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Transport of mycotoxins across human gastric NCI–N87 and intestinal Caco-2 cell models

M. Madalena.C. Sobral[a], Miguel A. Faria[a,*], Sara C. Cunha[a], Bojana Miladinovic[b], Isabel MPLVO. Ferreira[a]

[a] LAQV/REQUIMTE, Departamento de Ciências Químicas, Laboratório de Bromatologia e Hidrologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal
[b] Department of Pharmacy, Faculty of Medicine, University of Niš, Niš, Serbia

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ABSTRACT

Aflatoxin B1 (AFB1), deoxynivalenol (DON), fumonisin B1 (FB1), and ochratoxin A (OTA) are prevalent mycotoxins co-occurring in food, and their oral intake is conceivable to occur in the gastrointestinal epithelium. The intestinal absorption of some mycotoxins has been studied but only considering their isolated intake, while their gastric absorption in humans has not been explored. This study evaluated the bidirectional *in vitro* transport of four mycotoxins, isolated and in mixture, across gastric NCI–N87 and intestinal Caco-2 monolayers.

AFB1 and DON were bidirectionally transported, more rapidly for AFB1; whereas OTA and FB1 were only transported in the absorptive direction, the first in both monolayers, and the second only in the gastric epithelium. The mixture of four mycotoxins exhibited some differences in cell uptake/excretion ratios. AFB1 presented the highest fraction absorbed (> 96%) isolated and in mixture, followed by DON (72.8 and 82.9%); and OTA (11 and 66%) when transported isolated and in mixture, respectively. Different absorptive patterns on both epithelia were found when mycotoxins are transported isolated or in mixture. Further investigation on combined ingestion of toxins and their mixed transport should be considered for the proper evaluation of human absorption and toxicity of those mycotoxins considering their frequent co-occurrence and consequent co-exposure.

1. Introduction

Aflatoxin B1 (AFB1), deoxynivalenol (DON), fumonisin B1 (FB1) and ochratoxin A (OTA) are toxic metabolites produced by filamentous fungi recognized as food contaminants worldwide (Pereira et al., 2014). AFB1 is a well-known human carcinogen to humans, belonging to Group 1 in the International Agency for Research on Cancer (IARC) classification, while FB1 and OTA belong to Group 2B and DON to Group 3 due to the lack of clear evidence concerning their carcinogenicity to humans (Ostry et al., 2017). Thus, these mycotoxins were reviewed by Pinotti et al. (2016) as the most prevalent and considered of great concern. According to the European Food Safety Authority (EFSA), the daily exposure of European adults to the above mentioned mycotoxins ranges from 0.03 to 1.3 ng/kg bodyweight (b.w) for AFB1, 0.77–2.4 μg/kg b.w for DON, 2.14–8.57 ng/kg b.w for OTA, and lower than 0.25 μg/kg b.w for FB1 (Chain et al., 2017; EFSA, 2006; 2007; JECFA, 2017). Moreover, these metabolites are likely to naturally co-occur in food and feedstuff (Martins et al., 2018). As shown by Rodrigues and Naehrer (2012), 48% of the analyzed samples were contaminated with 2 or more mycotoxins, among them AFB1, DON, FB1, and OTA. Therefore, the human co-exposure to multiple mycotoxins is a real problem, which increases the concern about their combined impact on health.

The gastrointestinal epithelium is the first target of mycotoxins upon ingestion, mostly under higher doses (Yang et al., 2017). High mycotoxin bioaccessibilities after *in vitro* digestion have been reported (González-Arias et al., 2013), suggesting a long time exposure (4 h) at gastric and intestinal levels, promoting the absorption of these toxins. Moreover, the long exposure period, even at low doses, may exert toxic effects along the gastrointestinal tract (Maresca and Fantini, 2010).

Mycotoxins have been studied regarding their absorption at intestinal level, however ignoring stomach as the first barrier met by toxins to exert their toxicity and to be absorbed. These mycotoxins has been reported to rapidly reach blood circulation suggesting that their absorption takes place in the upper part of gastrointestinal tract (Grenier and Applegate, 2013). However, knowledge concerning human gastric absorption of mycotoxins is missing, being only found information on the absorption of patulin and OTA from rat stomach (Galtier, 1977; Rychlik et al., 2004) and ergot alkaloids in ruminant gastric tissues (Hill et al., 2001).

* Corresponding author. Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal.
E-mail address: mfaria@ff.up.pt (M.A. Faria).

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According to EFSA reports AFB1, DON, and OTA are rapidly absorbed, distributed and excreted after administration using animal absorption models (Chain et al., 2017; EFSA, 2006; 2007), while FB1 is poorly absorbed, following a rapid distribution and excretion (EC, 2000). Moreover, the bioavailability of some of these compounds differs among animal species (Grenier and Applegate, 2013), and knowledge concerning mycotoxins humans' absorption is very limited. Concerning *in vivo* absorption evaluation in humans data is not available to DON, FB1, however the International Agency for Research on Cancer (IARC) monographs include relevant information concerning AFB1 *in vivo* absorption in humans (IARC, 1993; 2002, 2012).

Human cell models, such as Caco-2 monolayers have been widely used to predict permeability and intestinal absorption of molecules (Hubatsch et al., 2007), including mycotoxins like DON and their acetylated metabolites, FB1, OTA, aflatoxin M1, and zearalenone (Berger et al., 2003; Caloni et al., 2012; De Angelis et al., 2005; Kadota et al., 2013; Sergent et al., 2006; Videmann et al. 2007, 2008). Whereas, concerning the gastric transport, differentiated NCI–N87 cells have been proposed by Lemieux et al. (2011) as an epithelial barrier model to perform permeability assays and predict the gastric absorption of molecules. Indeed, they are able to form cohesive monolayers at low pH by expressing E-cadherin, Zonula occludens-1 (ZO-1), producing high levels of gastric zymogens, and efficiently secreting gastric lipase and pepsinogen (Basque et al., 2001), but no studies were found concerning the use of this model to predict gastric absorption of mycotoxins.

The goal of the present work was to investigate the isolated and mixed transport of 4 mycotoxins – AFB1, DON, OTA, and FB1 - representing different chemical groups of mycotoxins, across the human-derived epithelial gastric NCI–N87 and intestinal Caco-2 cells, the first and second barrier met by mycotoxins after ingestion, where their absorption is conceivable to occur. To the best of our knowledge this study investigates for the first time the gastric transport of four prevalent mycotoxins on NCI–N87 cells as well as the mixed transport in both gastric and intestinal cells.

2. Material and methods

2.1. Reagents and materials

AFB1 (1 mg, > 98% purity), dimethyl sulfoxide (DMSO), 0.4% tryptan blue stain solution, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Hanks Balanced Salt Solution (HBSS), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Bovine serum albumin (BSA), and anhydrous magnesium sulphate ($MgSO_4$) were all purchased from Sigma-Aldrich corp. (St Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, High glucose Dulbecco's modified Eagle's medium (DMEM), minimum essential medium non-essential amino acids (MEM NEAA) 100x, GlutaMAX™ 100x, foetal bovine serum (FBS), 0.25% Trypsin-EDTA solution, and penicillin/streptomycin 100x solution (10.000 Units ml^{-1}/ 10.000 µg ml^{-1}) were purchased from Gibco/Life technology (Paisley, United Kingdom). DON (1 mg, 95% purity), FB1 (1 mg, 98% purity), OTA (1 mg, 97% purity), and OTA-d5 (0.5 mg, purity 95%) were purchased from Toronto Research Chemicals (Ontario, Canada). Syringe filters (PES, 0.2 µm × 33 mm) were purchased from TPP (Zollstarsse, Switzerland). Transwell permeable polycarbonate supports and inserts (24 mm diameter, 0.4 µm pore size) for the transport assay were obtained at Corning (Corning, NY, USA).

Acetonitrile and methanol, acetic and formic acids were of high-performance liquid chromatography (HPLC) grade, and ammonium acetate (P.A.) were from Merck (Darmstadt, Germany). Sodium chloride (NaCl) was purchased from VWR and the ultrapure water purified by Milli-Q gradient system (18.2 mΩcm^{-1}) from Millipore (Milford, MA, USA).

2.2. Cell culture

Human-derived gastric cells were purchased from ATCC/LGC Standards (Barcelona, Spain), while the intestinal cells were provided by the "Molecular Physical-Chemistry" group of the University of Coimbra. NCI–N87 cells were grown in 75-cm^2 culture flasks in complete medium (CM) constituted by RPMI with 10% heat inactivate FBS, and 1% penicillin/streptomycin and incubated at 37 °C with 5% CO_2. When the cells were 80% confluent, these were trypsinized and seeded in 96-well plates (TPP, Trasadingen, Switzerland) to perform the cytotoxic assays or seeded on transwell inserts for differentiation and transport studies. In the case of Caco-2 cells, these were grown in 75-cm^2 culture flasks in CM constituted by DMEM with 10% heat inactivate FBS, 1% NEAA, 1% glutaMAX and 1% penicillin/streptomycin and incubated at 37 °C with 5% CO_2. Cytotoxicity and permeability assays were performed under passages 6–23 and 41–50 for NCI–N87 and Caco-2 cells, respectively.

2.3. Cytotoxic assay

The cytotoxicity of AFB1, DON, FB1 and OTA and their mixture on NCI–N87 and Caco-2 cells was evaluated after 3 h, using the MTT assay. Proliferating NCI–N87 cells (10000 cells/well) and Caco-2 cells (10000 cells/well) were seeded in 96-well plates (TPP; Trasadingen, Switzerland), allowing 24 h for cell adherence at 37 °C, 5% CO_2, and then exposed to mycotoxins at the concentrations used for transport assay: 1.69 µM (DON), 1.60 µM (AFB1), 0.69 µM (FB1), and 1.24 µM (OTA) over 3 h. Cells treated with CM alone and with maximum methanol content (0.1%) present in all samples with mycotoxins were used as controls to ensure viability of cells during the experiments.

2.4. Transport study assay

NCI–N87 and Caco-2 cells were seeded at 1×10^5 cells/cm^2 in 24 mm 6-well Transwell with pore size of 0.4 µm and growth area of 4.67 cm^2 (Corning incorporated, NY, USA). During differentiation the medium was changed every two days and the cells were used in its 28th post-confluence day to perform the transport assay. For transport experiments, the NCI–N87 cell medium was replaced by acidified HBSS (pH 3) and HBSS (pH 7.4) in the apical and basolateral compartments, respectively; while for Caco-2 cells, the apical compartment was replaced by HBSS with 25 mM HEPES and the basolateral compartment by HBSS with 25 mM HEPES and 0.5% (w/v) BSA. BSA was added to prevent plastic binding of compounds (Hubatsch et al., 2007).

Mycotoxins at the initial concentrations of 1.69 µM (DON), 1.60 µM (AFB1), 0.69 µM (FB1), and 1.24 µM (OTA) (*i.e.* in equal mass ratio of approximately 500 µg/L each) were prepared in HBSS medium and introduced in the apical or basolateral compartments isolated or in mixture. After 15, 30, 60, 120 and 180 min, 200 µL aliquots were taken from the apical or basolateral compartments and replacing the same amount (200 µL) by fresh medium. The initial mycotoxins concentrations were also determined in the treatment solution as well as the final concentrations in both apical or basolateral compartments. These high concentrations are not expected to be found neither in food nor along the gastrointestinal tract considering the low concentrations of these toxins reported in literature and the strict regulations used to control maximum contents of these mycotoxins in food (EC, 2006); It was decided to use this concentration to allow the quantification of mycotoxins at all stages of transport assay. The transepithelial electric resistance (TEER) values were determined at 37 °C using the Millicell ERS-2 Voltohmmeter (Merck Millipore, Darmstadt, Germany) at the beginning and at the end of the experiment after monolayer washing with HBSS to check the barrier integrity. The resistance, expressed as Ω cm^2, was calculated by multiplying the cell monolayer resistance (Ω) by area of the filter (cm^2). All transport assays were performed in triplicate.

The gastric and intestinal permeabilities of mycotoxins were determined using transport rates across NCI–N87 and Caco-2 cell monolayers, as described elsewhere (Hubatsch et al., 2007). Permeability coefficient (P_{app}) at both directions (Apical→Basolateral (AB) and Basolateral→Apical (BA)) was calculated according to the following equation, described for experiments under non-sink conditions (Cabrera-Pérez et al., 2016; Hubatsch et al., 2007):

$$CR(t) = \frac{M}{V\,D + VR} + \left(CR0 - \frac{M}{V\,D + VR}\right)e^{-PappA\left(\frac{1}{VD}+\frac{1}{VR}\right)t}$$

where C_R (t) is the time-dependent mycotoxin concentration in the receiver compartment, M is the amount of mycotoxin in the system, V_D and V_R are the volumes of the donor and receiver compartment, respectively, C_{R0} is the concentration of the mycotoxin in the receiver compartment at the start of the time interval, A is the area of the filter, and *t* is the time from the start of the interval. P_{app} was obtained from nonlinear regression, minimizing the sum of squared residuals $\Sigma(C_{R,i,obs} - C_{R,i,calc})^2$ where $C_{R,i,obs}$ is the observed receiver concentration at the end of the interval and $C_{R,i,calc}$ is the corresponding concentration calculated according to the previous equation (Tavelin et al., 2002).

The mass balance was calculated using the equation:

$$\text{Mass balance } (\%) = [(V_R \times C_{R(final)}) + (V_D \times C_{D(final)})]/(V_{D(0)} \times C_{D(0)}) \times 100$$

Where C_R and C_D are the concentrations on the receiver (R) and donor (D) sides of the monolayer at the beginning (0) and end (final) of the experiment, and V is used for each of the respective volumes. Uptake (U_R) and efflux (E_R) ratios were calculated as the quotient of absorptive (to plasma) ($P_{app\ A\to B}/P_{app\ B\to A}$) and secretory (to intestinal lumen) ($P_{app\ B\to A}/P_{app\ A\to B}$) permeabilities, respectively.

Apical to basolateral P_{app} data on Caco-2 cells were used to estimate the calculated human fraction absorbed, *FA (%)*, accordingly to the nonlinear regression model described by Skolnik et al. (2010) and Tavelin et al. (2003):

$$\text{FA } (\%) = \frac{100}{1 + e^{((-5.74 - PappX)/0.39))}}$$

where 100 equals the minimum + maximum – minimum of % FA values constrained to 1 and 100%; −5.74 is the log $P_{appA\to B}$ value at 50% of absorption in humans, P_{appX} is the log $P_{app\ A\to B}$ for Caco-2 cells of mycotoxins obtained in the present study, and 0.39 is the slope that derived from the model fit.

2.5. Analytical analysis

2.5.1. Extraction procedure

Each aliquot (200 μL) took during transport assay (section 2.4) was transferred into a conic microtube and 20 μL of *d5*-OTA (200 μg/L) was added as internal standard according to Sobral et al. (2019). Then, the same amount of extracting solvent (ACN with 1% formic acid (v/v)) was added along with $MgSO_4$ anhydrous salt (70 mg) and NaCl (10 mg) immediately vortexing the tube for 10 s. Tubes were centrifuged (13,000 rpm for 5 min), the organic phase transferred to a 2 mL vial, evaporated to dryness under a stream of nitrogen (SBH CONC/1 sample concentrator from Stuart® (Staffordshire, OSA, USA)), reconstituted in 60 μL of mobile phase B (methanol: water: acetic acid (97:2:1) with 5 mM ammonium acetate) and analysed by LC-MS/MS.

2.5.2. LC-MS/MS analysis

The analysis of the target mycotoxins was performed on a Waters 2695 HPLC system (Water, Milford, MA, USA) coupled to a Micromass Quattro micro API™ triple quadrupole detector (Waters, Manchester, UK), equipped with the MassLynx 4.1 software for data processing. The HPLC conditions were according to Cunha et al. (2018) with the full description available in supplementary material along with Table S1 that lists the optimized MS/MS parameters for target mycotoxins.

Linearity was evaluated by matrix-matched calibration curves with six calibration points in the range of 0–600 μg/L that is 3.2 nM to 1.92 μM for DON, 3.3 nM to 2.02 μM for AFB1, 1.4 nM to 0.83 μM for FB1, and 2.5 nM to 1.49 μM for OTA. Precision was determined by repeatability (intraday precision) and intermediate precision (interday precision) of a spiked sample, at three concentration levels, using five replicates (twice injected), on each precision day. Limit of detection (LOD) and limit of quantification (LOQ) were determined by successive analyses of chromatographic extracts of sample solutions spiked with decreasing amounts of the analytes until signal-to-noise ratios of 3:1 or 10:1 were reached, respectively.

2.6. Statistical analysis

Statistical analysis was performed using XLSTAT for Windows version 2016.02 (Addinsoft, Paris, France). Normal distribution of variables was checked by Shapiro–Wilk's test. One-way ANOVA and Tukey's or Tamhane's T2 post hoc tests, with 5% significance level, was applied for mean comparison, depending on their homogeneity of variances. Data is expressed as mean ± SD of three independent experiments. GraphPad Prism version 7.00 for windows was used to build all graphs (Graphpad Software, La Jolla California USA).

3. Results

3.1. Analytical performance

Good linear response was verified for the 4 mycotoxins ($R^2 > 0.9916$). The LODs and LOQs ranged from 1.04 to 2.53 nM and 1.4–3.3 nM. The percentages of recovery were higher than 89% and the %RSD values for interday and intraday precision were lower than 9%. Therefore, the validated method was further applied to continue the study. All validation method parameters are presented in supplementary material in Table S2.

3.2. Quality control of cell monolayers

To confirm that the transport study was performed under recommended conditions two different "quality control" measurements were considered to check the monolayer integrity: i) measurement of TEER; and ii) determination of mycotoxins cytotoxicity in proliferating cells at the concentrations studied. TEER values of NCI–N87 and Caco-2 monolayers were in agreement with values previously reported by Lemieux et al. (2011) ($> 500\ \Omega\,cm^2$) and Melo et al. (2016) ($> 1000\ \Omega\,cm^2$) for NCI–N87 and Caco-2, respectively. No significant differences ($p < 0.05$) were observed on TEER values for both cell lines, measured before (0 h) and after transport experiments (3 h), after exposure to mycotoxins, individually or in mixture (MIX) (Fig. 1A–D). A decrease in TEER values could mean an increase in permeability of tight junctions (Hurni et al., 1993) due to cells detachment. Moreover, DON (1.69 μM), AFB1 (1.60 μM), FB1 (0.69 μM), OTA (1.24 μM), isolated or in mixture, did not exert significant cytotoxic effect in proliferating NCI–N87 and Caco-2 cells after 3 h exposure, not influencing cell viability (Fig. 1E and F) measured by the MTT assay and expressed as % of control. These results indicate that the monolayer integrity of differentiated NCI–N87 and Caco-2 cells was not compromised during transport assays.

3.3. Transport studies

The bidirectional transport of isolated DON, AFB1, FB1, OTA, as well as their mixture was assessed in NCI–N87 and Caco-2 monolayers through a concentration gradient. These models mimic the bidirectional transport occurring in gastric and intestinal absorption and allow the calculation of the uptake and efflux ratios.

Figs. 2 and 3 show the results concerning transport of each

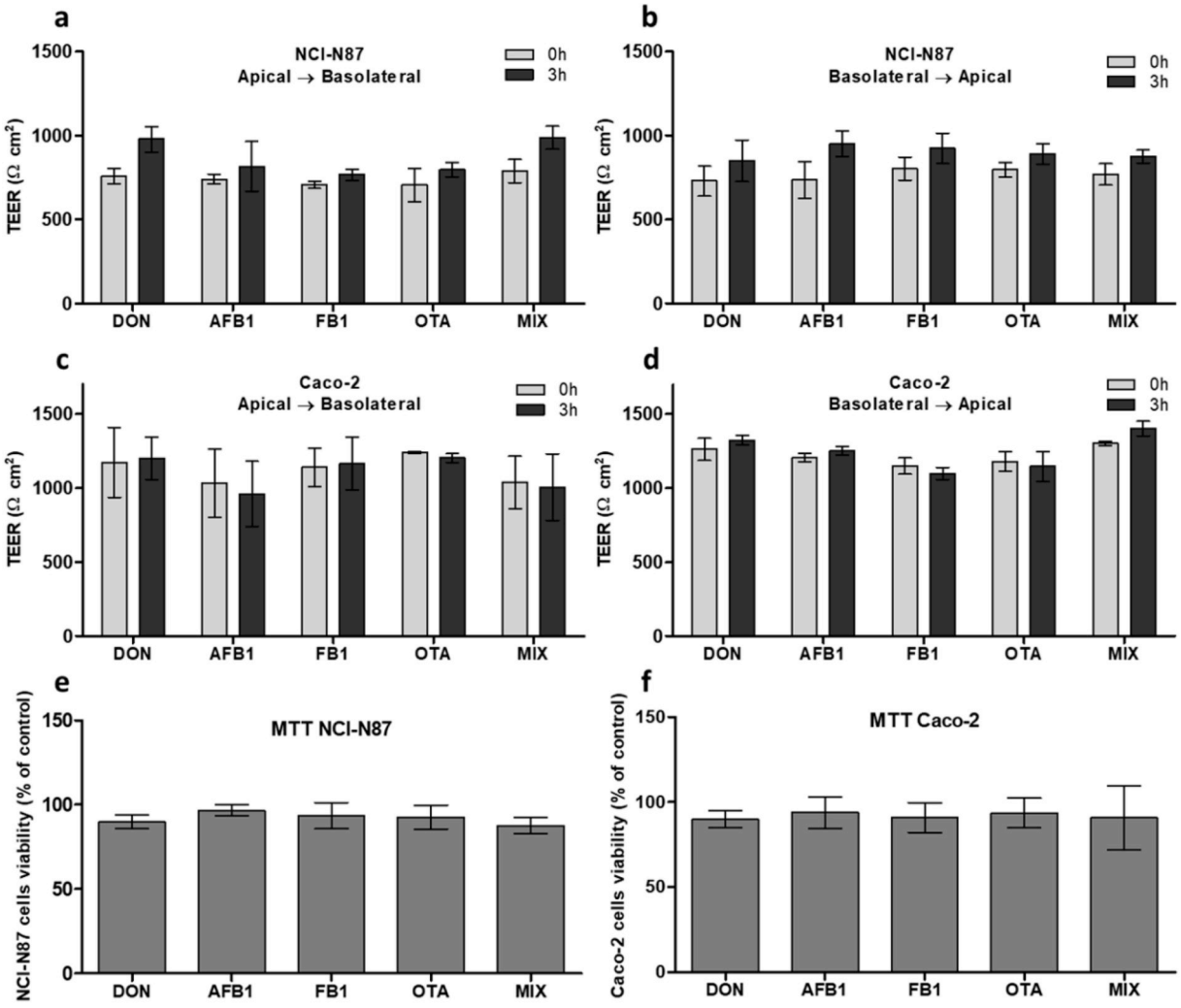

Fig. 1. Transepithelial electrical resistance (TEER) values (Ω cm^2) of DON, AFB1, FB1, OTA, and their mixture (MIX) before (0 h) and after (3 h) the transport experiment for: NCI–N87 cells in the apical→basolateral direction (a) and in the basolateral→apical direction (b); and Caco-2 cells in the apical→basolateral direction (c) and in the basolateral→apical direction (d). Fig. 1e–f shows the % of viability of proliferating NCI–N87 and Caco-2 cells, respectively, after 3 h exposure to mycotoxins isolated and in mixture. Data expressed as mean ± SD of 3 independent experiments (n = 3). AFB1 – Aflatoxin B1; DON – deoxynivalenol; FB1 – Fumonisin B1; MIX – mixture of four mycotoxins; and OTA – Ochratoxin A.

mycotoxin isolated and in mixture, expressed as mass transported over time, across NCI–N87 and Caco-2 monolayers, respectively. Different transport rates were observed concerning the type of mycotoxins, the type of transport (isolated or mixed), the cell line, and the flux direction. Regarding the gastric absorption (Fig. 2), all mycotoxins were transported, either isolated or in mixture through NCI–N87 cells in the AB direction, except the FB1 that was not transported in mixture; whereas, only DON and AFB1 were transported in the opposite direction, either isolated or in mixture. In the case of intestinal absorption, FB1 was not transported in any direction, OTA was absorbed in AB direction and not transported in the opposite direction, and DON and AFB1 were transferred in both directions through Caco-2 monolayers; moreover, for some mycotoxins different transport profiles were observed between isolated and mixed transport (Fig. 3).

Individually, the transfer of DON isolated or in mixture to the receiver compartments was linear with time in both cells and transport directions (AB and BA). In NCI–N87 cells, DON was similarly transported isolated or in mixture in both directions, achieving maximum percentages around 8% (Fig. 2). On the other hand, at intestinal level, a faster bidirectional transport of DON was observed in mixture (orange lines), mostly in the AB direction achieving maximum percentages of 14%, while in the same direction the isolated transport showed maximum percentages around 8%. This suggests that the presence of other mycotoxins may promote intestinal transport of DON (Fig. 3).

AFB1 transport rate was found non-linear being rapidly transferred to the receiver compartment and decreasing its rate with time until stabilization, either when transported isolated or in mixture. This phenomenon occurred in both cell lines and both transport directions, increasing rapidly in the first 60 min and more slowly until 180 min. AFB1 basolateral uptake isolated or in mixture differed across NCI–N87 monolayers, showing higher percentage of transport over time in the mixed transport; while, in the opposite direction, the isolated transport was faster, achieving higher transport rates. Concerning Caco-2 transport, AFB1 was equally transported either isolated or in mixture in the AB direction, however, in the opposite direction a faster and higher transport was observed in mixture, suggesting that the intestinal transport of AFB1 in the presence of other mycotoxins is beneficiated in the efflux direction.

OTA transport across NCI–N87 cells was similar if transported isolated or in mixture, and only happened in the AB direction; however, OTA transport isolated or in mixture highly differed across Caco-2 monolayers, being poorly transported isolated and more rapidly transferred when in mixture.

FB1 was only poorly transported at gastric level regarding

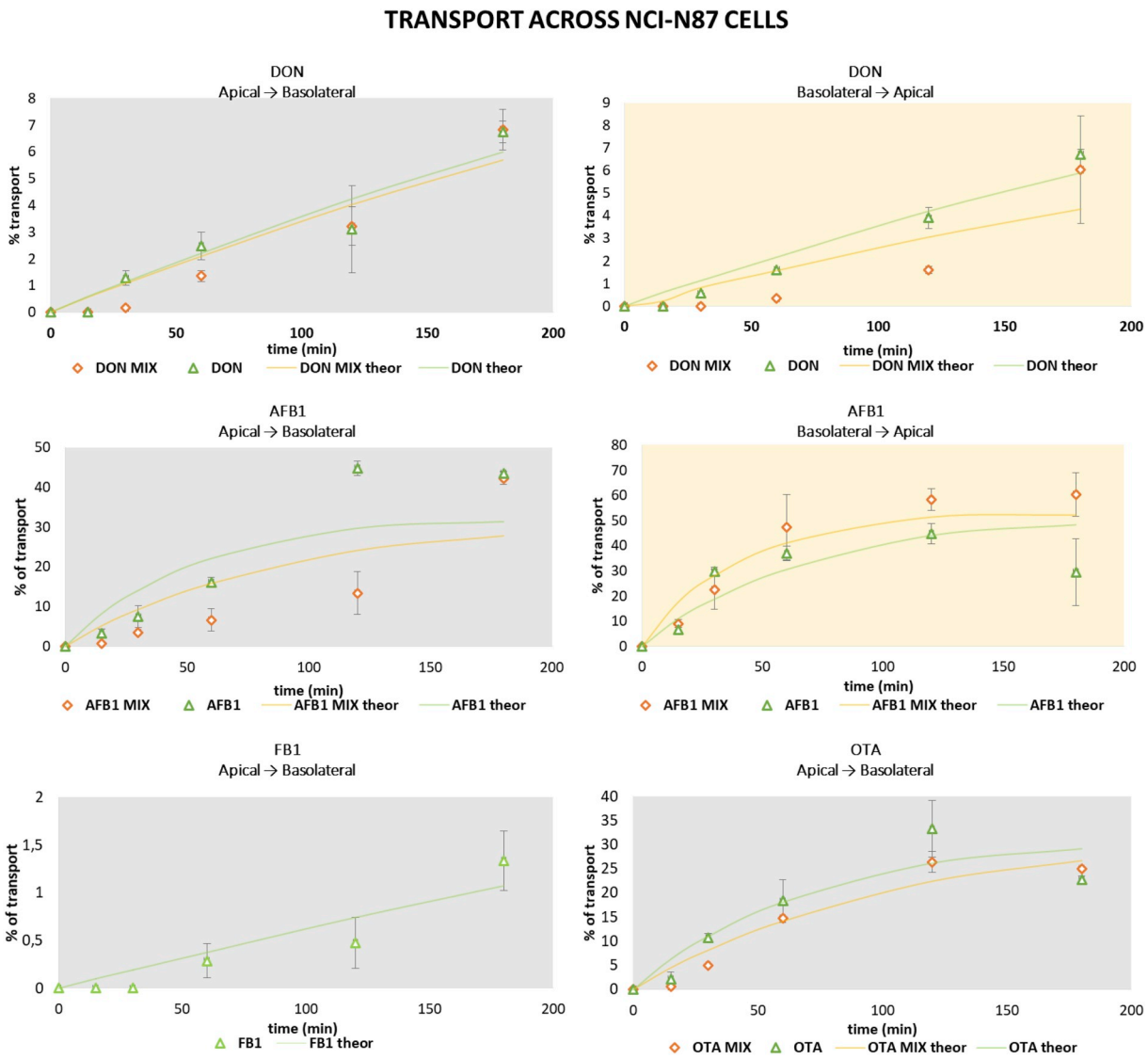

Fig. 2. The percentage of DON, AFB1, OTA, and FB1 transferred to the receiver compartment over 180 min across monolayers of NCI–N87 in the apical→basolateral and basolateral→apical directions when transported isolated (green dots and lines) or in mixture (orange dots and lines). The dots with SD represent the experimental values obtained in this study, while the line links the theoretical values obtained from the equation shown in section 2.4. Data expressed as mean ± SD of 3 independent experiments (n = 3). AFB1 – Aflatoxin B1; DON – deoxynivalenol; FB1 – Fumonisin B1; MIX – Mixture; and OTA – Ochratoxin A. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

basolateral uptake with a maximum flux of 1%, not being transported in mixture, neither in the opposite direction nor across Caco-2 monolayers in both directions.

Beyond expressing the amount of mycotoxin transported over time, we also determined the apparent permeability (P_{app}), expressed as cm/s, to accurately predict the absorption of mycotoxins via gastrointestinal tract. As AFB1 and OTA were rapidly absorbed at the beginning with the transport rate decreasing with time, it can be inferred that the sink conditions were not verified during the experiment contributing for a shallower concentration gradient between donor and receiver compartments, instead of a linear fitting between mycotoxin content in the donor and receiver compartment. Therefore, the permeability values were calculated using the equation mentioned in section 2.4 for nonlinear curve fitting developed for non-sink conditions (Hubatsch et al., 2007; Tavelin et al., 2002). Tavelin et al. (2002) reported that P_{app} obtained with non-sink conditions are in better accordance with the real permeability coefficients of the epithelial cell monolayer *in vivo*. Thus, Fig. 4 shows the P_{app} values calculated from the curves of the transport of isolated mycotoxins present in Figs. 2 and 3.

As expected by the isolated transport rates observed in Figs. 2 and 3, in general, AFB1 exhibits the highest P_{app}, followed by DON, and then with the lowest permeabilities are FB1 and OTA in those directions to which transport was observed (apart from AB flux of OTA in gastric monolayers) (Fig. 4). Comparing the different mycotoxins, the gastric P_{app} in the AB direction was significantly ($p < 0.05$) higher for AFB1 and OTA in comparison with DON and FB1, while all intestinal transport in AB direction differed among mycotoxins with AFB1 having the highest and OTA the lowest P_{app}. From those mycotoxins that were transported in the opposite direction (BA) the permeabilities obtained also significantly differed ($p < 0.05$) in both cell lines with AFB1 having higher P_{app} rather than DON which was verified in both cell lines.

The transport of the four mycotoxins in mixture showed significant differences ($p < 0.05$) in the P_{app} (Table 1). Concerning the gastric transport, the co-transport of mycotoxins significantly decreased the P_{app} of AFB1 and blocked the AB flux of FB1; while for the intestinal flux an increase of P_{app} was observed for DON and OTA when simultaneously transported (Table 1).

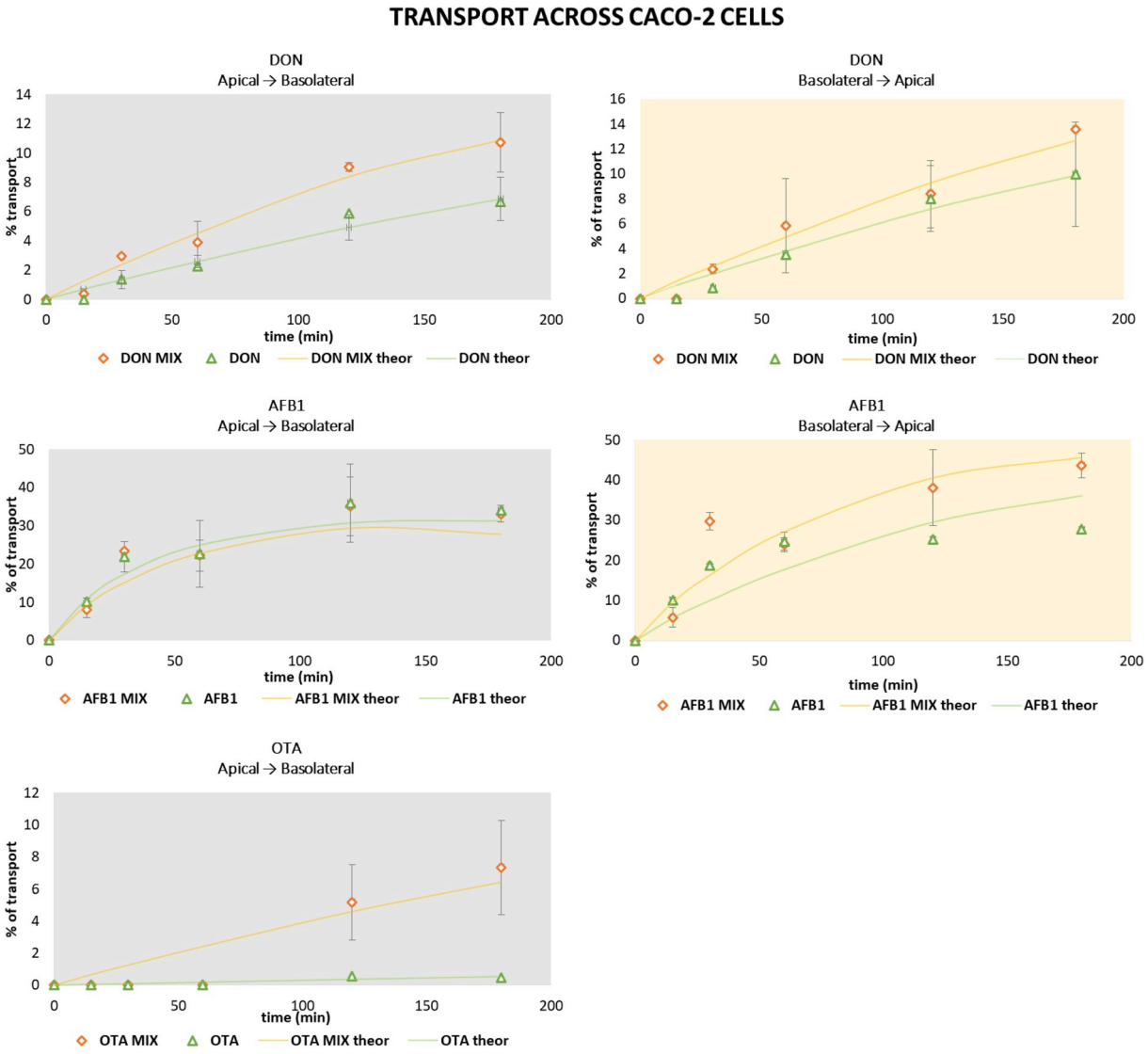

Fig. 3. The percentage of DON, AFB1, OTA, and FB1 transferred to the receiver compartment over 180 min across monolayers of Caco-2 in the apical→basolateral and basolateral→apical directions when transported isolated (green dots and lines) or in mixture (orange dots and lines). The dots with SD represent the experimental values obtained in this study, while the line links the theoretical values obtained from the equation shown in section 2.4. Data expressed as mean ± SD of 3 independent experiments (n = 3). AFB1 – Aflatoxin B1; DON – deoxynivalenol; MIX – Mixture; and OTA – Ochratoxin A. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Regarding the mass balance values in both transport directions (*i.e.* the sum of mycotoxin recovered from the donor and receiver compartments at the end of the experiment divided by the initial amount in donor compartment) ranged from 70% to 116% for NCI–N87 and Caco-2 cells, supporting acceptable approximation of the P_{app} values (Hubatsch et al., 2007).

Other parameters that are usually calculated to evaluate the transport of substances across monolayers are the ratio of uptake (U_R) and efflux (E_R). An asymmetric transport through cell monolayers (*i.e* U_R or E_R higher than 2) usually suggests the involvement of transporters mediating the passage of molecules. As only DON and AFB1 were transported in both flux directions the calculation of uptake/efflux ratios was possible only for these two mycotoxins (Table 1).

A high uptake ratio (1.74) and low efflux ratio (0.57) was observed for DON individually transported through NCI–N87 cells, meaning that this mycotoxin is preferably transported in the AB direction in the stomach, while similar values of U_R (1.08) and E_R (0.93) were observed in the intestinal model suggesting no preferable AB or BA flux.

Individually transported AFB1 showed higher uptake ratios (1.28 and 3.76) than efflux ratios (0.78 and 0.28) for both gastric and intestinal cells, respectively, suggesting that AFB1 transport is preferable in the AB direction, and in the case of intestinal absorption the $U_R > 2$ may suggest the presence of an uptake transporter in the apical membrane (FDA, 2006). In fact, intestinal transporters such as organic cation transporter (OCT) and organic anion transporters (OAT) were found to mediate the uptake of aflatoxin B1 (Tachampa et al., 2008; Volk, 2014). When exposed in mixture, the transport of AFB1 resulted in a drastic decrease of U_R (0.45 and 1.74) and increase of E_R (2.24 and 0.58), mainly in gastric transport where the inverted ratios suggest that the exposure to the mixture of mycotoxins might beneficiate the efflux of AFB1.

The use of Caco-2 cell model to predict the human fraction absorbed has often been used by correlating the AB apparent permeabilities across Caco-2 monolayers of molecules with the experimental human fraction absorbed data, resulting in a sigmoidal relationship between the human fraction absorbed and the log (P_{app}) of molecules (Skolnik et al., 2010; Tavelin et al., 2003). Thus, with this correlation, the *in vitro* permeability of a compound in Caco-2 cells can be used to predict the

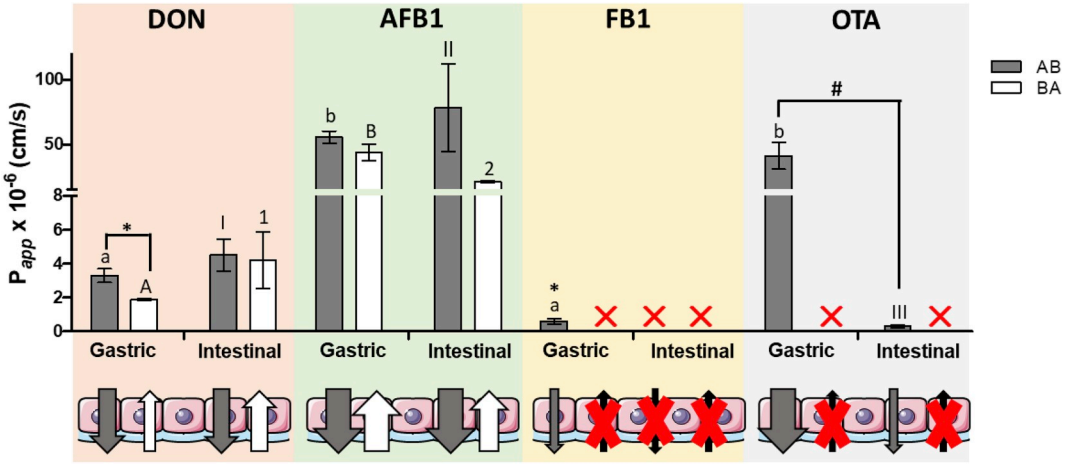

Fig. 4. Apparent permeabilities (P_{app}) of isolated DON, AFB1, FB1, and OTA transported across NCI–N87 (gastric) and Caco-2 (intestinal) cells over 3 h in both AB and BA directions. Grey and white arrows indicate AB or BA transport, respectively, and their thickness mean higher or lower permeability; the red "X" indicates no transport across the monolayer. Data expressed as mean ± SD of 3 independent experiments (n = 3). AFB1 – Aflatoxin B1; DON – deoxynivalenol; FB1 – Fumonisin B1; and OTA – Ochratoxin A. Values with different letters or numbers differ significantly ($p < 0.05$) from the given mean (lowercase letters for AB gastric transport, uppercase letters for BA gastric transport, Roman numerals for AB intestinal transport, and Arabic numbers for BA intestinal transport).* Significant difference ($p < 0.05$) between AB and BA permeabilities for the same mycotoxins in the cell line. # Significant difference ($p < 0.05$) between gastric and intestinal permeabilities for the same compound. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

absorption in humans. The fraction absorbed (FA%) values are considered more intuitive than the P_{app} values for understanding intestinal absorption of compounds offering the additional benefit of assessing permeability ranking as sparingly (0–20%), intermediately (20–80%), and completely (80–100%) absorbed. Fig. 5a–b shows the FA% of each mycotoxin isolated and in mixture as well as their position in the sigmoidal curve according to their percentage of absorption. AFB1 is the mycotoxin with the highest FA percentage (> 96%) both isolated and mixed transported; followed by DON with FA% of 72.8 and 82.9 when transported isolated and in mixture; and finally, OTA with significant difference ($p < 0.05$) between isolated (11%) and mixed (66%) transport. In this sense, AFB1 is classified as completely absorbed either isolated or in mixture, DON is intermediately absorbed isolated and completely absorbed in mixture, and OTA has the greatest difference being sparingly absorbed isolated and intermediately absorbed in mixture (Fig. 5a–b).

4. Discussion

Transport processes across absorptive epithelia may occur through several routes: passive (transcellular and/or paracellular), active transcellular (transporter-mediated) or transcytosis (Tavelin et al., 2002). This study focused on the passive transport of four prevalent mycotoxins across gastric and intestinal monolayers.

Previous research on the mycotoxins mechanism and routes of intestinal uptake propose AFB1 and DON as passively transported (DON using a paracellular route), OTA also passively transported through simple diffusion, and the FB1 entero-hepatic circulation was explained by its interaction with cholesterol or bile salts facilitating its intestinal transport (Berger et al., 2003; Mahfoud et al., 2002; Sergent et al., 2006; Tomkova et al., 2002). The passive bidirectional transport evaluated herein might be pH-dependent with transport rates differing between gastric and intestinal transport, if we consider the pH-partition hypothesis in which ionisable compounds diffuse through biological membranes primarily in their non-ionized form (Yu et al., 1996). In gastric transport the pH difference between apical (pH 3.0) and basolateral (pH 7.4) compartments seem also to influence the transport direction of FB1 and OTA being only transported in the AB direction. As these mycotoxins are weak acids (pK_a FB1/OTA 3.49/4.40), and according to Henderson-Hassel Balch equation, a pH near its pK_a suggests that 50% of the molecule is non-ionized, its capacity to pass through the cell membranes is thus higher on the acidic than on the neutral side of the monolayer (Antonissen et al., 2015; Nistor et al., 2017). Moreover, Afsah-Hejri et al. (2013) reviewed that in animals OTA is usually absorbed in the stomach due to its lipid-soluble, nonionized, and acidic properties, which is in agreement with the present results as OTAs transport was higher across NCI–N87 monolayers than Caco-2 monolayers. Similarly, as intestinal transport through Caco-2 monolayers is performed at physiological pH (7.4) in both sides, it was not expected FB1 to be transported in any direction by passive non-ionic transcellular diffusion because at duodenum pH the FB1 is mainly negatively charged (Antonissen et al., 2015). There are no reports on gastric absorption of FB1 to discuss our results, but the non-transport of FB1 across Caco-2 monolayer is in agreement with De Angelis et al. (2005). Also, EFSA reports show a poor absorption of FB1 from animal experiments, which agrees with the poor FB1 found in the present study (lower than 2%, and happening only in gastric transport) (EC, 2000). In the case of OTA, concerning the pK_a of phenolic hydroxyl group of 7.1 it would be expected some transport in the BA direction at gastric level, as well as bidirectional transport of this mycotoxin across Caco-2 monolayers, but it was only observed a weak AB transport of OTA (P_{app} of 0.29 ± 0.07 (x10^{-6}) cm/s) (Fig. 4 and Table 1). Bidirectional transport of OTA using pH 7.4 was reported by Berger et al. (2003), with low transport levels in AB direction and high transport in the opposite side. However, authors verified that the addition of BSA in the basolateral compartment, as performed here, can have the effect of drastically decrease the transport in BA direction. This is in agreement with EFSA reporting that when OTA reaches the systemic circulation it extensively binds to plasma proteins, *e.g.* serum albumin, suggesting that only a small fraction remains in its free form (EFSA, 2006). The strategy of adding BSA to the basolateral compartment, despite its influence on the transport results, increases the reliability of the model as the serum contains equally a high levels of proteins. In the same study, Berger et al. (2003) also demonstrated that decreasing the pH in the apical side to pH 6.0 increased the AB flux. In addition, Qi et al. (2017) reported that, considering both pK_a's (4.4 and 7.1) of OTA, it is expected most OTA in nature being present as organic anion, supporting the thesis of transmembrane transporters as significant pathways for the uptake and efflux of OTA. Several transporters have been described to mediate OTAs uptake and efflux across epithelial cells as the organic anion-transporting polypeptides 1A2, 1B1, 2B1 (OATP1A2, OATPA1B1, OATP2B1), which mediate the uptake of OTAs and the breast cancer

Table 1
Apparent permeabilities (x 10^{-6} cm/s) and efflux/uptake ratios of DON, AFB1, FB1, and OTA isolated and in mixture in the Apical → Basolateral (AB) and Basolateral → Apical (BA) directions. % MB shows the mass balance recoveries of transport experiments for each mycotoxin.

		NCI–N87				Caco-2				
		AB (%MB)	*BA (%MB)*	E_R	U_R	*Sig.*	*AB (%MB)*	*BA (%MB)*	E_R	U_R	*Sig.*
DON	**isolated**	3.30 ± 0.40 *(87.4 ± 6.3)*	1.89 ± 0.05 *(106 ± 30.9)*	0.57	1.74	*NS*	4.53 ± 0.94* *(76.3 ± 2.9)*	4.21 ± 1.65 *(100 ± 18.8)*	0.93	1.08	*$p < 0.05$
	mixture	3.41 ± 0.32 *(106 ± 0.6)*	1.58 ± 0.55 *(81.9 ± 9.7)*	0.46	2.16		*7.65 ± 0.98* (74.2 ± 6.7)*	5.08 ± 0.89 *(88.2 ± 3.1)*	0.66	1.51	
AFB1	**isolated**	55.5 ± 4.72* *(79.4 ± 1.2)*	43.5 ± 6.21 *(103 ± 31.1)*	0.78	1.28	*$p < 0.05$	78.4 ± 34.1 *(86.7 ± 4.8)*	20.9 ± 0.58 *(104 ± 27.9)*	0.27	3.76	*NS*
	mixture	33.5 ± 4.22* *(72.9 ± 3.3)*	75.1 ± 21.6 *(114 ± 2.1)*	2.24	0.45		63.9 ± 25.9 *(81.9 ± 25.7)*	36.8 ± 8.24 *(97.9 ± 8.4)*	0.58	1.74	
FB1	**isolated**	0.59 ± 0.17* *(89.1 ± 4.2)*	0 *(99.8 ± 20.9)*	0	–	*$p < 0.05$	0 *(91.6 ± 12.5)*	0 *(70.1 ± 9.6)*	–	–	*NS*
	mixture	0* *(70.0 ± 1.8)*	0 *(74.3 ± 3.5)*	–	–		0 *(76.9 ± 1.1)*	0 *(73.2 ± 8.0)*	–	–	
OTA	**isolated**	41.2 ± 10.6 *(70.0 ± 1.1)*	0 *(116 ± 8.2)*	0	–	*NS*	0.29 ± 0.07* *(93.5 ± 18.9)*	0 *(78.7 ± 9.6)*	0	–	*$p < 0.05$
	mixture	28.5 ± 1.89 *(70.0 ± 13.1)*	0 *(79.1 ± 8.1)*	0	–		3.99 ± 1.77* *(91.3 ± 2.3)*	0 *(88.6 ± 17.5)*	0	–	

Abbreviations: AFB1 – Aflatoxin B1; DON – Deoxynivalenol; E_R – Efflux ratio; FB1 - Fumonisin B1; OTA – Ochratoxin A; U_R – Uptake ratio. Data expressed as mean ± SD of 3 independent experiments (n = 3) * significant difference ($p < 0.05$) between isolated and mixed transport of mycotoxins.

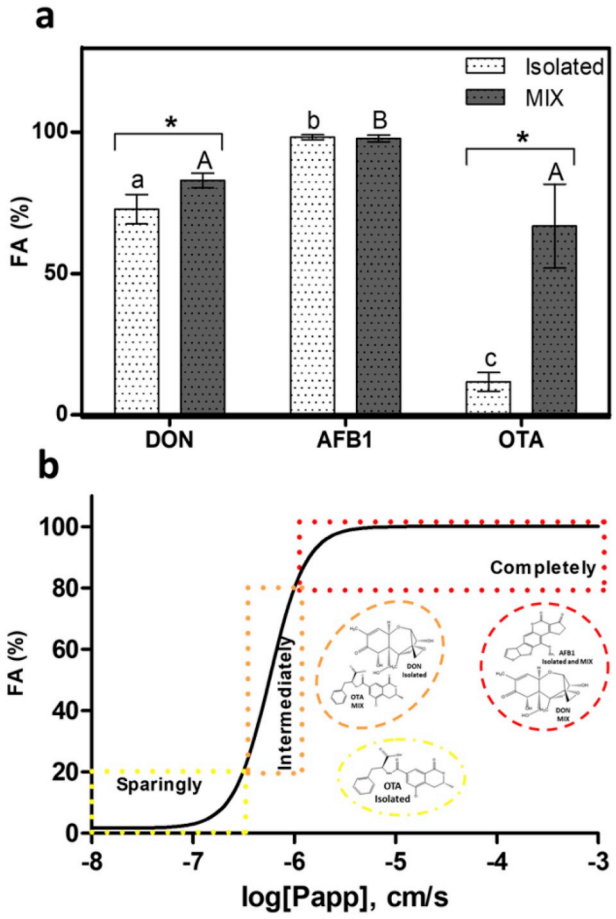

Fig. 5. Fraction absorbed at intestinal level (FA%) of DON, AFB1, and OTA, isolated and in mixture (a); as well as their position in the sigmoidal curve according to their FA% – completely (red), intermediately (orange), and sparingly (yellow) absorbed (b). The sigmoidal curve was built according to (Skolnik et al., 2010; Tavelin et al., 2003). Data expressed as mean ± SD of 3 independent experiments (n = 3). AFB1 – Aflatoxin B1; DON – Deoxynivalenol; and OTA – Ochratoxin A. Values with different letters differ significantly ($p < 0.05$) from the given mean (lowercase letters for isolated transport, uppercase letters for transport in mixture. * significant difference ($p < 0.05$) on FA% between mycotoxins transported isolated or in mixture. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

resistance protein (BCRP) and the multidrug resistance protein 2 (MRP2) transporters known to mediate the efflux of OTA out of the epithelial cells back into lumen (De Angelis et al., 2005; Qi et al., 2017; Santos et al., 2016; Schrickx et al., 2006). The limited intestinal absorption of OTA was justified by the presence of MRP2 transport at the apical side of Caco-2 cells (Sergent et al., 2008). Thus, the use of physiological pH, the addition of BSA in the basolateral, and the presence of efflux transporters may justify the low transport in AB direction and the absence of transport in the A direction in Caco-2 cells.

Concerning AFB1 and DON bidirectional transports, these were found to be insensitive to pH gradients (Figs. 2 and 3) as each mycotoxin was equally transported in both sides at gastric and intestinal level. There are no literature reports on AFB1 and DON gastric absorption, while for intestinal absorption higher transport rates were reported for AFB1 rather than DON (Gratz et al., 2007; Kadota et al., 2013). Gratz et al. (2007) and Mata et al. (2004) studied AFB1 absorption across Caco-2 in AB direction reporting lower P_{app} (~20 × 10^{-6} cm/s) values than those observed herein, probably because our experiment was made under non-sink conditions and the respective equation used to calculate the permeabilities usually results

in higher P*app* values (Tavelin et al., 2002). Moreover, higher permeability values (105.1 ± 7.89 × 10^{-6} cm/s), using the non-sink equation, for AFM1 across Caco-2/TC7 cells were reported by Caloni et al. (2012). In the case of DON, similar Caco-2 P_{app} 3.3 ± 0.61 (x10^{-6}) and 5.02 × 10^{-6} cm/s were reported by Kadota et al. (2013) and Sergent et al. (2006), respectively. In the BA direction, the P*app* value of AFB1 obtained in the present study is similar to the P_{app} reported by Mata et al. (2004), as well as DON P_{app} that is also in accordance with Sergent et al. (2006) and Kadota et al. (2013). These results suggest that AFB1 is more rapidly absorbed than DON, either at gastric or intestinal level. The faster intestinal uptake of AFB1 could also be explained by its mediated transport through OAT or OCT transporters for the uptake (Tachampa et al., 2008; Volk, 2014) or MRP transport for the efflux transport of AFB1 (Loe et al., 1997; Sergent et al., 2008). AFB1 was found in plasma of lactating dairy cows 5 min after ingestion of contaminated meals suggesting a rapid absorption of AFB1 through gastrointestinal tract of cows (Gallo et al., 2008). Additionally, Jubert et al. (2009) studied the pharmacokinetics of AFB1 in human volunteers showing a fast absorption of AFB1 into systemic circulation (peak concentration after 1 h). This may justify the higher transport rate of AFB1 observed in comparison with DON. Sergent et al. (2006) studied the mechanisms of DON transport across Caco-2 cells suggesting that this mycotoxin can be either transcellular or paracellular passive transported, and that after animal ingestion DON may be absorbed in the proximal part of the small intestine considering its early occurrence in plasma. Moreover, after 30 min of DON oral administration to mice, this toxin has been detected in several organs as spleen, kidney, liver, large intestinal and plasma, and after 1 h in the small intestine, suggesting a fast absorption and distribution (Chain et al., 2017).

As discussed until know, the uptake/excretion of toxins is usually evaluated considering a single exposure of these contaminants. However, multiple toxins may be ingested at once and these specific mycotoxins often co-occur in food (Lee and Ryu, 2017; Rodrigues and Naehrer, 2012) and increase the toxicity in target organs when in combination (Sobral et al., 2018), thus transport monitoring of mycotoxins in mixture is of huge importance as, for example, DONs presence has proved to influence the absorption of several nutrients by inhibiting the uptake of sugars, amino acids, lipids and vitamins (Grenier and Applegate, 2013), and the present study shows that mycotoxin co-transportation may differ from their individual transport (Figs. 2 and 3, and Table 1).

Artursson et al. (2001) proposed that P*app* values greater than 1 × 10^{-6} cm/s indicate high permeability coefficients, thus the P_{app} values obtained for mycotoxins suggest that AFB1, DON, and OTA were efficiently absorbed in NCI–N87 and Caco-2 cells either isolated or in mixture, excepting OTA isolated in intestinal uptake.

The influence of transporting mycotoxins in mixture was also noticed in the uptake/efflux ratios of DON and AFB1. In the case of DON a similar efflux ratio (0.86) in Caco-2 cells was reported by Kadota et al. (2013) when transported individually, but its simultaneous transport with other mycotoxins increased the U_R of DON and decreased the E_R, while decreased U_R of AFB1 and increased the E_R. This may suggest that the co-transportation of mycotoxins might beneficiate the uptake of some mycotoxins to the detriment of other across the gastric and intestinal monolayers. The U_R and E_R ratios of DON were all lower than 2 showing that active transport was negligible under the experimental conditions and indicating that DON absorption/excretion is not modulated by P-glycoprotein (PgP) and multidrug resistance associated proteins (MRPs) (Sergent et al., 2006). On the other hand, AFB1 exhibited high U_R (3.76 and 1.74) when transported isolated and mixture across Caco-2 monolayers and a high E_R (2.24) when transported in mixture across NCI–N87 monolayers, which may suggest the involvement of transporters as AFB1 was previously mentioned as a substrate of uptake and efflux transporters. The changing of these ratios when simultaneously transported might suggest that the co-ingestion of multiple mycotoxins may influence their cell uptake and the ability of cells to excrete some xenobiotics. A relevant finding of the present work shows that the fraction absorbed, calculated to predict the final % of intestinal uptake (Skolnik et al., 2010; Tavelin et al., 2003), is significantly increased for OTA when exposure occurs in mixture (Fig. 5a). Berger et al. (2003) and Schrickx et al. (2006) proposed OTA as substrate of efflux transporters (MRP2 and BCRP) decreasing its uptake in Caco-2 cells. However, the co-exposure of OTA with some polyphenols increased OTAs transport across Caco-2 monolayers probably due to the competition for the MRP2 efflux pump (Sergent et al., 2005). Moreover, the co-administration of OTA and DON in pigs resulted in the double of concentration of OTA in liver, muscles and kidney in comparison with OTAs single administration (Lusky et al., 1998; Sergent et al., 2008). This results corroborate the findings of the present study suggesting both absorptive epithelia in the gastrointestinal tract show different absorptive patterns when mycotoxins are transported isolated or in mixture, which may be justified concerning some of their shared absorptive pathways as AFB1 and OTA that share the same uptake/efflux transporters. However, further investigation on combined ingestion of toxins and their mixed transport should be included in such evaluations, concerning the frequent co-occurrence and consequent co-exposure of these mycotoxins. Moreover, this data should be taken into consideration in future assessment of human toxicity by these compounds.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110595

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