Fusarium mycotoxins and in vitro species-specific approach with porcine intestinal and brain in vitro barriers: A review

Review

Fusarium mycotoxins and *in vitro* species-specific approach with porcine epithelial barriers: a review

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Abstract

Fusarium mycotoxins, such as fumonisins, trichothecenes, zearalenone and emerging fusariotoxins, common contaminants of feed and food, have received increased interest, due to the possible impact on animal and human health.

In this context, it is urgent to focus our attention on fusariotoxins adverse effects, considering and analysing data in relation to their species-specificity.

The *in vitro* approach for fusariotoxins risk assessment evaluation, through porcine epithelial barriers model, allowed to collect information on their absorption profile, bioavailability and toxicity.

The aim of this review is to give an overview on *Fusarium* mycotoxins and their interactions with porcine intestinal and brain *in vitro* barriers, because they represent a direct target organ of toxicity and as a tool to evaluate their permeability and transport.

Keywords: epithelial barriers, species-specificity, porcine, intestinal barrier, brain barrier, fusariotoxins

Highlights

- Porcine intestinal epithelial cell lines (IPEC) are widely used as species-specific model of the intestinal barrier;
- Primary Porcine Brain Capillary Endothelial Cells (PBCEC) are commonly used for neurotoxicity studies.
- Effects of the fusariotoxins, zeralanone, Fumonisin B1, beauvericin, enniatins, and T2 toxin, on IPEC and PBCEC are described;

1. Introduction

Fumonisins, trichothecenes, and zearalenone, *Fusarium* mycotoxins of primary concern (Bakker, 2018), together with so-called emerging fusariotoxins*, i.e.* beauvericin and enniatins, on which attention has recently increased because of their frequent isolation in food and feed commodities (Fraeyman et al., 2017; Streit et al., 2013), are raising concern for possible adverse effects on animal and human health (D'Mello et al., 1999; Ferrigo et al., 2016; Bakker et al., 2018).

In this context, it is urgent to collect information on toxicological effects, through a cell-based assay strategy, with a species-specific approach.

Regarding emerging and traditional *Fusarium* mycotoxins, *in vitro* data on absorption, bioavailability, transport and effects, as the results of exposure to predictive *in vitro* models of epithelial barriers, are well focused mainly on porcine epithelial biological barrier of intestine and brain (Krug et al., 2018; Nossol et al., 2015).

Indeed, some mycotoxins have the epithelial barriers as their targets (Pinton et al., 2010), whereas others, crossing it, are able to reach their target organs and exert their toxicity (Behrens et al., 2015; Fraeyman et al., 2017).

In vitro epithelial barriers have found many applications in research and industrial settings, either as stand-alone method, or as a part of an Integrated Testing Strategy (Gordon et al., 2015; Rovida et al., 2015), mainly as human tissue/cell based.

The aim of this review is to collect and summarize data on *Fusarium* mycotoxins, traditional and emerging ones, and their interactions on the principal porcine epithelial barriers, in the optic of species-specific toxicity.

2. Porcine intestinal barriers (IPEC-1 and IPEC-J2)

Regarding the intestinal barrier, various techniques have been studied to obtain suitable polarised gut columnar epithelium *in vitro* and several animal intestinal cell lines are available. Among these, the intestinal

porcine epithelial cell lines IPEC-1 and IPEC-J2, derived from pig small intestine and cultured on permeable support membranes that allow the creation of an apical and a basolateral compartment, are one of the most used species-specific model of the intestinal barrier and are considered valuable tools for the *in vitro* investigation of intestinal function and morphology (Schierack et al., 2006; Diesing et al., 2011; Nossol et al., 2011). Both immortalized cell lines were obtained from piglets less than 12 hold (Berschneider, 1989) and are non-transformed, not carcinoma derived and maintain most of their original epithelial nature (Mariani et al., 2009). IPEC-J2 is columnar epithelial cell line that was isolated for the first time in 1989 by Berschneider *et al.* (1989) from mid-jejunum of neonatal unsuckled piglets (Berschneider, 1989), whereas IPEC-1 were isolated from jejunum and ileum, a more distal part of the intestinal tract, thus they represent a slightly different cell population (Schierack et al., 2006). IPEC-J2 undergo spontaneous differentiation during i*n vitro* culture on inserts with the formation, within 1-2 weeks, of a polarized monolayer. The transepithelial electrical resistance (TEER) is one of the most used parameters to evaluate the formation of the tight junctions and thus the functional integrity of the epithelial monolayer. Interestingly, this parameter is influenced by the type of serum used in the culture medium: higher TEER values (therefore low active transport rates) are obtained when the cells are cultured in medium added with fetal bovine serum (FBS) (Geens and Niewold, 2011), whereas porcine serum (PS) induces higher transport rates and thus lower TEER values, a condition that is comparable with the *in vivo* situation (Zakrzewski et al., 2013), with cells showing smaller horizontal dimension and higher vertical diameter. IPEC-1 cell line, as IPEC-2, also shows spontaneous *in vitro* polarization and apical-basolateral electrical resistance after differentiation (that occur within 10 days of culture). The first studies using IPEC-J2 grown in monolayer investigated the transepithelial ion transport and cell proliferation (Kandil et al., 1995; Rhoads et al., 1994) but then this cell line, as well as IPEC-1, have proven to be very useful also in toxicity studies including mycotoxicity studies. Acute and chronic toxicity caused by long-term exposure to mycotoxins have been investigated using these cell lines, thus they are accurate models of the *in vivo* pattern of cellular responses in the intestinal epithelium (Mariani et al., 2009). Moreover, the gastrointestinal system that, together with the integumentary system, represents the major barrier between the organism and the external environment, is often the route of exposure to potentially toxic substances, such as mycotoxins (Marin et al., 2013). Thus, toxicity studies involving these cell lines are

particularly useful to understand, under a species-specific point of view, absorption, cell transport systems involved, distribution, biotransformation, and excretion of mycotoxins, but also their interactions in cases of co-exposure as well as the efficacy of mycotoxin detoxifying agents (Devreese et al., 2012).

2.1. Zearalenone

Zearalenone (ZEA) is a mycotoxin produced by species of *Fusarium* genus (especially *F. graminearum*) which is a natural contaminant of crops in temperate climate geographical areas (Tabuc et al., 2009). This mycotoxin is characterized by a chemical structure that allows it to bind to the estrogenic receptors of mammalians, acting as an endocrine disruptor.

Regarding the intestinal effect, just few studies have been performed; lately ZEA's actions on the intestinal barrier had been investigated to evaluate the potential correlation between exposure to this mycotoxin and human chronic intestinal inflammatory diseases (Maresca and Fantini, 2010). A study performed by Taranu *et al.* (2015) used IPEC-1 to evaluate whether the exposure to low concentration of ZEA (Taranu et al., 2015) could cause differences in genome wide expression, in order to have a better overview on the mechanisms of action at the intestinal level. Cell viability after exposure and inflammatory mediator production were also analysed, finding that exposure of 80% confluent IPEC-1 to 0-100 µM ZEA for 24 h, caused a decrease of the cell viability in a concentration-dependent manner: concentrations up to 10 µM did not exert any detrimental effect on cell viability, whereas with concentrations of 100 µM, only 67% of the cells were still viable. Moreover, increasing concentrations of ZEA (namely 10, 50 and 100 µM) decreased cell proliferation in a dose-dependent manner and exposure to ZEA at 10 µM for 24 h induced up-regulation of inflammatory cytokine genes in undifferentiated IPEC- 1 (IL-6, IL-8, IL-10, IL- 18, TNF-α, MCP-1, IL-12p40 and CCL20). Interestingly, an up-regulation of the TLR-s gene was also found: this gene is responsible for the activation of IL-17C, a cytokine produced by intestinal epithelial cells involved in early innate immunity mechanisms which can activate a downstream signalling for the induction of genes encoding for a pro-inflammatory molecule but also for peptides that can exert a protective effect against inflammatory stimuli (Song et al., 2011). Other

affected genes (over a total of 1954 found to have an altered profile) were those involved in signalling and transcriptional regulation pathways with alterations in immune response to pathogens. Among them, an over expression of ITGB5, involved for example in the inhibition of the attachment and adhesion phases of enterotoxigenic *Escherichia coli* (ETEC) on porcine intestinal cells, was detected. ZEA was also found to induce oxidative stress on the intestinal cells, via up-regulation of glutathione peroxidase enzymes and to exert a potential carcinogenic action at the intestinal level, since genes responsible for initiating of tRNA transcription in eukaryotes (GTF3C4) and signal transducers and activators of transcription gene were highly expressed compared to the controls (Taranu et al., 2015).

The effects of ZEA on the intestinal barrier were also studied by Goossens *et al.* (2012). In this study IPEC-J2 were used to investigate the cytotoxic effects of ZEA and other mycotoxins. After 72 h of exposure to 0-20 μg/ml ZEA (the molar concentration corresponding with 1 μg/ml ZEA is 3.14 μM), a cytotoxic effect (concentration-dependent) was recorded when cells were exposed to concentrations greater or equal to 9 μg/ml. These results are in agreement with those of Wan *et al.* (Wan et al., 2013), who tested the effect of ZEA (2.5-40 μM) alone and in combination with other mycotoxins on IPEC-J2 and found a reduction in cell viability when ZEA was applied alone at dosages of 40 μM, whereas, interestingly, a dose of 10 μM showed significant increases in cell viability, compared to the control. Regarding the effects of mycotoxin mixtures on the intestinal barrier, all of them were more cytotoxic than the mycotoxins taken individually, revealing a non-additive interaction in all mixtures except for DON-ZEA-FB1which did not significantly affect cell viability. The association FB1–ZEA exerts a greater cytotoxic effect than FB1 or ZEA alone. Also, mixtures of ZEA and other mycotoxins at the individually non-toxic concentrations, exerted a cytotoxic effect on the cells, namely ZEA-DON, ZEA-FB1, ZEA-DON-nivalenol (NIV) and ZEA-DON-NIV-FB1. The sensitivity differences of this cell line to DON, NIV, ZEA and FB1 as well as their mechanism of action is not fully understood, and may involve, as demonstrated *in vivo*, metabolic transformation of the toxins. ZEA is reported to be biotransformed into glucuronide conjugates, α-zearalenol (the predominant form) and β-zearalenol (Malekinejad et al., 2006). The accumulation of α-ZEA can be responsible for the inhibition of cell proliferation also in this *in vitro* model, via the activation of apoptotic mechanisms of cell death (Luongo et al., 2006). Also, the intestinal cell

synthesis of defensins (antimicrobial peptides involved in the innate immune defence of mammals) was investigated using the IPEC-J2 cell line. Wan *et al.* (2013) evaluated the effect of *Fusarium* mycotoxins alone (ZEA, DON, NIV, FB1) and in combination on the mRNA expression, protein secretion, and the corresponding antimicrobial effects of porcine beta-defensins 1 and 2 (pBD-1 and pBD-2). After exposure to mycotoxins an induction of mRNA for both defensins was recorded, but this increase was not followed by a correspondent increase in the encoded protein levels. Moreover, the exposure to mycotoxins caused a reduction in the antibacterial properties of the cell supernatant. It has been found that the upregulation of defensins is one of the first protective responses of the intestine against bacterial infections, tissue injuries/inflammation (Veldhuizen et al., 2008) and that any perturbance in the delicate mechanisms of the defensin production may cause the alteration of the intestinal homeostasis predisposing animals to various diseases.

2.2. Fumonisin B_1

Fumonisins (FBs) are a group of cytotoxic and carcinogenic mycotoxins produced by many *Fusarium* species, predominantly *F*. *verticillioides* and *F*. *proliferatum* (Yazar and Omurtag, 2008) that occur worldwide primarily in maize. Among the fumonisin homologues (the known are at least 28, up to date), the most important is the B group, that include fumonisin B1 (FB1), B2 (FB2), B3 (FB3) that are the most relevant molecules of the group due to their toxicological activities (EFSA, 2005). The ability of FB1 to exert toxic effects on intestinal epithelial cell lines have been reported by several authors (Bouhet et al., 2004; Loiseau et al., 2007). In a study conducted by Bouhet *et al.* (2004) the cytotoxic effects of FB1 on IPEC-1 were investigated by measuring lactate dehydrogenase (LDH) release in the culture medium after 48 h of exposure to different concentrations of FB1 (2 to 700 μM), whereas the effects on cell proliferation and viability were assessed via a colorimetric assay (MTS bioassay). The analysis of the cellular cycle was performed by flow cytometry after exposure to concentrations 5 to 100 μM for 48 h, and the evaluation of the integrity of tight junctions was investigated by measuring the TEER during exposure to 0, 50, 200, and 500 μM FB1. They found that, at the lower concentrations employed (2 to 20 μM), FB1 did not show any cytotoxic effects on dividing IPEC-1, while at higher concentrations (>50 μM) the cytotoxic effect was dose-dependent. On confluent nondividing cells,

FB1 induced LDH release only when applied at the highest concentration of 700 μM. Regarding the effect of FB1 on cell proliferation, the mycotoxin had a negative effect (inhibition) in a dose-dependent manner. Cell growth was significantly affected already at FB1 concentrations (10 and 20 μM) proven to be noncytotoxic for IPEC-1 and in the same dose-dependent manner, exposure to FB1 (starting from concentration of 5 μM) blocked the IPEC-1 cell cycle in the G0/G1 phase. Moreover, TEER evaluation demonstrated that FB1 affected the monolayer integrity in a dose-dependent manner, and this effect was not influenced by the differentiation stage in which the cells were tested. Interestingly, when cells were cultured for another 16 days in a medium FB1-free after the exposure to FB1, a significant increase in TEER was recorded, even if it remained lower than those of the control cells, thus indicating a partially reversibility of the effect of FB1.

Loiseau *et al.* (2007) using the same cell line (IPEC-1), evaluated the trans-epithelial passage post-differentiation of FB1 from the apical to the basolateral cell compartment finding a linear correlation (r^2 = 0.98) between passage rate and dose (2 and 100 μM). At this FB1 concentration (100 μM), the cell permeability was increased threefold compared to those of the cells exposed to a lower concentration (50 μM). In addition, comparing these results with TEER data, they observed that the mycotoxin passage was significantly increased with low values of TEER, indicating a tight junction dysfunction linked to a damage of these structures. They also found a dose- and time-dependent effect of FB1 on the quantity of free sphingolipids in the cells with a significant increase in sphinganine concentration after 48 h of FB1 exposure at concentrations higher than 100 μM, and from day 6 of FB1 exposure with concentration >20 μM. Thus, it is likely that most of the effects produced by FB1 on the intestinal cells are mediated via inhibition of the ceramide synthase (enzyme that plays a key role in the de novo biosynthesis of sphingolipids), causing an accumulation of free sphingoid bases (sphinganine and, to a lesser extent, sphingosine) and a depletion of complex sphingolipids and ceramide (Wang et al., 1991). Sphingolipids represent a significative part of the cell membrane lipids (10-20%) and act as regulators in many cellular functions and pathways such as growth, differentiation, apoptosis, *etc*. Alterations regarding these molecules can affect many cell functions, i.e., specifically for the intestinal cells, the folate uptake since the transporter is a protein that requires

sphingolipids and cholesterol to exert its function (Merrill Jr et al., 2001). Moreover, the accumulation of sphingoid bases can inhibit protein kinase C and alter the cellular membrane architecture (Smith et al., 1997).

Also, the cell line IPEC-J2 has been used in studies for the evaluation of *in vitro* effects of FB1. In a study performed by Goossens *et al.* (2012) (Goossens, et al., 2012), the cytotoxic effects of FB1, among other *Fusarium* mycotoxins, were investigated. After 72 h of exposure to 0-15 μg/ml FB1 (the molar concentration corresponding with 1 μg/ml FB1 is 1.39 μM), no cytotoxic effect on the cells were observed, and this is in agreement with findings reported using IPEC-1 (Bouhet et al., 2004). Another evaluation of the cytotoxicity of FB1 on IPEC-J2, alone and in combination with other *Fusarium* mycotoxins (deoxynivalenol, nivalenol and zearalenone) was performed by Wan and co-workers (2013) (Wan et al., 2013). The dose-response effect of each mycotoxin alone on cell viability was assessed using MMT test and cytotoxic and non-cytotoxic concentrations were obtained. The IPEC-J2 proved to be a cell line that was generally resistant to FB1 (concentrations ranging from 2.5 and 40 μM), with cytotoxic and non-cytotoxic concentration values of 20 and 40 μM, respectively. Regarding mycotoxin combination, mixtures of FB1 and ZEA proved to be more cytotoxic than either FB1 or BEA alone. For mycotoxin combinations at individually non-cytotoxic concentrations, all mixtures showed cytotoxic effects, and the lowest cell viability (48.5 ± 23.0%) was recorded when all the four mycotoxins were mixed together. Moreover, the results showed a non-additive interaction in mixture of DON-NIV ($p = 0.008$), DON-ZEA ($p = 0.028$) and ZEA-FB1 ($p = 0.042$), DON-NIV-ZEA ($p < 0.001$) and DON-NIV-ZEA-FB1 ($p < 0.001$), confirming also with this cell line the presence of interactions in terms of toxic effects between mycotoxins and underlying the need to evaluate co-exposure and not only the effects of individual toxins.

2.3. Deoxynivalenol

Deoxynivalenol (DON), also known as vomitoxin, belongs to the large family of trichothecenes, that include more than 150 *Fusarium* mycotoxins that are potent inhibitors of protein synthesis. DON is produced by many Fusarium moulds but mainly by *F. graminearum* and *F. culmorum* and it is a common contaminant of wheat.

DON is a good example of a mycotoxin whose action is primarily associated with the disruption of the intestinal integrity (Pestka, 2010; Akbari et al., 2017) thus numerous studies have been performed on its actions on the intestinal barrier, both *in vitro* and *in vivo*. On polarized IPEC-1, DON induces a concentration- and time- dependent decrease in TEER values: after 24 h of exposure to 50 µM DON, a significant decrease of TEER values was recorded (60%) and after 14 days of exposure to concentration of DON at 5, 10, 20 and 50 µM, TEER values decreased by 58, 69, 75 and 97%, respectively. Likely this reduction was not due to cell death, since the exposure to these concentrations (0-50 µM) for 48 h did not significantly affect the cell viability (assessed by LDH release assay), even if exposure to higher concentrations of DON (200 µM) resulted in decreased cell viability. The impact of DON on cell permeability was also assessed using FITC-dextran: the cells were treated for 48 h with 0-50 µM DON and then FITC-dextran was added to the apical compartment. At the end of the experiment a dose-dependent increase of permeability was found for DON concentration of 5 and 20 µM, a finding which is in line with the TEER decrease, confirming TEER as a good indicator of the epithelium integrity. The alteration of the barrier function of the IPEC-1 after 48 h of exposure to 0-50 µM DON was also demonstrated by an increase of the *E. coli* translocation (*E. coli* was placed in the apical compartment and after 1-4 h the basolateral medium was collected to assess bacterial translocation). All these results clearly demonstrate that DON can act by modulating the paracellular pathway thus altering intestinal barrier function. Since tight junctions play a fundamental role in the integrity and functionality of the intestinal barrier, the actions of DON on the expression of these proteins were also assessed. Differentiated IPEC-1 cells, were treated with 0 and 30 µM DON for 48 h, then stained for ZO-1, occludin, claudin-3 or 4, which are common tight junction proteins (for review see Zhang et al., 2018). While ZO-1 and occludin staining pattern and localization were not affected by DON, the intensity of the staining for claudin-3 and claudin-4 was significantly decreased and the western blot analysis confirmed a reduced signal for claudin-3 (-67%) as well as for claudin-4 (-60%) in the cells exposed to DON, compared to the non-exposed cells (Pinton et al., 2009). Since it is well known that DON acts as an activator of mitogen-activated protein kinases (MAPKs) via the so-called mechanism of the ribotoxic stress response (Pestka et al., 2004) and that the tight junction function is regulated by MAPK proteins, it could be hypothesized that the mechanism of action of this toxin at the intestinal level may involve the DON-mediated activation of MAPKs with a

consequent negative effect on the expression of the tight junction proteins. This hypothesis was investigated in a study performed by Pinton *et al.* (2010) (Pinton et al., 2010), that investigated the role played by MAPKs in the DON-induced loss of barrier functionality using IPEC-1 cell line (which is considered to be very sensitive to DON). Results showed that DON induced MAPK activation at a dose of 30 µM for 1 h. Moreover, the inhibition of MAPK (obtained with a pretreatment of the cells with 0.5 µM of U0126, which is a MAPK inhibitor) avoids the DON-induced alteration of intestinal barrier and the DON-induced decreased expression of claudin-4 but has no effect on claudin-4 mRNA expression, thus it can be hypothesized that the pathway involved in the regulation of claudin-4 is not transcriptional. In a study performed by De Walle *et al.* (2010) on Caco-2 quantification of mRNA after exposure to DON showed an increase in the transcript level of claudin-4. Thus, the decreased claudin-4 protein expression, that has also been described in DON-exposed Caco-2 cells, may be due to the ability of this toxin to inhibit protein neosynthesis. Even if claudin-4 is not a specific target of the protein synthesis inhibition mediated by DON, the high turnover that characterized this protein may be responsible for its particular sensitivity to this mycotoxin. Indeed, it is well known that trichothecenes bind to ribosomal 60 S subunit in eukaryotic cells, interfering with peptidyl transferase activity (Cope, 2018). In particular, DON acts on synthesis inhibition during the elongation and termination phases (Ehrlich and Daigle, 1987), with the simultaneous onset of ribotoxic stress which cause the activation of cellular kinases involved in signal transduction pathways. Therefore, it has been hypothesized that tissues characterized by a high protein turnover or highly proliferating cells (*i.e.*, intestinal cells), are particularly sensitive to this mycotoxin (Desjardins, 2006).

IPEC-J2 were also used as a model to investigate investigate whether *Bacillus subtilis* could improve intestinal barrier functionality impairment due to exposure to DON (Gu et al., 2014). Before DON-exposure, heat-inactivated *B. subtilis* or lipoteichoic acid (LTA-BS) from the same bacteria were added for 1 h to the cell culture medium. Results showed that *B. subtilis* and LTA-BS were able to increase the expression of tight junction proteins (ZO-1 and occludin) and, when the cells were incubated with DON (2 µg/ml) for 48 h, both the group treated with *B. subtilis* and with LTA-BS showed an increased TEER value compared with non-treated group, thus demonstrating that these substances may have a protective effect on permeability in

IPEC-J2 exposed to DON. Regarding tight junction protein expression, pre-treatment with *B. subtilis* induced better expression of ZO-1, whereas pre-treatment with LTA-BS or no pre-treatment caused loss of cell-cell association. This data study suggests that *B. subtilis* may enhance the intestinal barrier resistance towards the detrimental effect of DON.

Based on these results, it has been hypothesized that the treatment of the toll-like receptor 2 (TLR2), that is expressed in the IPEC-J2, with several TLR2 ligands (LTA-BS, *B. subtilis*-derived peptidoglycan -PGN-BS, *S. aureus*-derived LTA -LTA-SA, synthetic TLR2 ligand Pam3CSK4 -PCSK and *E. coli*-derived lipopolysaccharide -LPS), may have a protective effect against the damages induced by DON on the intestinal barrier (Gu et al., 2016). TRL2 and, more in general, TRL, are a family of receptors that are constitutively expressed on intestinal epithelial cells that are involved in the discrimination between self- and microbial non-self, playing an important role in the activation of antimicrobial pathways, for the elimination of pathogens (Cario et al., 2004). Moreover, TRL2 are able to redistribute ZO-1 (by the activation of a protein kinase) increasing the epithelial barrier resistance, also towards ribotoxic stress induced-damages, promoting the maintenance of the "belt-like" cell-cell contacts of ZO-1 in the tight junction areas. Ribotoxic stress-induced response (Lebrun et al., 2015) consists in the activation of many MAPKs (*i.e.*, p38, that mediate apoptosis; ERK-1/ERK-2 that induce proinflammatory cytokine expression; *etc.*). The overall result of these pathways activation and interaction depends on concentrations and duration exposure to DON.

All the TLR2 ligands evaluated in the study significantly increased TEER values in a dose- and time-dependent manner after 24 and 72 h of treatment, only LPS had little or no effect. When PCSK and LTA-BS (two TLR2 ligands), were used as a pre-treatment, adding them to the culture medium for 24 h and then treating the cells with DON (2 µg/ml) for 48 h, the integrity of the barrier was maintained, as demonstrated by normal TEER values (comparable to those of the control cells) and tight junction expression was higher than those of the group treated only with DON. Moreover, pre-treating cells with LTA-BS, cell viability after DON exposure was significantly greater than those of the group treated only with DON. Gu *et al.* (Gu et al., 2016) demonstrated that DON-induced upregulation of MCP-1 expression was suppressed when a pre-treatment with TLR2 ligand was applied.

In order to better represent the intestinal environment, a modification of IPEC-J2 model was performed, co-culturing these cells with peripheral blood mononuclear cells (PBMCs) on the basis of the hypothesis that damage to IPEC-J2 exposed to DON could affect immune cells due to the close connection between intestinal epithelial cells and the lamina propria which is proximal to blood vessels. In this system, IPEC-J2 were cultivated on inserts, whereas the PBMCs were cultivated at the bottom of the wells, in order to avoid a direct contact between the two cell types. It was found that DON caused a decreased expression of CD163 by $CD172a^+$ monocytes, with a high production of tumor necrosis factor-α, showing that DON exposure could stimulate an inflammatory response. Moreover, PBMC viability was affected by DON-exposure (46.7% of viable cells in DON-treated group and 64.9% in the control group) with percentages of apoptotic and necrotic PBMC cells significantly greater in the DON-treated groups, compared with the control. Conversely, TLR2 ligand pre-treatment showed a decreased apoptosis of PBMCs after DON treatment, an increased percentage of viable cells and an increased proliferation of $CD172a^+$ cells compared with the non-pre-treated group (Gu et al., 2016). This study is an interesting application of an intestinal barrier model that has proven to be a good representation of the complexity of the intestinal environment and to be flexible enough to allow many applications.

2.3.T-2 Toxin

As DON, T-2 toxin (T2) is a trichothecene and like the other members of this family, T2 inhibits protein, RNA and DNA synthesis (Hossam, 2013). This mycotoxin is a natural contaminant of many crops including wheat and corn, and is mainly produced by *F. sporotrichioides* and *F. langsethiae*.

The cytotoxic effects of T2 have been evaluated with the IPEC-J2 cell line, using flow cytometric technique and exposing the cells for 72 h to increasing doses of T2 (0-10 ng/ml, the molar concentration corresponding to 1 ng/ml T2 is 2.14 nM). A concentration-dependent toxicity was demonstrated and, among other *Fusarium* mycotoxins (ZEA, DON, FB1), T2 appeared to be the most toxic on IPEC-J2, with a lower percentage of viable cells, even at very low concentration (ng/ml range), whereas DON and ZEA exert their toxicity at higher doses

(μg/ml range) and FB1 did not show any toxicity at concentrations of 0-15 μg/ml. Regarding the toxic effect of T2, comparing the percentages of viable, apoptotic and necrotic cells, the necrotic ones are the most numerous, and this is in line with the clinical findings of skin and gastrointestinal necrosis that are manifestations of T2 toxicosis (Harvey et al., 1990; Adhikari et al., 2017). Moreover, at cytotoxic concentrations (10 and 100 ng/ml), T2 caused a significant decrease in TEER values of differentiated IPEC-J2 after 72 h of exposure. On the contrary, exposure to non-cytotoxic concentrations did not cause any alterations in TEER values. Disruption of the epithelial integrity after 72 h of exposure to cytotoxic concentrations of T2 (10 and 100 ng/ml) was also proven by an increased passage of doxycycline and paromomycin across the polarized monolayer (Goossens et al., 2012). Regarding the mechanism of action, one of the T2 target sites includes the 60S ribosomal unit, where T2 acts impeding polypeptide chain initiation (Devreese et al., 2013). Since actively proliferating cells (like intestinal and skin cells) are more sensitive to this inhibitory effect (Rai et al., 2011), IPEC-J2 cell line could represent a good and sensitive model to study the effect of this toxin.

2.5. Beauvericin and Enniatins

Beauvericin (BEA), as well as enniatin (ENN), are cyclic depsipeptides which are characterized by the presence of free electron pairs giving these molecules the ability to act as nucleophiles (Hamill et al., 1969) and a high instability which lead to the tendency to form complexes with many molecules, especially metallic cations thus acting as ionophores (Kouri et al., 2003). IPEC-J2 were used to examine the effect of ENNs (A, A1, B, B1) and BEA, alone and in combination, on the intestinal barrier integrity and function.

Exposure to concentration of 2.5 μM ENN A for 72 h did not affect the TEER values, whereas concentration of 5 μM, had no effect at 24 and 48 h of exposure, but significantly reduced TEER (-70%) after 72 h. ENN A1 at 5 μM had no effect on TEER, while concentrations of 10 μM caused a reduction of the TEER values after 24, 48 and 72 h of -29, -64 and -74% respectively. ENN B caused a higher TEER alteration: at concentrations of 1.5, 2.5, and 5 μM ENN B had no effect on TEER values after 24 h of exposure, but concentrations of 2.5 and 5 μM ENN B after 48 and 72 h of exposure caused significant decreases in TEER values (-55% and -68%, respectively). ENN B1 had no effect on TEER values at a dosage of 2.5 μM over 72 h but induced a significant

decrease at 5 µM after 48 h and 72 h (- 44 and -58%, respectively). Regarding co-exposure, a mixture of all the enniatins, (with concentrations of 1.5-3 µM for each toxin), caused significant TEER value decreases after 24 h (1.5 µM: −30%; 3 µM: −33%), 48 h (1.5 µM: −64%; 3 µM: 72%) and 72 h (1.5 µM: −75%; 3 µM: −82%). These data showed that ENN alone had no effect on TEER values, but in a mixture significantly affected TEER values, thus the co-exposure appear to exert an additive effect. Co-exposure with all the ENNs and DON (with concentration of 1.5 or 3 µM for each toxins), significantly reduced TEER values starting from 24 h of exposure (1.5 µM: −33%; 3 µM: −41%). At 48 h TEER reduction was −69% for 1.5 µM concentrations and −75% for 3 µM concentrations, while at 72 h was −80% (1.5 µM) and −83% (3 µM). Summarizing, ENNs exerted a strong effect on TEER values, that was even stronger in case of co-exposure, and this effect was not due to a cytotoxic effect, since neutral red test demonstrated that cell viability was not affected by ENN or DON exposition, alone or in combination, at all the used concentrations.

It can be hypothesized that the effect of ENNs on TEER values could be due to the ionophoric properties of these compounds that allow the passage of ions (i.e., K and Ca^{2+}) across the cytoplasmic membrane that lead to a decreased tight junction resistance via protein kinase C as it has been demonstrated in T84 cells by Tai *et al*. (1996) (Tai et al., 1996).

Also, the effects of BEA (1.5–10 µM) on the intestinal barrier were examined using a IPEC-J2 cell model. While BEA at all the concentrations tested did not affect cell viability, the exposure to 5 and 10 µM BEA significantly reduced TEER values after 24 (-59%), 48 (-74%) and 72 h (-80%). Co-exposure with 2.5 µM BEA and 1.5 or 3 µM DON did not cause a significant TEER reduction. Because of their similar ionophoric properties, it could be hypothesized that BEA shares the same mechanism of action to those of ENNs. Another mechanism of action could involve BEA-mediated induction of the phosphorylated MAPK ERK44/42 (Lu et al., 2016) as demonstrated for DON indicating that intestinal barrier function is altered via impairment of the expression of claudin-3 and 4 through the phosphorylation of p44/42 (Springler et al., 2016).

In vitro intestinal barrier studies are very important, especially with regard to emerging mycotoxins, because of the lack of knowledge of these mycotoxins, for example on their effect on the intestinal barrier function. Thus, the ability to study a crucial toxicological target of these compounds, alone and in combination, using

a sensitive *in vitro* model that has strong morphological and functional similarities with the intestinal epithelial cells *in vivo* (Nossol et al., 2015) could be very useful to fill the knowledge gap on how these mycotoxins act.

3. Primary Porcine Brain Capillary Endothelial Cells

Primary Porcine Brain Capillary Endothelial Cells (PBCEC) is a well characterized, flexible and validated *in vitro* predictive model, formed using porcine brain capillary endothelial cells, seeded on polycarbonate inserts, becoming an effective tool for neurotoxicity and transport studies between the blood and brain (Franke H et al., 2000; Weidner et al., 2013). This PBCEC barrier has several advantages including handling and correlation *in vitro/in vivo* (Behrens et al., 2015).

PBCEC was exposed to some traditional fusariotoxins, trichothecenes, and to emerging ones, Enniatin B, Enniatin B1, and Moniliformin, and results of these studies are summarized below.

3.1 Tricothecenes

According to work conducted by Behrens and coworkers (2015) (Behrens et al., 2015), fusariotoxins T-2 toxin (T2) and H-T2, showed very high cytotoxicity at very low concentrations and both toxins were able to cross the PBCEC barrier, after application from either apical or basolateral site, suggesting translocation in the brain is possible. Also, a direct effect on the blood-brain barrier (BBB) tight junctions was reported with a loss of occludin; T-2 seemed more potent and more rapidly transported in comparison with HT-2. On the other hand, DON was slowly transferred through the barrier, but it drastically impaired barrier integrity and decreased TEER values (Behrens et al., 2015). DON cytotoxicity was observed at higher concentrations (10 µM) when compared to the cytotoxic nanomolar levels of T-2 and H-T2, and PBCEC seems permeable to DON although to a limited extent in relation to its polarity (Behrens et al., 2015). Because T2, HT2 and DON were not found enriched in either of the two compartments during active transfer evaluations, it was hypothesized that their transport did not involve an efflux protein. Additional research will be required to fully elucidate the mechanisms of transport for these tricothecene mycotoxins.

3.2 Enniatins and Moniliformin

Moniliformin (MON) exposure for 48 h (10 μM) did not exert any detrimental effects on cell viability, integrity of cytoplasmic membranes or permeability of PBCEC monolayer. Due to its small molecular size it is likely to cross cellular membranes, but its high polarity represents an important factor that can limit this passage, thus MON showed a transfer rate across the BBB similar to that of DON, which was 3-4 times more rapid than ^{14}C sucrose which was used as a negative control (Behrens et al., 2015). This rate indicates that MON is able to cross BBB but in a limited extent. Moreover, an active transfer study was performed, and MON was not found to be more concentrated in one of the two compartments, suggesting efflux proteins are not likely involved in MON transfer.

The treatment of PBCEC with ENN B and ENN B1 caused a decrease in cell viability (ENN B>5 μM, whereas ENN B1 showed a slightly higher cytotoxic effect starting from concentration of 2.5 μM). Passive transport (from apical to basolateral side) studies were also performed, exposing PBCEC with 1 μM ENNB and ENN B1 (concentrations that did not show any cytotoxic effects on PBCEC and did not cause changes in TEER values). Permeability coefficient for ENN B was of 14.9×10^{-6} cm/s, similar to that of ENN B1, thus both showed a transport kinetic characterized by a very rapid and high transfer rate, similar to those of molecules that are known cross the BBB (Patabendige et al., 2013).

4.Conclusions

The high contamination of fusariotoxins in feed and food, demonstrate how is urgent a risk assessment evaluation, and *in vitro* epithelial barriers, human and animal based, represent a very promising model either as a stand alone method, or in the context of an Integrated Testing Strategy, to assess toxicological effects (Gordon et al., 2015; Rovida et al., 2015; Akbari et al., 2016).

Fusariotoxins mechanisms of action, direct or indirect target organ toxicity, toxicokinetic, with *in vitro* barrier species-specific approach, from intestine to brain, were here extensively reviewed.

The *in vitro* porcine intestinal epithelial barriers IPEC-1 and IPEC-J2 and Brain Capillary Endothelial Cells (PBCEC), represent predictive tool for absorption, transport and direct impairment of xenobiotics and many

fusariotoxins have been tested on these models, and useful information on their pathway of toxicity, and finally their risk, in the perspective of the species-specificity, were provided.

6. References

- Adhikari, M., Negi, B., Kaushik, N., Adhikari, A., Al-Khedhairy, A.A., Kaushik, N.K., Choi, E.H., 2017. T-2 mycotoxin: toxicological effects and decontamination strategies. Oncotarget. 8(20), 33933-33952. doi:10.18632/oncotarget.15422
- Akbari, P., Braber, S., Varasteh, S., Alizadeh, A., Garssen, J., Fink-Gremmels, J., 2017. The intestinal barrier as an emerging target in the toxicological assessment of mycotoxins. Arch Toxicol. 91(3), 1007-1029. doi: 10.1007/s00204-016-1794-8
- Bakker, M.G., Brown, D.W., Kelly, A.C., Kim, H.-S., Kurtzman, C.P., Mccormick, S.P. O'Donnell, K.L., Proctor, R.H., Vaughan, M.M., Ward, T.J., 2018. Fusarium mycotoxins: a trans-disciplinary overview. Can. J. Plant. Pathol. 40, 161-171. doi: 10.1080/07060661.2018.1433720
- Behrens, M., Hüwel, S., Galla, H.J., Humpf, H.U., 2015. Blood-Brain Barrier Effects of the Fusarium Mycotoxins Deoxynivalenol, 3 Acetyldeoxynivalenol, and Moniliformin and Their Transfer to the Brain. PLoS One 23, 10(11):e0143640. doi: 10.1371/journal.pone.0143640
- Berschneider, H., 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Abstract of the Annual Meeting of the American Gastroenterological Association.
- Bouhet, S., Hourcade, E., Loiseau, N., Fikry, A., Martinez, S., Roselli, M., Galtier, P., Mengheri, E., Oswald, I.P., 2004. The mycotoxin fumonisin B1 alters the proliferation and the barrier function of porcine intestinal epithelial cells. Toxicol. Sci. 77, 165-171.
- Cario, E., Gerken, G., Podolsky, D.K., 2004. Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. Gastroenterology 127, 224-238.
- Cope, R.B., 2018. Trichothecenes, in: Gupta, R.C. (Ed.), Veterinary toxicology, basic and clinical principles. Academic Press, London, UK, pp. 1043-1053.

- D'Mello, J.P.F., Placinta, C.M., Macdonald, A.M.C., 1999. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Anim. Feed Sci. Tech. 80, 183-205.
- De Walle, J.V., Sergent, T., Piront, N., Toussaint, O., Schneider, Y.J., Larondelle, Y., 2010. Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. Toxicol. Appl. Pharmacol. 245, 291-298.
- Desjardins, A.E., 2006. Fusarium mycotoxins. Chemistry, genetics and biology. The American Phytopathological Society Press, USA.
- Devreese, M., De Backer, P., Croubels, S., 2013. Different methods to counteract mycotoxin production and its impact on animal health. Vlaams Diergen. Tijds. 82, 181–190.
- Devreese, M., Pasmans, F., De Backer, P., Croubels, S., 2012. An in vitro model using the IPEC-J2 cell line for efficacy and drug interaction testing of mycotoxin detoxifying agents. Toxicol. In Vitro 27(1), 157-163. doi: 10.1016/j.tiv.2012.09.020
- Diesing, A.-K., Nossol, C., Panther, P., Walk, N., Post, A., Kluess, J., Kreutzmann, P., Dänicke, S., Rothkötter, H.-J., Kahlert, S., 2011. Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicol. Lett. 200, 8-18.
- EFSA (European Food Safety Authority), 2005. Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to Fumonisins as undesirable substances in animal feed. EFSA J. 235, 1-32.
- Ehrlich, K.C., Daigle, K.W., 1987. Protein synthesis inhibition by 8-oxo-12,13 epoxytrichothecenes. Biochim. Biophys. Acta 923, 206-213.
- Ferrigo, D., Raiola, A., Causin, R., 2016. Fusarium Toxins in Cereals: Occurrence, Legislation, Factors Promoting the Appearance and Their Management. Molecules, 21, 627.
- Fraeyman, S., Croubels, S., Devreese, M., Antonissen, G., 2017. Emerging Fusarium and Alternaria Mycotoxins: Occurrence, Toxicity and Toxicokinetics. Toxins (Basel) 9(7), pii: E228. doi: 10.3390/toxins9070228

- Franke, H. Galla, H., Beuckmann, C.T., 2000. Primary cultures of brain microvessel endothelial cells: a valid and flexible model to study drug transport through the blood-brain barrier in vitro. Brain Res. Brain Res. Protoc. 5(3), 248-256.
- Geens, M.M., Niewold, T.A., 2011. Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization. Cytotechnology 63(4), 415-423. doi:10.1007/s10616-011-9362-9
- Goossens, J., Pasmans, F., Verbrugghe, E., Vandenbroucke, V., De Baere, S., Meyer, E., Haesebrouck, F., De Backer, P., Croubels, S., 2012. Porcine intestinal epithelial barrier disruption by the Fusarium mycotoxins deoxynivalenol and T-2 toxin promotes transepithelial passage of doxycycline and paromomycin. BMC Vet Res. 8, 245. doi: 10.1186/1746-6148-8-245
- Gordon, S., Daneshian, M., Bouwstra, J., Caloni, F., Constant, S., Davies, D.E., Dandekar, G., Guzman, C.A., Fabian, E., Haltner, E., Hartung, T., Hasiwa, N., Hayden, P., Kandarova, H., Khare, S., Krug, H.F., Kneuer, C., Leist, M., Lian, G., Marx, U., Metzger, M., Ott, K., Prieto, P., Roberts, M.S., Roggen, E.L., Tralau, T., van den Braak, C., Walles, H., Lehr, C.M., 2015. Non-animal models of epithelial barriers (skin, intestine and lung) in research, industrial applications and regulatory toxicology. ALTEX 32(4), 327-378. doi:10.14573/altex.1510051
- Gu, M.J., Song, S.K., Lee, I.K., Ko, S., Han, S.E., Bae, S., Ji, S.Y., Park, B., Song, K., Lee, H., Han, S.H., Yun, C., 2016. Barrier protection via Toll-like receptor 2 signaling in porcine intestinal epithelial cells damaged by deoxynivalnol. Vet. Res. 47, 25.
- Gu, M.J., Song, S.K., Park, S.M., Lee, I.K., Yun, C.-H., 2014. Bacillus subtilis Protects Porcine Intestinal Barrier from Deoxynivalenol via Improved Zonula Occludens-1 Expression. Asian-Australas. J. Anim. Sci. 27(4), 580-586. doi:10.5713/ajas.2013.13744
- Hamill, R.L., Higgens, C.E., Boaz, H.E., Gorman, M., 1969. The structure of beauvericin, a new depsipeptide antibiotic toxic to Artemiasalina. Tetrahedron Lett. 49, 4255-4258.

- Harvey, R.B., Kubena, L.F., Corrier, D.E., Huff, W.E., Rottinghaus, G.E., 1990. Cutaneous ulceration and necrosis in pigs fed aflatoxin- and T-2 toxin- contaminated diets. J. Vet. Diagn. Invest. 2, 227-229.
- Hossam E.-D.M.O., 2013. Mycotoxins-Induced Oxidative Stress and Disease, in: Makun H.A. (Ed.), Mycotoxin and Food Safety in Developing Countries. InTech, Croatia, pp. 63-92.
- Kandil, H.H., Argenzio, R.A., Chen, W., Berschneider, H.M., Stiles, A.D., Westwick, J.K., Rippe, R.A., Brenner, D.A., Rhoads, J.M., 1995. L-Glutamine and L-asparagine stimulate ODC activity and proliferation in a porcine jejunal enterocyte line. Am. J. Physiol. 269, G591-599.
- Kouri,K., Lemmens, M., Lemmens-Gruber, R., 2003. Beauvericin-induced channels in ventricular myocytes and liposomes. Biochim. Biophys. Acta 1609, 203-210.
- Krug, I., Behrens, M., Esselen, M., Humpf, H.U., 2018. Transport of enniatin B and enniatin B1 across the blood-brain barrier and hints for neurotoxic effects in cerebral cells. PLoS One 13(5), e0197406. doi: 10.1371/journal.pone.0197406
- Lebrun, B., Tardivel, C., Félix, B., Abysique, A., Troadec, J.D., Gaigé, S., Dallaporta, M., 2015. Dysregulation of energy balance by trichothecene mycotoxins: mechanisms and prospects. Neurotoxicology 49, 15-27.
- Loiseau, N., Debrauwer, L., Sambou, T., Bouhet, S., Miller, J.D., Martin, P.G., Viadere, J.L., Pinton, P., Puel, O., Pineau, T., Tulliez, J., Galtier, P., Oswald, I.P., 2007. Fumonisin B1 exposure and its selective effect on porcine jejunal segment: sphingolipids, glycolipids and trans-epithelial passage disturbance. Biochem. Pharmacol. 74, 144-152.
- Lu, C.L., Lin, H.I., Chen, B.F., Jow, G.M., 2016. Beauvericin-induced cell apoptosis through the mitogen-activated protein kinase pathway in human nonsmall cell lung cancer A549 cells. J. Toxicol. Sci. 41, 429-437.
- Luongo, D., Severino, L., Bergamo, P., De Luna, R., Lucisano, A., Rossi, M., 2006. Interactive effects of fumonisin B1 and a-zearalenol on proliferation and cytokine expression in Jurkat T cells. Toxicol. In Vitro 20, 1403-1410.

- Malekinejad, H., Maas-Bakker, R., Fink-Gremmels, J., 2006. Species differences in the hepatic biotransformation of zearalenone. Vet. J. 172, 96-102.
- Maresca, M., Fantini, J., 2010. Some food-associated mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon 56, 282-294.
- Mariani, V., Palermo, S., Fiorentini, S., Lanubile, A., Giuffra, E., 2009. Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. Vet. Immunol. Immunopathol. 131, 278-284.
- Marin, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. Food Chem. Toxicol. 60, 218-237. doi: 10.1016/j.fct.2013.07.047
- Merrill, A.H. Jr, Sullards, M.C., Wang, E., Voss, K.A., Riley, R.T., 2001. Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. Environ. Health Perspect. 109(2), 283-289.
- Nossol, C., Barta-Böszörményi, A., Kahlert, S., Zuschratter, W., Faber-Zuschratter, H., Reinhardt, N., Ponsuksili, S., Wimmers, K., Diesing, A.K., Rothkötter, H.J., 2015. Comparing TwoIntestinal Porcine Epithelial Cell Lines (IPECs): Morphological Differentiation, Function and Metabolism. PLoS One 10(7), e0132323. doi: 10.1371/journal.pone.0132323
- Nossol, C., Diesing, A.K., Walk, N., Faber-Zuschratter, H., Hertig, R., Post, A., Kluess, J., Rothkötter, H.J., Kahlert, S., 2011. Air-liquid interface cultures enhance the oxygen supply and trigger the structural and functional differentiation of intestinal porcine epithelial cells (IPEC). Histochem. Cell. Biol. 36, 103-115.
- Patabendige, A., Skinner, R.A., Abbott, N.J., 2013. Establishment of a simplified in vitro porcine blood–brain barrier model with high transendothelial electrical resistance. Brain Res. 1521, 1-15. doi: 10.1016/j.brainres.2012.06.057
- Pestka, J.J., 2010. Deoxynivalenol-induced proinflammatory gene expression: mechanisms and pathological sequelae. Toxins (Basel) 2, 1300-1317. doi:10.3390/toxins2061300

- Pestka, J.J., Zhou, H.R., Moon, Y., Chung, Y.J., 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol. Lett. 153, 61-73.
- Pinton, P., Braicu, C., Nougayrede, J.P., Laffitte, J., Taranu, I., Oswald, I.P., 2010. Deoxynivalenol impairs porcine intestinal barrier function and decreases the protein expression of claudin-4 through a mitogen-activated protein kinase-dependent mechanism. J. Nutr. 140(11), 1956-1962. doi: 10.3945/jn.110.123919
- Pinton, P., Nougayrède, J.P., Del Rio, J.C., Moreno, C., Marin, D.E., Ferrier, L., Bracarense, A.P., Kolf-Clauw, M., Oswald, I.P., 2009. The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. Toxicol. Appl. Pharmacol. 237(1), 41-8. doi: 10.1016/j.taap.2009.03.003
- Rai, R.B., Rahman, S., Dixit, H., Rai, S., Singh, B., Kumar, H., Damodaran, T., Dhama, K.,2011. Analysis of feed ingredients for Afla and T-2 mycotoxins by ELISA in rural areas of Uttar Pradesh. Ind. J. Vet. Pathol. 35, 238-240.
- Rhoads, J.M., Chen, W., Chu, P., Berschneider, H.M., Argenzio, R.A., Paradiso, A.M., 1994. L-glutamine and L-asparagine stimulate Na+ -H+ exchange in porcine jejunal enterocytes. Am. J. Physiol. 266, G828-838.
- Rovida, C., Alépée, N., Api, A.M., Basketter, D.A., Bois, F.Y., Caloni, F., Corsini, E., Daneshian, M., Eskes, C., Ezendam, J., Fuchs, H., Hayden, P., Hegele-Hartung, C., Hoffmann, S., Hubesch, B., Jacobs, M.N., Jaworska, J., Kleensang, A., Kleinstreuer, N., Lalko, J., Landsiedel, R., Lebreux, F., Luechtefeld, T., Locatelli, M., Mehling, A., Natsch, A., Pitchford, J.W., Prater, D., Prieto, P., Schepky, A., Schüürmann, G., Smirnova, L., Toole, C., van Vliet, E., Weisensee, D., Hartung, T., 2015. Integrated Testing Strategies (ITS) for safety assessment. ALTEX 32(1), 25-40. doi: 10.14573/altex.1411011
- Schierack, P., Nordhoff, M., Pollmann, M., Weyrauch, K.D., Amasheh, S., Lodemann, U., Jores, J., Tachu, B., Kleta, S., Blikslager, A., Tedin, K., Wieler, L.H., 2006. Characterization of a porcine

intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem. Cell. Biol. 125, 293-305.

- Smith, E.R., Jones, P.L., Boss, J.M., Merrill, A.H.Jr., 1997. Changing J774A.1 cells to new medium perturbs multiple signaling pathways, including the modulation of protein kinase C by endogenous sphingoid bases. J. Biol. Chem. 272, 5640-5646.
- Song, X., Zhu, S., Shi, P., Liu, Y., Shy, Y., Levin, S.D., Qian, Y., 2011. IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. Nat. Immunol. 12, 1151-1158.
- Springler, A., Hessenberger, S., Schatzmayr, G., Mayer, E., 2016. Early Activation of MAPK p44/42 Is Partially Involved in DON-Induced Disruption of the Intestinal Barrier Function and Tight Junction Network. Toxins (Basel) 8(9). doi: 10.3390/toxins8090264
- Streit, E., Schwab, C., Sulyok, M., Naehrer, K., Krska, R., Schatzmayr, G., 2013. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. Toxins (Basel) 5(3), 504-523. doi: 10.3390/toxins5030504
- Tabuc, C., Marin, D., Guerre, P., Sesan, T., Bailly, J.D., 2009. Molds and mycotoxin content of cereals in southeastern Romania. J. Food Prot. 72, 662-665.
- Tai, Y.H., Flick, J., Levine, S.A., Madara, J.L., Sharp, G.W., Donowitz, M., 1996. Regulation of tight junction resistance in T84 monolayers by elevation in intracellular Ca2+: A protein kinase C effect. J. Membr. Biol. 149, 71-79.
- Taranu, I., Braicu, C., Marin, D.E., Pistol, G.C., Motiu, M., Balacescu, L., Beridan Neagoe, I., Burlacu, R., 2015. Exposure to zearalenone mycotoxin alters in vitro porcine intestinal epithelial cells by differential gene expression. Toxicol Lett. 232(1), 310-325. doi: 10.1016/j.toxlet.2014.10.022
- Veldhuizen, E.J.A., Rijnders, M., Claassen, E.A., van Dijk, A., Haagsman, H.P., 2008. Porcine beta-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria. Mol. Immunol. 45, 386-394.

- Wan, L.Y.M., Turner, P.C., El-Nezami, H., 2013. Individual and combined cytotoxic effects of Fusarium toxins (deoxynivalenol, nivalenol, zearalenone and fumonisins B1) on swine jejunal epithelial cells. Food Chem. Toxicol. 57, 276-283. doi:10.1016/j.fct.2013.03.034
- Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T., Merrill, A.H.Jr., 1991. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. J. Biol. Chem. 266, 14486-14490.
- Weidner, M., Hüwel, S., Ebert, F., Schwerdtle, T., Galla, H.-J., Humpf, H.-U., 2013. Influence of T-2 and HT-2 Toxin on the Blood-Brain Barrier In Vitro: New Experimental Hints for Neurotoxic Effects. PLoS One 8(3), e60484. doi:10.1371/journal.pone.0060484
- Yazar, S., Omurtag, G., 2008. Fumonisins, trichothecenes and zearalenone in cereals. Int. J. Mol. Sci. 9, 2062-2090.
- Zakrzewski, S.S., Richter, J.F., Krug, S.M., Jebautzke, B., Lee, I.F., Rieger, J., Sachtleben, M., Bondzio, A., Schulzke, J.D., Fromm, M., Gunzel, D., 2013. Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. PLoS One 8(11), e79643. doi:10.1371/journal.pone.0079643
- Zhang, L., Feng, T., Spicer, L.J., 2018. The role of tight junction proteins in ovarian follicular development and ovarian cancer. Reproduction 155, R183-R198. doi: 10.1530/REP-17-0503