on mucus was assessed *in vitro*, using HT29-MTX cells alone or in co-culture with Caco-2 cells (Wan et al. 2014). Recently Lewczuk et al. (2016) reported that in pigs fed ZEN-contaminated feed for 1-6 weeks, the % goblet cells in duodenum remained unchanged.

*ZEN and intestinal microbiota*

Piotrowska et al. (2014) determined the cultivable bacterial populations, present in ascending colon of pigs fed ZEN-containing feed. The community structure, which includes

diversity and richness, was estimated using the BIOLOG method. This method analyzes*in vitro* the consumption patterns of individual carbon substrates by the microbial communities. Data showed that ZEN reduced the levels of total cultivable aerobic bacteria. A summary of the effects of ZEN on intestinal epithelium, mucus and microbiota is presented in Table 3.

***Ochratoxin A (OTA)***

Ochratoxin A (OTA) is produced by the fungi *Aspergillus* section *Flavi*, *Penicillium verrucosum* and *Penicillium nordicum* (Table 1). This mycotoxin contaminates a wide variety of commodities and bio-accumulates in blood and milk of mammals exposed to OTA. In several animal species, OTA is a potent renal carcinogen by decreasing kidney function and inducing renal adenomas or carcinomas. The International Agency for Research on Cancer (IARC) thus considers OTA as possibly carcinogenic to humans (Group 2B) (Clark and Snedeker 2006; Pfohl-Leszkowicz & Manderville 2007; Kőszegi & Poór 2016).

*OTA and intestinal epithelium*

Although the kidney is the main target for OTA-induced toxicity, histological abnormalities and lesions of the GIT were reported. In particular, in chickens, a decrease in villi height was observed, in combination with an increase in necrosis and apoptosis of intestinal epithelial cells with prismatic epithelium and alterations of brush border (Solcan et al. 2015). Interestingly, a rise in crypt depth was also detected, as a compensatory process to shortening of villi.

Several *in vitro* studies showed that OTA inhibited cell growth, enhanced apoptosis, diminished TEER and increased permeability to mannitol or FITC-dextran (Maresca et al. 2001; Lambert et al. 2007; Ranaldi et al. 2009). When human Caco-2 cells were exposed to OTA, translocation of commensal *E. coli* K12 strain was facilitated (Maresca et al. 2008). A reduction in expression of TJ proteins, namely claudins 3 and 4, was noted and these adverse effects on intestinal permeability were associated with an elevation in OTA absorption and, thus, toxicity on systemic organs (Maresca et al. 2001; McLaughlin et al. 2004; Lambert et al. 2007).

Treatment of epithelial esophageal cells (Het-1A) with OTA induced DNA double strand breaks and chromosome aberrations with the observation of dicentric chromosome, ring chromosome and chromosome breaks and gaps in a concentration-dependent manner (Liu et al. 2015). Further, these changes were associated to G2 cell cycle arrest and cellular apoptosis (Cui et al. 2010; Liu et al. 2015).

*OTA and intestinal mucus*

In an early study, Lee et al. (1984) investigated the localization of OTA in the gut of mice given orally a single dose of mycotoxin. OTA was predominantly found to be present in the surface mucus of stomach. To the best of our knowledge, no other apparent data are available on this mycotoxin.

*OTA and intestinal microbiota*

Guo et al. (2014) investigated in rats the effects of a 28-day OTA administration on fecal microbiota composition. Bacterial composition was determined by 16S rRNA levels. In parallel, shotgun sequencing was performed to evaluate changes in functional genes. OTA treatment was shown to enhance relative abundance of members of the *Lactobacilliaceae* family, accompanied by an increase in facultative anaerobes and a decrease in microbial α-diversity. Guo et al. (2014) suggested that absorption, rather than hydrolysis, was the main route

by which *Lactobacillus* species was able to detoxify OTA. Such modifications of gut bacteria composition, together with alterations in specific functional genes, including signal transduction, sugar and amino acid transport systems clearly indicated that chronic exposure to OTA may be involved in shaping gut microbiota. A summary of the effects of OTA on intestinal epithelium, mucus and microbiota is given in Table 4.

***Fumonisin B1 (FB1)***

Fumonisins are produced by the fungi *F. verticillioides, F. proliferatum* and some related species from the *Fusarium* section *Liseola* (Norred, 1993; Marasas 2001) (Table 1). These mycotoxins mainly contaminate maize in the field. The family of fumonisins includes more than 22 congeners classified into 4 groups: A, B, C and P. Among fumonisins, the most prevalent that contaminates feed and food is fumonisin B1 (FB1). FB1 toxicity was described in several animal species, including horses where it induced encephalomalacia leading to death (Marasas 2001). Several studies demonstrated hepatotoxic, nephrotoxic, carcinogenic, immunotoxic and reprotoxic effects attributed to FB1 (Norred, 1993; Riley et al. 1994). In South Africa, a correlation was established between high human esophageal cancer rates and fumonisin-contaminated diet (Sun et al. 2007) and, since 2002, FB1 is considered by IARC as possibly carcinogenic to humans (Group 2B). At the molecular level, FB1 acts as a potent inhibitor of ceramide synthase, a key enzyme in the *de novo* synthesis and turnover of complex sphingolipids, leading to decrease in concentration of sphingomyelin and glycosphingolipids, which participate to the composition of cellular membranes (Wang et al. 1991).

*FB1 and intestinal epithelium*

FB1 was shown to (1) alter viability and proliferation of intestinal epithelial cells, (2) modulate the production of pro-inflammatory cytokines (IL8) and (3) affect the intestinal barrier function (Bouhet et al. 2007). In fact, in porcine IPEC-1 cells, FB1 decreased the survival rate in a concentration-dependent manner, which was also reported in human Caco-2 and HT29 cells (Kouadio et al. 2005; Wan et al. 2013a; Minervini et al. 2014). Through the inhibition of the ceramide synthase and thus disruption of lipid metabolism as first molecular event, FB1 increased lipid peroxidation and free sphinganine levels in intestinal epithelial cells, inducing a block of cells in G0/G1 phase which resulted in cell growth inhibition and induction of apoptosis (Bouhet et al. 2004; Minervini et al. 2014). In piglets, exposure to FB1 elevated the ratio of sphinganine to sphingosine and altered distribution of glycoproteins in the jejunum (Loiseau et al. 2007). An increase in transepithelial passage of FB1 was also observed in IPEC-1 cells (Loiseau et al. 2007). In addition, FB1 was found *in vivo* and *ex vivo* to alter intestinal barrier integrity, diminishing the expression levels of TJ proteins (E-cadherin, occludin) while enhancing transcellular permeability and bacterial translocation (Lessard et al. 2009; Lallès et al. 2009; Bracarense et al. 2012; Basso et al. 2013).

*FB1 and intestinal mucus*

Brown et al. (1992) first reported that FB1 at high doses was responsible for intestinal goblet cell hyperplasia in intoxicated broiler chickens. Bracarense et al. (2012) in piglets fed fumonisin (FB1/FB2 mixture) noted a fall in number of goblet cells in the ileum, while in the jejunal segment, the number remained unchanged. In contrast, in a study on piglet jejunal explants exposed to FB1, Basso et al. (2013) observed a reduction in number of goblet cells. In broiler chickens fed a FB1/FB2 mixture-contaminated diet for 2 weeks, the mRNA expression level of *MUC2* was lowered in the duodenum, which was accompanied by significant differences in the mucin monosaccharide composition with an increase in GalNac and NeuAc proportions, and decrease in Gal proportion (Antonissen et al. 2015b). To the best of our knowledge, the only study *in vitro*, demonstrating the impact of FB1 on mucus through

MUC5AC and MUC5B, was reported by Wan et al. (2014) on HT29-MTX cells, alone or in combination with Caco-2 cells. In particular, in the Caco-2/HT29-MTX (90/10) co-culture, FB1 significantly upregulated *MUC5AC* and *MUC5B* mRNA levels. In the 70/30 co-culture, significant downregulation of *MUC5AC* and *MUC5B* mRNA expression was found. In the 90/10 co-culture, FB1 significantly increased the MUC5AC protein level in cell lysate but led to a decreased level in cell supernatant. Conversely, no changes in the MUC5B protein level in cell lysate were observed. In the 70/30 co-culture, FB1 significantly decreased the total mucin level in cell lysate.

*FB1 and intestinal microbiota*

The only apparent study reported was in pigs by Burel et al. (2013) who observed a transient change in similarity between microbial CE-SSCP profiles after a fumonisin-based feeding. A summary of the influence of FB1 on intestinal epithelium, mucus and microbiota is presented in Table 5.

***Aflatoxin B1 (AFB1)***

Aflatoxins, which are mainly produced by *Aspergillus flavus*, *A. parasiticus* and some related species from *Aspergillus* section *Flavi*, represent a large family of mycotoxins present in a wide variety of cereals and tropical and sub-tropical food/feed stuffs (Table 1). The most abundant form of aflatoxins is aflatoxin B1 (AFB1), which exerts a potent genotoxic and carcinogenic effect both in humans and animals. Indeed, AFB1 is the most potent hepatic carcinogen known in mammals and has been classified by IARC in Group I (carcinogenic to humans). AFB1 targets the liver and increases the development of hepatic cancers and acute aflatoxicosis. Aflatoxicosis is an acute hepatitis associated with vomiting, abdominal pain, edema and may lead to death. In 2004 in Kenya, an aflatoxicosis outbreak, associated with excessive aflatoxin concentrations in maize, resulted in 317 cases and 125 deaths (Azziz-Baumgartner et al. 2005). Among animal species, poultry is extremely sensitive to AFB1, resulting in decreased growth and egg production as well as increased susceptibility to diseases and other adverse effects including immunotoxicity (Jakhar & Sadana 2004).

*AFB1 and intestinal epithelium*

Few studies have to date reported the effect of AFB1 on intestinal epithelium. In human Caco-2 cells, AFB1 decreased TEER (Gratz et al. 2007), altered cell viability and growth, accompanied by increased lactate dehydrogenase (LDH) activity release and DNA damage (Zhang et al. 2015). Several *in vivo* studies in broilers noted that dietary exposure to AFB1 at low doses reduced the density of whole intestine (weight/length), suggesting a diminished absorptive area. However, at higher doses, no histological changes were observed (Ledoux et al. 1999; Kana et al. 2011; Yunus et al. 2011). The number of apoptotic cells in the jejunum and expression levels of *Bax* and *caspase-3* genes were elevated in chickens fed AFB1-contaminated diet in comparison with controls. This was associated with a lower jejunal villi height and less proliferation of cell nuclear antigen-positive cells (Peng et al. 2014; Zhang et al. 2014). In rodents, AFB1 induced intestinal lesions in the duodenum and ileum, characterized by a leucocytic and lymphocytic infiltration (Akinrinmade et al. 2016).

*AFB1 and intestinal microbiota*

In a study conducted in rats following AFB1 chronic exposure, Wang et al. (2016), using 16S rRNA sequencing, observed a reduction of microbial diversity in the colon. An adverse effect on lactic acid bacteria population after treatment was noted while the proportion of *Firmicutes* and *Bacteroidetes* remained unchanged. Using a cultural method, Galarza-Seeber et al. (2016) examined the total aerobic, facultative anaerobe (coliforms) and lactic acid bacterial

populations in cecum of broilers exposed to AFB1. At the highest dose tested, the coliform population was 10-fold higher whereas, for other microbial populations under study, only a numerical non-significant rise was observed. A summary of the influence of AFB1 on intestinal epithelium and microbiota is given in Table 6. To the best of our knowledge, there are no apparent data available to date on intestinal mucus.

***Patulin (PAT)***

Patulin (PAT) is an unsaturated heterocyclic lactone, mainly produced by several fungal species belonging to *Penicillium*, *Aspergillus*, *Paecilomyces* and *Byssochlamys* (Table 1). Patulin is frequently detected in moldy fruits, fruit products, barley, wheat, corn and their processed products. PAT was originally identified for its antibiotic properties but mycotoxin-mediated neurotoxicity led to its abandonment as a therapeutic substance (Puel et al. 2010). This mycotoxin is highly reactive towards thiol groups of proteins and glutathione (GSH) and produces structural DNA damage (Glaser & Stopper 2012). Since 1986, PAT is classified by IARC in Group 3 (not classifiable as to its carcinogenicity to humans). High ingested-doses induce acute symptoms including kidney alterations, ulceration, convulsions, pulmonary congestion, edema, GIT disturbances, inflammation and vomiting (WHO 1998).

*PAT and intestinal epithelium*

Ingestion of PAT leads to intestinal disorders and, in children, outbreaks of diarrhea were attributed to the consumption of contaminated fruits (Lewis et al. 1995). In rats, mice and hamsters, PAT induced histopathological lesions in the GIT, including epithelial degeneration, hemorrhage and ulceration of gastric mucosa, as well as exudation and epithelial desquamation in the duodenum (McKinley & Carlton 1980a; 1980b; McKinley et al. 1982). A 24-h exposure of proliferating Caco-2 cells markedly decreased the number of cells and viability rate in a dose-dependent manner due to necrosis but did not involve apoptosis (Tannous et al. 2017). PAT-mediated acute toxicity on differentiated cells was partly due to destruction of TJ proteins and was associated with a reduction in TEER in human HT29 and Caco-2 cells without affecting intestinal absorption and secretion (Mahfoud et al. 2002; Assuncao et al. 2014). In Caco-2 cells, Kawauchiya et al. (2011) suggested that the decrease in TEER was correlated with enhanced phosphorylation and subsequent degradation of ZO-1. In the same *in vitro* model, McLaughlin et al. (2009) reported a reduced expression of ZO-1 and occludin, which was accompanied by marked changes in the distribution but not levels of claudins. Translocation of non-invasive commensal *E. coli*, together with associated inflammatory responses, was also observed (Maresca et al. 2008). In rats, the influence of PAT on permeability, ion transport and morphology in isolated colonic mucosa were assessed *ex vivo* (Mohan et al. 2012). High doses of PAT diminished TEER, elevated permeability to mannitol and induced pronounced morphologic effects including cell detachment, epithelial desquamation and sub-mucosal swelling. For pig jejunal explants exposed to lower doses of PAT, no significant differences were found in lesional scores and villi height (Maidana et al. 2016). According to Mahfoud et al. (2002), the toxicity attributed to PAT involved, among other potential mechanisms, inactivation of the active site of protein tyrosine phosphatases, which regulate the function of TJ proteins.

*PAT and intestinal mucus*

In a recent study, Maidana et al. (2016) investigated the impact of PAT exposure on intestinal mucus using pig jejunal explants. PAT significantly lowered number of goblet cells in both villi and crypts. No other apparent data are to date available on this mycotoxin. A summary of the influence of PAT on intestinal epithelium is provided in Table 7. To the best of our knowledge, there are no apparent data available to date on intestinal microbiota.

## Conclusions and next challenges for the future

### *Mycotoxins impair the integrity of intestinal epithelial barrier.*

Dietary exposure of humans and animals to mycotoxins is of growing concern due to their frequent prevalence in food and feed commodities (Streit et al. 2013). The intestinal mucosa is the first physical, chemical and biological line of defense for the host and thus plays a pivotal role for maintaining intestinal homeostasis under mycotoxin-exposure stress. Evidence indicates that DON, ZEN, OTA, FB1, AFB1 and PAT when present individually markedly affects epithelial cells through various mechanisms as follows: (i) increase in cell death and cytotoxicity, (ii) enhanced permeability of the cell monolayer, (iii) decreased transepithelial electrical resistance (TEER), (iv) impaired expression, intracellular localization and function of TJ proteins and (v) subsequent translocation of harmful stressors, in particular bacteria (commensals and pathogens), from gut lumen to internal environment. Due to these epithelium-targeted alterations, it is worthwhile to unravel the significance of mycotoxin exposure in the initiation of inflammatory reactions and clinical manifestations such as intestinal disorders. This is of utmost importance as, especially for DON, PAT and ZEN, the differences between the health-based guidance values for mycotoxins and their effective doses inducing effects on the intestine are lower than the usual safety factor of 100 (Maresca & Fantini, 2010). Owing to apparent lactational transfer of various mycotoxins (Omar, 2012) and, as already proposed by Akbari et al. (2017), evaluation of an early life exposure, associated to an immature intestinal barrier function, also warrants further attention.

### *Intestinal mucus and microbiota are emerging targets for mycotoxins.*

Even though the epithelial monolayer is a major functional element of the intestinal barrier, increasing evidence suggests that other constituents are involved such as mucus and microbiota, and these are emerging targets for mycotoxins. Regarding mucus, among the various mycotoxins described in this review, DON is undoubtedly the most frequently documented. *In vivo*, DON toxicological impact on mucus has generally only been assessed through goblet cell number determination. The absence of a clear consensus concerning a deleterious or non-effect, viewed with respect to the sole “goblet cell number”, is at least to some extent due to differences in the experimental conditions used: intestinal region of interest, administration mode, and particularly duration, dose, frequency and type of animal species (rodents, pig, chicken) which exhibit varying susceptibilities to mycotoxin exposure. In addition to this “goblet cell number” criterion, evaluation of the mycotoxins/mucus interplay through other various indicators such as mucin expression, mucin/mucus composition, mucin O-glycosylation, thickness and physico-chemistry/penetrability of the mucus network also warrants special attention.

Concerning microbiota, few studies have to date been reported for all mycotoxins reported here, with demonstration of slight or moderate deleterious effects. However, especially during chronic exposure, the continuing presence of mycotoxins within the intestine may reasonably produce marked changes in the composition of microbiota, together with abundance and metabolic activity of specific groups or genera. Therefore, this area of research needs to be further addressed. In particular, one of the major challenges for the future might be to analyze mycotoxin-induced influence not only on fecal microbiota but also on mucosal microbiota, in close proximity to the epithelium or embedded within the mucus layer. Moreover, evaluating the variations of microbial functions such as signal transduction, metabolism and transport of complex sugars and proteins, employing transcriptomics and/or metabolomics, might be a great opportunity to assess the impact of mycotoxins on health.

***New mycotoxin-based associations may impair intestinal barrier integrity.***

New mycotoxins (emergent, modified and masked mycotoxins), as well as mycotoxin mixtures present in food and feed, warrant further attention. In particular, emergent mycotoxins, including beauvericin, enniatins and alternariol (Gruber-Dorninger et al. 2016), and also modified forms of major mycotoxins such as the acetylated forms of DON (Payros et al. 2016) require to be carefully examined since these are known to induce deleterious effects on intestinal functions. Similarly, simultaneous exposure to several mycotoxins may lead to additive, synergistic or antagonist toxic effects, which have been poorly evaluated through the prism of gut homeostasis (Alassane-Kpembi et al. 2015; 2017a).

***Mycotoxins may compromise gut homeostasis by influencing crosstalk between epithelium, mucus and microbiota.***

Another emerging challenge in the understanding of mycotoxin/intestinal barrier interplay is to take into account crosstalk between epithelium, mucus and microbiota. As illustrated in Figure 1, these three gut players act not only as individual but also as tightly-connected entities within the small intestine and colon to ensure host homeostasis. Regarding the epithelium/microbiota crosstalk, in various adult germ-free animal models such as rat, pig or zebrafish, inoculation of complex microbiota was shown to induce expression of genes involved in intestinal cell proliferation, differentiation and mucus biosynthesis as well as in innate immune response and nutrient metabolism (Jones et al. 2007; Cheesman et al. 2011; Tomas et al. 2015). The role of specific bacteria was also assessed (Bry et al. 1996; Hooper et al. 1999). Reciprocally, the host epithelium may regulate and shape the microbiota, notably through Paneth-cell mediated secretion of antimicrobial peptides (Bevins & Salzman 2011).

Epithelium and mucus are also intrinsically connected components since enterocytes/colonocytes are interspersed by goblet cells all along the GIT. However, few studies to date investigated whether and how these constituents act in concert and are reciprocally regulated. The sole apparent study noted by Da Silva et al. (2014) occurred in rats subjected to chronic psychological stress. In an attempt to dissect the underlying "orchestrated" mechanisms, Lu et al. (2011) investigated colonic gene expression patterns of mucin Muc2 knockout ($Muc2^{-/-}$) mice. Expression of genes involved in cell structure related pathways was significantly altered. In particular, expression of genes regulating cell growth was enhanced, in conjunction with increased crypt length and epithelial proliferation. Interestingly, the TJ-associated gene claudin-10 was upregulated, whereas claudin-1 and claudin-5 were downregulated.

Finally, concerning the mucus/microbiota interplay, the outer colonic mucus layer is the natural habitat for commensal bacteria (Johansson et al. 2011), due to the presence of mucin-derived carbohydrates that are utilized as an energy source (Bäckhed et al. 2005; Sonnenburg et al. 2005; El Kaoutari et al. 2013) or preferential binding sites through bacterial adhesins (Juge 2012). This may explain, at least to some extent, the regio-specific colonization of bacteria in the gut (Robbe et al. 2004). Specific bacterial mediators driving adhesion to mucins were identified, including pili (Von Ossowski et al. 2010; Le et al. 2013), mucus-binding proteins (MacKenzie et al. 2010) and other cell surface proteins (Pretzer et al. 2005). Numerous mucolytic bacteria have also been described ( Tailford et al. 2015), such as *A. muciniphila* (Derrien et al. 2004), *Bacteroides thetaiotaomicron* (Bäckhed et al. 2005), *Bacteroides fragilis* (Huang et al. 2011), *Ruminococcus gnavus* and *Ruminicoccus torques* (Png et al. 2010; Crost et al. 2013). Some of these were suggested to be considered as biomarkers in gut health and disease (Malinen et al. 2010; Png et al. 2010; Everard et al. 2013). In particular, *A. muciniphila* prevents obesity and associated metabolic disorders, underlying the important crosstalk

between host and "his" microbiota (Plovier et al. 2017). Further, Li et al. (2015) recently showed that bacterial species present in mucus exhibit differential proliferation and resource utilization compared to their counterparts in the lumen and this is not restricted to mucolytic capability such as iron harvesting. Reciprocally, commensal bacteria may shape the mucus phenotype, as shown in rodents (Jakobsson et al. 2015; Johansson et al. 2015). The mucus/microbiota symbiotic system was recently expanded by identification of "sentinel" goblet cells in charge of protecting the colonic crypts from bacterial intruders that would have overwhelmed the inner mucus barrier (Birchenough et al. 2016).

All the issues raised here highlight the necessity to determine the deleterious effects of ingested mycotoxins on epithelium, mucus and microbiota, which are depicted as a "ménage à trois", in which each component interacts with each other. In conclusion, a broad range of questions and future challenges still need to be addressed for a healthy gut by improving risk assessment for exposure of humans and animals to dietary mycotoxins.

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## Declaration of interest

The authors report no conflicts of interest.

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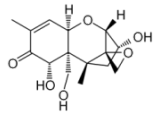
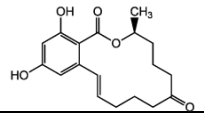
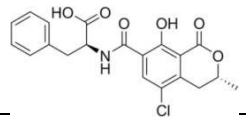
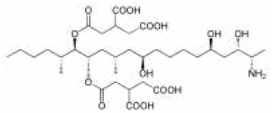
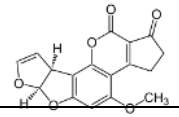
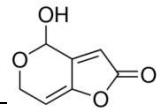
Table 1. Major mycotoxins: formula, producing species, affected crops, adverse effects and health-based guidance values (adapted from Karlovsky et al. (2016)).

| Mycotoxin | Chemical structure | Major producing fungi | Main contaminated crops | Main adverse effects & mode of action | Health-based guidance value |
|---|---|---|---|---|---|
| Deoxynivalenol (DON) |  | *Fusarium graminearum, F. culmorum* | Wheat, maize, barley, oats, rye | Feed refusal, emesis<br>Reduction of growth<br>Immunotoxicity<br>Not classifiable as to carcinogenic to humans<br>Binds to ribosomes and activates MAP Kinases | ARfD[a] 8 µg/kg bw/day<br>TDI[b] 1 µg/kg bw/day<br>group-PMTDI[b] (for DON and its acetylated forms) 1 µg/kg bw/day |
| Zearalenone (ZEN) |  | *F. graminearum, F. culmorum* | Wheat, maize, barley, oats, rye | Endocrine disruptor (interaction with estrogen-receptors) | PMTDI[c] 0.5 µg/kg bw/day<br>TDI[b] 0.25 µg/kg bw/day |
| Ochratoxin A (OTA) |  | *Aspergillus* section *Circumdati* or section *Nigri, Penicillium verrucosum, P. nordicum* | Cereals, nuts, dried fruits, coffee, cocoa | Nephrotoxic (renal tumors)<br>Carcinogenic to animals and possibly to humans | PTWI[d] 100-120 ng/kg bw/day |
| Fumonisin B1 (FB1) |  | *Fusarium* section *Liseola, Aspergillus niger* | Maize (*Fusarium* spp.)<br>Grapes *(A. niger)* | Inhibition of sphingolipid biosynthesis<br>Induction of apoptosis in liver<br>Tumorigenic in rodents<br>Possibly carcinogenic to humans | TDI[b] 2 µg/kg bw/day |
| Aflatoxin B1 (AFB1) |  | *Aspergillus* section *Flavi* | Maize, peanuts, nuts, pistachious, other dried fruits | Genotoxic carcinogen<br>Carcinogenic to humans | Because of carcinogenicity, exposure should be kept as low as reasonably achievable. No official health-based guidance value |
| Patulin (PAT) |  | *Byssochlamys nivea, Penicillium expansum, Aspergillus* section *Clavati* | Fruits especially apples, silage | Gastrointestinal ulceration<br>Immunotoxicity<br>Neurotoxicity | PMTDI[c] 0.4 µg/kg bw/day |

[a]: ARfD: Acute Reference Dose (for 1-day exposure)
[b]: TDI: Tolerable Daily Intake
[c]: PMTDI: Provisional Maximum Tolerable Daily Intake
[d]: PTWI: Provisional Tolerable Weekly Intake

Table 2. Effects of deoxynivalenol on intestinal epithelium, mucus and microbiota

| Intestinal partner | Experimental model | Conditions | Effects | References |
|---|---|---|---|---|
| Epithelium | Human and piglet cells | Various doses and durations | Decrease in cell survival<br>Increase in cell apoptosis<br>Decrease in TEER and increase in permeability<br>Decrease in expression of TJ proteins<br>Increase in bacterial translocation | (Pinton & Oswald 2014)<br>(Ghareeb et al. 2015)<br>(Akbari et al. 2017)<br>(Payros et al. 2016) |
| | Rat IEC-6 cells | 0 – 50 µM<br>4 - 8 h | Increase in DNA damage | (Payros et al. 2017) |
| | Piglet jejunal explants | Various doses and durations | Increase in cell apoptosis<br>Increase in lesional scores<br>Decrease in TEER and increase in permeability<br>Decrease in expression of TJ proteins | (Pinton & Oswald 2014)<br>(Cheat et al. 2016)<br>(Payros et al. 2016) |
| | Piglet, chicken, rodent | Various administration modes (contaminated diet, oral gavage) doses and durations | Increase in cell apoptosis<br>Increase in lesional scores<br>Decrease in TEER and increase in permeability<br>Increase in bacterial translocation | (Pinton & Oswald 2014)<br>(Ghareeb et al. 2015)<br>(Akbari et al. 2017)<br>(Payros et al. 2016) |
| | Pig | Water-soluble capsules<br>12 µg/kg bw/day<br>1 - 6 weeks | No change in the thickness of the mucosa, the height of the villi and the villus-to-crypt length ratio in the duodenum | (Lewczuk et al. 2016) |
| | Rat | Contaminated diet<br>10 mg/kg feed<br>4 weeks | Increase in jejunal lesions<br>Decrease in villi height | (Payros et al. 2017) |
| | Rat | Contaminated diet<br>2 - 10 mg/kg feed<br>1 - 4 weeks | Increase in DNA damage at adulthood | (Payros et al. 2017) |
| Mucus | Human HT29-16E cells | 0.1 - 100 µM<br>6 - 48 h | Decrease in mucin production<br>Decrease in *MUC1, MUC2* and *MUC3* mRNA | (Pinton et al. 2015) |
| | Human HT29-MTX and/or Caco-2 cells | 2 µM<br>48 h | Modulation of mRNA and protein of MUC5AC and MUC5B and mucin-like glycoprotein | (Wan et al. 2014) |
| | Piglet jejunal explants | 10 µM<br>4 h | Decrease in the number of goblet cells | (Basso et al. 2013) |
| | Pig | Contaminated diet<br>3 mg/kg feed 5 weeks | Decrease in the number of goblet cells | (Bracarense et al. 2012) |
| | | Water-soluble capsules<br>12 µg/kg bw/day<br>1 - 6 weeks | No change in the percentage of goblet cells in the epithelium covering the villi and forming the crypts in the duodenum | (Lewczuk et al. 2016) |
| | Chicken | Contaminated diet<br>4.6 mg/kg feed<br>2 weeks | Decrease in mRNA expression of *MUC2* in the duodenum<br>Significant increase in monosaccharide composition (GalNac and NeuAc)<br>Decrease in Gal proportion in the duodenum | (Antonissen et al. 2015b) |
| Microbiota | Pig | Contaminated diet<br>2.8 mg/kg feed<br>4 weeks | Increase in cultivable total aerobic bacteria<br>Change in intestinal microflora (CE-SSCP profiles) | (Waché et al. 2009) |
| | Rat | Oral gavage<br>100 µg/kg bw/day<br>4 weeks | Increase in *Bacteroides*/*Prevotella* group<br>Decrease in *Escherichia coli* | (Saint-Cyr et al. 2013) |
| | Rat | Contaminated diet<br>10 mg/kg feed<br>4 weeks | No change in the composition and the diversity of the gut microbiota | (Payros et al. 2017) |

Table 3. Effects of zearalenone on intestinal epithelium, mucus and microbiota

| Intestinal partner | Experimental model | Conditions | Effects | References |
|---|---|---|---|---|
| Epithelium | Porcine IPEC-1 cells | 10 - 100 µM<br>10 days | Decrease in survival rate<br>No effect on TEER | (Marin et al. 2015) |
| | | 10 µM<br>24 h | No effect on cell viability<br>Up-regulated expression of proliferative genes (*BMP4, CD67*)<br>Down-regulated expression of tumor suppressor genes (*DKK-1, PCDH11X, TC5313860*) | (Taranu et al. 2015) |
| | Porcine IPEC-J2 cells | 0 - 40 µM<br>48 h | Decrease in survival rate | (Wan et al. 2013a,b) |
| | Pig | Water-soluble capsules<br>40 µg/kg bw<br>1 - 6 weeks | No change in the thickness of the mucosa, the height of the villi and the villus-to-crypt length ratio in the duodenum | (Lewczuk et al. 2016) |
| Mucus | Human HT29-MTX and/or Caco-2 cells | 40 µM<br>48 h | Modulation of mRNA and protein of MUC5AC, MUC5B and mucin-like glycoprotein | (Wan et al. 2014) |
| | Pig | Water-soluble capsules<br>40 µg/kg bw<br>1 - 6 weeks | No change in the percentage of goblet cells in the epithelium covering the villi and forming the crypts in the duodenum | (Lewczuk et al. 2016) |
| Microbiota | Pig | Oral gavage<br>40 µg/kg bw/day<br>6 weeks | Reduction of total cultivable aerobic bacteria | (Piotrowska et al. 2014) |

Table 4. Effects of ochratoxin A on intestinal epithelium, mucus and microbiota

| Intestinal partner | Experimental model | Conditions | Effects | References |
|---|---|---|---|---|
| Epithelium | Human Caco-2 cells | 100 µM<br>24 h | Decrease in expression of claudins 3 and 4<br>Removal of claudins from microdomains to intracellular compartments | (Lambert et al. 2007) |
| | | 100 µM<br>24 h | Increase in permeability to FITC-dextran<br>Decrease in TEER | (McLaughlin et al. 2004) |
| | | 40 µM – 1 mM<br>48 h | Increase in paracellular permeability to mannitol<br>Intracellular redistribution of claudin 4<br>Increase in cell apoptosis | (Ranaldi et al. 2007) |
| | | 10 mM | Up-regulation of *MT2A* gene expression<br>Increase in cell apoptosis | (Ranaldi et al. 2009) |
| | Human Caco-2 and HT29 cells | 0 - 100 µM<br>72 h | Decrease in TEER in a dose-dependent manner<br>Decrease in cell growth | (Maresca et al. 2001) |
| | | 10 µM 48 h | Potentialization of its own absorption by increasing paracellular permeability | |
| | Human Het 1-A cells | 0 - 100 µM<br>24 h | Induction of DNA strand breaks and chromosome aberrations<br>G2 cell cycle arrest<br>Down-regulation of Cdc2 and cyclin B1 protein expression<br>Increase in apoptosis and activation of the cleavage of caspase 3 | (Liu et al. 2015) |
| | Human GES-1 cells | 0 - 100 µM<br>24 h | G2 cell cycle arrest | (Cui et al. 2010) |
| | | Long-term exposure<br>40 weeks | Induction of oxidative damage (ROS production)<br>Increase in proliferation, migration and invasion<br>Activation of Wnt/βcatenin pathway<br>Induction of anchorage-independent growth of cells in soft agar | (Jia et al. 2016) |
| | Chicken | Oral gavage<br>1 - 50 µg/kg bw/day<br>2 or 4 weeks | Decrease in body weight gain<br>Decrease in small intestine villi height<br>Small intestine crypt hypertrophy<br>Induction of cells with multiple nuclei rich in dispersed chromatin | (Solcan et al. 2015) |
| Mucus | Mice | Oral gavage<br>25 mg/kg bw | Presence in the surface mucus of stomach | (Lee et al. 1984) |
| Microbiota | Rat | Oral gavage<br>70 and 210 µg/kg bw<br>28 days | Enhancement of *Lactobacilliaceae* family<br>Increase in facultative anaerobes<br>Decrease in microbial α-diversity | (Guo et al. 2014) |

Table 5. Effects of fumonisin B1 on intestinal epithelium, mucus and microbiota

| Intestinal partner | Experimental model | Conditions | Effects | References |
|---|---|---|---|---|
| Epithelium | Porcine IPEC-1 cells | 0 - 40 µM<br>48 h | Decrease in survival rate | (Wan et al. 2013a) |
| | | > 100 µM 48 h<br>> 20 µM 6 days | Increase in sphinganine concentration in a dose-dependent manner | (Loiseau et al. 2007) |
| | | 200 µM 16 days | Increase in transepithelial flux of FB1 | |
| | | 2 - 700 µM<br>48 h | Decrease in survival rate of proliferative and differentiated cells<br>G0/G1 cell cycle arrest<br>Decrease in TEER in a dose-dependent manner | (Bouhet et al. 2004) |
| | Human HT29 cells | 0 - 69 µM<br>12 - 72 h | Inhibition of cell proliferation<br>Increase in microviscosity of the cellular membrane<br>Increase in lipid peroxidation | (Minervini et al. 2014) |
| | Human Caco-2 cells | 1 - 150 µM<br>72 h | Decrease in cell viability | (Kouadio et al. 2005) |
| | Pig jejunal explants | 100 µM<br>4 h | Decrease in expression of E-cadherin | (Basso et al. 2013) |
| | | 10 µM<br>2 h | Increase in TEER<br>Increase in permeability to horseradish peroxidase | (Lallès et al. 2009) |
| | Pig | Bolus oral administration<br>1.5 mg/kg bw/day<br>9 days | Increase in production of stress proteins : COX-1, nNOS, hsp70 and HO-2 | (Lallès et al. 2010) |
| | | Oral gavage<br>1.5 mg/kg bw/day<br>7 days | Increase in ratio of sphinganine to sphingosine<br>Changes in the distribution of glycoproteins in the jejunum | (Loiseau et al. 2007) |
| | | Oral gavage<br>2.8 µmol/kg bw/day<br>2 weeks | Reduction of villi height<br>Increase in lesional scores | (Grenier et al. 2012) |
| | | Contaminated diet<br>5.9 mg (4.1 mg FB1 + 1.8 mg FB2)/kg feed<br>5 weeks | Reduction of cell proliferation in the jejunum<br>Decrease in expression of E-cadherin and occludin<br>Reduction of villi height and induction of atrophy and fusion of villi | (Bracarense et al. 2012) |
| | Rodent | Contaminated diet<br>810 mg/kg bw/day<br>90 days | Increase in number of mitotic cells in the small intestine | (Theumer et al. 2002) |
| | | Contaminated diet<br>10 mg/kg feed<br>60 days | Increase in necrosis and immune cell infiltrate | (Casado et al. 2001) |
| Mucus | Human HT29-MTX and/or Caco-2 cells | 40 µM<br>48 h | Modulation of mRNA and protein of MUC5AC, MUC5B and mucin-like glycoprotein | (Wan et al. 2014) |
| | Pig jejunal explants | 100 µM<br>4 h | Reduction of the number of goblet cells | (Basso et al. 2013) |
| | Pig | Contaminated diet<br>5.9 mg (4.1 mg FB1 + 1.8 mg FB2)/kg feed<br>5 weeks | Reduction of the number of goblet cells in the ileum | (Bracarense et al. 2012) |
| | Chicken | Contaminated diet<br>300 mg/kg bw<br>2 weeks | Hyperplasia of goblet cells | (Brown et al. 1992) |
| | | Contaminated diet | Decrease in mRNA expression of *MUC2* in the duodenum | (Antonissen et al. 2015b) |

| | | | | |
|---|---|---|---|---|
| | | 25.4 mg (FB1+FB2)/kg feed 2 weeks | Significant increase in monosaccharide composition (GalNac and NeuAc) Decrease in Gal proportion in the duodenum | |
| Microbiota | Pig | Contaminated diet 11.8 mg/kg feed 9 weeks | Global effect on gut microbiota (CE-SSCP profiles) | (Burel et al. 2013) |

Table 6. Effects of aflatoxin B1 on intestinal epithelium and microbiota

| Intestinal partner | Experimental model | Conditions | Effects | References |
|---|---|---|---|---|
| Epithelium | Human Caco-2 cells | 150 µM<br>24 - 72 h | Decrease in TEER<br>Increase in DNA damage | (Gratz et al. 2007) |
| | | 0.01 - 1 µg/mL<br>24 - 72 h | Alterations of cell viability and growth<br>Increase in DNA damage | (Zhang et al. 2015) |
| | Broiler | Contaminated diet<br>4 mg/kg feed<br>21 days | Decrease in weight and length of the intestine<br>Decrease in absorptive area | (Ledoux et al. 1999) |
| | | Contaminated diet<br>0.3 mg/kg feed<br>21 days | Increase in apoptosis of jejunal cells<br>Increase in expression of *Bax* and *caspase-3* genes<br>Decrease in expression of *Bcl2* gene | (Peng et al. 2014) |
| | | Contaminated diet<br>0.3 mg/kg feed<br>7 - 21 days | Decrease in jejunal villi height<br>Decrease in PCNA positive proliferative cells<br>Shedding of epithelial cells on the top of villi<br>G2 cell cycle arrest | (Zhang et al. 2014) |
| | | Contaminated diet<br>0.07 - 0.75 mg/kg feed<br>28 days | No critical histological change | (Yunus et al. 2011) |
| | Rodent | Intraperitoneal injection<br>2.5 mg/kg<br>3 days | Intestinal lesions in the duodenum and ileum<br>Leucocytic and lymphocytic infiltration in intestinal tissues | (Akinrinmade et al. 2016) |
| Microbiota | Rat | Oral gavage<br>5 - 75 µg/kg bw<br>4 weeks | Reduction of microbial diversity in the colon<br>No change in the proportion of *Firmicutes* and Bacteroidetes<br>Adverse effect on lactic acid bacteria | (Wang et al. 2016) |
| | Broiler | Contaminated diet<br>1 - 2 mg/kg feed<br>21 days | Tenfold increase in facultative anaerobe bacteria (coliform) in the caecum<br>Slight increase in total aerobic and lactic acid bacterial populations in the caecum | (Galarza-Seeber et al. 2016) |

Table 7. Effects of patulin on intestinal epithelium

| Intestinal partner | Experimental model | Conditions | Effects | References |
|---|---|---|---|---|
| Epithelium | Human Caco-2 cells | 1 - 150 µM<br>24 h | Dose-dependent decrease in cell viability | (Assunção et al. 2016) |
| | | 0 - 100 µM<br>24 h | Decrease in cell viability | (Mahfoud et al. 2002) |
| | | 3 - 100 µM<br>5 or 24 h | Increase in cytotoxicity<br>Increase in cell death | (Tannous et al. 2017) |
| | | 50 µM<br>72 h | Decrease in TEER<br>Increased phosphorylation and degradation of ZO-1 | (Kawauchiya et al. 2011) |
| | | 100 µM<br>5 h | Decrease in TEER<br>Increase in permeability to FITC-dextran<br>Reduced expression of ZO-1 and occludin<br>Changes in the distribution of claudins | (McLaughlin et al. 2009) |
| | Human HT29 cells | 0 - 100 µM<br>24 h | Decrease in cell viability | (Mahfoud et al. 2002) |
| | Human HCT 116 cells | 0 - 10 µM<br>24 h | Increase in cell apoptosis<br>Increase in cleaved caspase 3<br>G2/M cell cycle arrest<br>Increase in ROS production<br>Increase in *ATF3* gene expression | (Kwon et al. 2012) |
| | Pig jejunal explants | 10 – 30 – 100 µM<br>4 h | No significant difference in lesional scores and villi height | (Maidana et al. 2016) |
| | | 100 µM<br>4 h | Increase in cell apoptosis | |
| | Rat colonic explants | 500 µM<br>24 h | Decrease in TEER<br>Increase in permeability to mannitol<br>Morphological alterations of the mucosa | (Mohan et al. 2012) |
| Mucus | Pig jejunal explants | 100 µM<br>4 h | Reduction of the number of goblet cells in villi and crypts | (Maidana et al. 2016) |

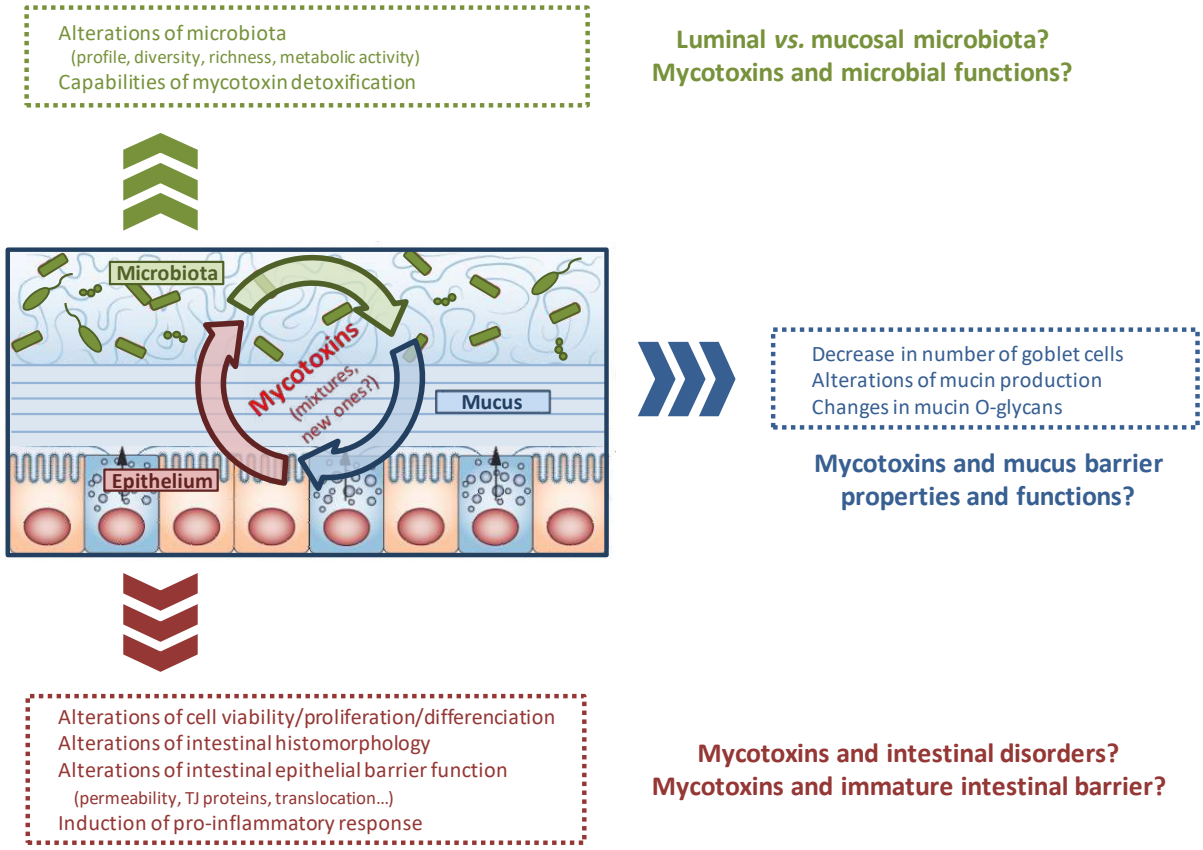



**Figure 1.** A schematic view of the three gut players involved in intestinal barrier integrity: example of the colon (adapted from Johansson et al. 2013). The two-layered mucus with different properties is coloured in blue. The bacteria residing in the outer mucus layer are indicated in green. The epithelium (in red) combines colonocytes and mucin-secreting goblet cells. For each constituent, main findings on the effects of mycotoxins are summarized. These three constituents act not only as individual but also as tightly-connected entities to ensure gut homeostasis. The consequences of mycotoxin exposure on this "orchestrated" triad should be evaluated as a challenging question for the future. Other questions to be addressed are also indicated.