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Hyperglycemic conditions modulate the response of colon cancer cells to the mycotoxins alternariol and deoxynivalenol *in vitro*☆

Karolina Kowalska [a,1,*], Eszter Borsos [a,b], Elisabeth Varga [c], Doris Marko [a,*]

[a] *Department of Food Chemistry and Toxicology, University of Vienna, Währingerstraße 38, 1090 Vienna, Austria*
[b] *Doctoral School in Chemistry, Faculty of Chemistry, University of Vienna, Währingerstraße 42, Vienna, Austria*
[c] *Unit Food Hygiene and Technology, Centre for Food Science and Veterinary Public Health, Clinical Department for Farm Animals and Food System Science, University of Veterinary Medicine, Vienna, Veterinärplatz 1, 1210 Vienna, Austria*

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ABSTRACT

A hyperglycemic condition, characterized by high glucose concentration in the bloodstream, is one of the most potent dietary risk factors for colorectal cancer (CRC). It regulates the response to oxidative stress, migration, and invasion of CRC cells, as well as affects the integrity of the intestinal barrier, which plays a pivotal role in protecting against chemicals and contaminants present in the diet.

Although the molecular mechanisms underlying the toxicity of the mycotoxins deoxynivalenol (DON) and alternariol (AOH) are quite well-known, no research so far has considered the possibility that high glucose concentration might affect the response of CRC cells to mycotoxins. In this study, we evaluated how high glucose condition (50 mmol/L) modulates the effect of two mycotoxins distinct in toxicity and underlying mechanisms: DON (0.1–1 μmol/L) and AOH (0.1–20 μmol/L). We evaluated the migration and invasion in Caco-2 and HT-29 CRC cell lines, impact on barrier integrity and metabolism of mycotoxins in an intestinal *in vitro* co-culture model comprising Caco-2/HT-29-MTX-E12/CCD-18Co. Our results showed that high glucose concentration indeed modulates the response of CRC cells to mycotoxins, affecting their metabolism and altering the cellular sensitivity towards them. This observation shed new light on the importance of the involvement of different physiological and pathological conditions when considering the toxicity of mycotoxins.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers among the Western population (Ferlay, Steliarova-Foucher, Lortet-Tieulent, et al., 2013), and its five-year survival rate is low due to metastases (Lin, Lee, Huang, et al., 2015). Recent years of research highlighted the role and the influence of the diet on carcinogenesis, linking obesity, metabolic syndrome, type 2 diabetes and hyperglycemia with CRC incidence. It is estimated that 30% of CRC patients present hyperglycemia (Vekic, Zeljkovic, Stefanovic, et al., 2021). Hyperglycemic condition modulates the metastatic potential of CRC cells by stimulation of the migration and invasion of cells (Lin et al., 2015). On the molecular level, high glucose induces oxidative stress, modulates apoptosis and inflammation (Lin et al., 2015), regulates the expression and activity of matrix metalloproteinases (MMPs), epithelial to mesenchymal transition (EMT) (Wu, Chen, Xi, et al., 2018), and proliferation-associated signaling pathways (Ma, Wang, Ren, et al., 2023). High glucose also affects the fundamental physiological function of the colon: the intestinal barrier. Hyperglycemic condition significantly decreases the integrity and increases the permeability of the epithelial monolayer, modulating the functional properties of the intestinal barrier (Dubois, Muñoz-Garcia, Heymann, & Renodon-Cornière, 2023). The intestinal barrier is crucial to protect the organism against chemicals, microbial metabolites, or foodborne contaminants, such as mycotoxins (Akbari, Braber, Varasteh, et al., 2017).

Mycotoxins are the most common natural pollutants that contaminate food and feed during cultivation, production, and storage (Yang, Song, & Lim, 2020). Depending on the reports, even more than 70% of feed samples might be contaminated with mycotoxins (Eskola, Kos, Elliott, et al., 2020). Deoxynivalenol (DON) produced by *Fusarium* species is one of the most common mycotoxins, present in half of all tested

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* Corresponding authors.
E-mail addresses: karolina.kowalska1@umed.lodz.pl (K. Kowalska), doris.marko@univie.ac.at (D. Marko).
[1] Current affiliation: Medical University of Lodz, Department of Cell Culture and Genomic Analysis, Zeligowskiego 7/9, 90–752 Lodz, Poland.

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food products in the European Union (EFSA Panel on the Contaminants in the Food Chain (CONTAM) et al., 2017). The toxicity of DON is based on its ability to bind to ribosomes and trigger ribosomal stress and protein synthesis arrest, which in consequence induces oxidative stress, apoptosis and DNA damage (Pestka, 2010). DON is also reported to modulate the colon barrier function by increasing the permeability and decreasing the integrity of the epithelial barrier (Pinton, Nougayrède, Del Rio, et al., 2009, Pinton et al., 2010). DON modulates tight junction proteins (TJs) expressions, nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling in colon cells (De Walle, Sergent, Piront, et al., 2010). The modulation of the barrier integrity in the intestine by DON might also affect the basic function of the intestine-the transport of glucose, mediated by glucose receptors. The modulation of the expression and function of glucose receptors is observed in many diseases like diabetes and intestinal inflammation (Koepsell, 2020). Glucose intake is especially important in cancer cells, where it accelerates the Warburg effect and where glucose receptors are directly regulated by the key proliferation and pro-survival pathways (Barron, Bilan, Tsakiridis, & Tsiani, 2016). So far, DON was reported to modulate the expression of glucose transporters in the porcine intestinal IPEC-J2 cells (Liao, Liao, Li, et al., 2017), and chickens were it significantly reduced the expression and activity of glucose receptors in intestine (Awad, Vahjen, Aschenbach, & Zentek, 2011). In human epithelial intestinal cell line HT-29-D4 the modulation of the expression of glucose receptors caused by DON was observed (Maresca, Mahfoud, Garmy, & Fantini, 2002), but not evaluated in other CRC cells so far.

Another mycotoxin, alternariol (AOH), produced by *Alternaria* species, is an emerging mycotoxin with moderate genotoxic, estrogenic and immunotoxic properties (Schmutz, Cenk, & Marko, 2019). AOH is known as a topoisomerase inhibitor, which affects the cell cycle, causes oxidative stress and DNA damage (Fehr, Pahlke, Fritz, et al., 2009). In colon cells, AOH was reported to modulate the inflammatory response (Schmutz et al., 2019), increase the transepithelial electrical resistance (TEER) value measured in differentiated Caco-2 cells, as well as cell permeability (Groestlinger, Seidl, Varga, et al., 2022). Similarly to DON, the possible modulation of glucose receptors by AOH in human intestinal cells was not evaluated so far. Thus, in this study we decided to incorporate these two different in mechanism mycotoxins, with the known modulation of the intestinal barrier, but not known effect on the glucose receptors.

What is more, none of the studies so far considered the fact that hyperglycemic conditions might modulate the response of CRC cells to mycotoxins and significantly change their effect. Thus, in this study, we decided to evaluate if and how the co-exposure of two mycotoxins with distinct toxicities and hyperglycemia modulates the migration and invasion of CRC cells, as well as affects epithelial barrier integrity and metabolism of mycotoxins.

2. Materials and methods

2.1. Cell culture and chemicals

Human colon adenocarcinoma cell lines Caco-2 (C2BBe1 clone, CRL-2102), HT-29 (HTB-38) (ATCC, Manassas, VA, USA) and HT-29-MTX-E12 (12040401) (EACC, Salisbury, United Kingdom) were cultured in phenol red containing Dulbecco's Modified Eagle's Medium (DMEM), high glucose supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Pen/Strep) mixture (10,000 U/mL), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (1 mol/L) and sodium pyruvate (100 mmol/L) under standard cell culture conditions (5% CO_2, 37 °C, humidified atmosphere). Human fibroblast cell line CCD-18Co (ATCC, CRL-1459) was cultured under normal conditions in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% Pen/Strep (10,000 U/mL). All cell culture media were purchased from ThermoFisher Scientific (Waltham, MA, USA). AOH (≥95% purity) and DON ([99.4 ± 0.6]% purity) were purchased from Biomol (Hamburg, Germany) and Biopure (Tulln, Austria), respectively and dissolved in dimethyl sulfoxide (DMSO) as aliquoted stock solutions with a concentration of 10 mmol/L. DMEM, high glucose without phenol red, FBS and antibiotics was used as experimental medium for mycotoxin treatment. Hyperglycemic condition was achieved by adding additional 25 mmol/L (in total 50 mmol/L) of D-glucose (Sigma Aldrich, Saint Louis, MO, USA) to culture medium for 120 h (5 days) prior to the mycotoxin exposure. D-glucose was dissolved as 1 mol/L- stock solution in experimental medium. For the liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements, CHROMASOLV LC − MS-grade acetonitrile (ACN) and methanol (MeOH) from Honey-well Riedel-de Haen (Seelze, Germany), as well as HiPerSolv CHROMANORM LC − MS-grade water from VWR International (Radnor, PA, USA) were used. The certified analytical standard of AOH was obtained from Romer Labs Diagnostic GmbH (Tulln, Austria). LC − MS-grade ammonium acetate, acetic acid, and ammonium hydroxide solutions were purchased from Sigma-Aldrich. In all experiments cells were used in passages: 6–22 (Caco-2), 14–20 (HT-29), 52–60 (HT-29-MTX-E12) and 1–6 (CCD-18Co). In all of the experiments the solvent control was not incorporated due to low (<1%) and insignificant content of DMSO in the highest tested concentration, with no biological effect on the tested cell lines, based on the preliminary and previous data obtained by us.

2.2. Cell viability assay

To evaluate the metabolic activity of the cells, the CellTiter-Blue® (CTB) (Promega Corporation, Madison, WI, USA) assay was used. Cells: 5×10^4 (HT-29) or 10×10^4 (Caco-2) per well were seeded onto 96-well plates and left overnight to adhere. The next day, cells were treated with mycotoxin concentrations: 0.01–100 μmol/L (AOH) and 0.001–5 μmol/L (DON), prepared in experimental medium for 24 or 48 h under standard conditions. The DMSO concentration in experimental medium for the highest tested doses of AOH (100 μmol/L) and DON (5 μmol/L) was 0.1 and 0.005%, respectively. Four hours before the end of incubation time, 10 μL of CTB was added to each well and incubated until the end of the 24 or 48 h period. Then, the fluorescence was measured with Synergy plate reader (Biotek, Shoreline, WA, USA) at 560 nm_{ex}/590 nm_{em}. For high glucose condition, cells were pre-treated with high glucose medium (addition of 25 mmol/L D-glucose) for 120 h (5 days) before seeding. The medium was exchanged every second day. Then, the cells were seeded in the respective media, left to adhere and treated with AOH (0.1–10 μmol/L) or DON (0.1–1 μmol/L) in experimental medium. The results were expressed as % of control (normal glucose, untreated cells). The experiment was conducted in at least three technical and three independent biological replicates.

2.3. Migration assay

For the analysis of the migration of cells, the scratch assay was incorporated. For hyperglycemia condition, the cells were pretreated with high glucose for 120 h prior mycotoxin exposure. Then, cells were seeded onto 12-well plates in the number of 0.8×10^6 cells (HT-29) or 1.6×10^6 (Caco-2) per well and left overnight to adhere. Then scratching was performed with 10 μL plastic tips and the medium was exchanged for the experimental one containing mycotoxins: AOH (0.1–10 μmol/L) and DON (0.1–1 μmol/L). The scratches were photographed, magnitude 10×, at 0 and 48 h of incubation time with Synergy plate reader (BioTek). The % of wound closure was calculated as a difference of the area between 48 h and 0 h in ImageJ software (National Institutes of Health, NIH, USA) and used for data analysis. The experiment was conducted with three biological replicates.

2.4. Invasion assay

For the invasion assay a modified Boyden chamber assay was used. Briefly, cells (Caco-2 and HT-29) were treated with normal and high

glucose concentration for 5 days. Then, cells were trypsinized and suspended in experimental medium. For the assay 24-well 8 μm PET inserts (Carl Roth, Karlsruhe, Germany) coated with thin layer of Geltrex (Thermo Fisher Scientific) were used. The cells in the number of 0.75×10^6 were seeded onto each insert in experimental medium containing mycotoxins: AOH (0.1–10 μmol/L) and DON (0.1–1 μmol/L), whereas experimental medium containing 10% of FBS was used for basolateral side. The cells were then incubated in standard cell culture conditions for 48 h. After that time, cells were stained with crystal violet (Sigma Aldrich) solution, the cells from the upper side of the insert were removed with a cotton pad. Inserts were photographed with a light microscope (Zeiss, Oberkochen, Germany) and stained cells were dissolved in 10% acetic acid. The absorbance was measured at 550 nm with Synergy plate reader. The results were normalized to control (untreated, normal glucose) and expressed as % of control. The experiment was conducted with three biological replicates.

2.5. Colony formation assay

To evaluate the clonogenic properties of the cells (Caco-2 and HT-29), the colony formation assay was used. Cells were treated with normal and hyperglycemic conditions for 120 h. Then, 5×10^2 cells per well were seeded onto 6-well plates and left overnight to adhere. The next day, cells were treated with mycotoxins: AOH (0.1–10 μmol/L) and DON (0.1–1 μmol/L) in experimental medium for 48 h. Then, the medium was exchanged for the normal one (25 mmol/L glucose), and cells were cultured in standard condition for additional 14 days. The medium was exchanged every 2–3 days. Then, cells were stained with crystal violet solution (1:3 in H_2O) and photographed. The stained cells were dissolved in 10% acetic acid and the absorbance was measured at 550 nm with Synergy plate reader. The experiment was conducted with three biological replicates.

2.6. Gelatin zymography assay

To assess the activity of metalloproteinase 2 and 9 (MMP-2 and MMP-9), the gelatin zymography assay was conducted. In this assay, a co-culture differentiated model was used. Caco-2: HT-29-MTX-E12 cells in the mixture of 9:1 in the number of 8.5×10^4 cells/cm^2 were seeded onto 12-well PET 0.4 μm cell culture insert in normal or hyperglycemic medium in both apical and basolateral side. The cells were treated for 7 days, and the medium was exchanged every second day. Then the apical medium was collected and immediately frozen in −80 °C until time of analysis. The protein content in the apical medium was measured with Bradford reagent (Carl Roth). Equal amounts of protein (6 μg) were separated onto polyacrylamide gels containing gelatin (Carl Roth) with electrophoresis (90 V, 4 °C). Then the gels were permeabilized with 2.5% Triton X-100 solution (2×30 min) and incubated in 37 °C for 48 h in the induction buffer (0.05 M TRIS, 0.01 M $CaCl_2$, 0.02 M NaCl, pH = 7.5). Then, the gels were stained with Coomassie Brilliant Blue R-250 solution in MeOH: acetic acid (5:2) mixture. The white bands were destained with destaining buffer (MeOH: acetic acid: H_2O, 5:2:3) and photographed. The intensity was measured with ImageJ software (NIH). The results are expressed as a fold change of control condition. The experiment was conducted with three biological replicates.

2.7. Real time quantitative polymerase chain reaction (RT-qPCR)

Cells were seeded onto 12-well plates, dependently on the experiment as monoculture or co-culture. For monoculture, the cells in the number of 0.5 or 1×10^6 cells (HT-29/Caco-2) per well were seeded. For co-culture model Caco-2: HT-29-MTX-E12 cells in the mixture of 9:1 in the number of 8.5×10^4 cells/cm^2 were used (*MMP-2* and *MMP-9* expression). Cells were left overnight to adhere and then treated with mycotoxins: AOH (0.1–10 μmol/L) and DON (0.1–1 μmol/L). Then, the cells were proceeded with RNA isolation (RNeasy Mini Kit, Qiagen) according to the manufacturer's protocol. RNA concentration and quality was measured spectrophotometrically with NanoDrop (ThermoFisher Scientific). One μg of RNA was reversely transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Ten ng/mL of cDNA was used per one reaction in RT-qPCR (Applied Biosystems). Power SYBR™ Green PCR Master Mix (Applied Biosystems) was used as a buffer of the reaction. Primers for *SGLT1, GLUT2* and *GLUT5* were provided by Qiagen, whereas the rest of the primers were designed in Primer-BLAST (National Library of Medicine, NIH, USA) and synthetized by Origene (Rockville, MD, USA) (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. Melting curve analyses were performed for each reaction. The ΔΔCt method was used for analysis of the data. The experiment was carried out in a technical duplicate with three independent biological replicates.

2.8. Western blot

Cells (Caco-2 and HT-29, 3.65×10^6) were seeded onto 60 mm Petri dishes and left overnight to adhere. On the next day cells were treated with different concentrations of mycotoxins: AOH (0.1–10 μmol/L) and DON (0.1–1 μmol/L) for 24 h. Then, the cells were scratched in cell culture medium, centrifuged at 277 x*g* 10 min, 4°C and cell pellets were proceed for isolation with radio-immunoprecipitation assay (RIPA) extraction buffer (Sigma Aldrich) supplemented with inhibitors: phenylmethylsulfonyl fluoride (1 mmol/L, PMSF, Roche, Basel, Switzerland), Complete Protease Inhibitor Cocktail (1 x, Roche) and PhosSTOP™ (1 x, Roche). The protein concentration was measured with BCA assay (ThermoFisher Scientific) according to the manufacturer's protocol. Twenty μg of protein was used for gel electrophoresis (SDS-PAGE 12% polyacrylamide gel, 120 V) and wet transfer ((polyvinylidene difluoride (PVDF) membranes, 0.45 μm, 400 mA, 110 min). Then the membranes were blocked for 1 h in non-fat milk in a tris-buffered saline with Tween 20 (TBST) buffer, washed 3 times with TBST (5 min) and incubated overnight with primary antibodies (1:1000 in 5% bovine serum albumin (BSA, Sigma Aldrich) in TBST): anti-GLUT2 (MA5–35162, ThermoFisher Scientific), anti-SGLT1 (PA5–28240, ThermoFisher Scientific), anti-GAPDH (#97166, Cell Signaling Technology, Danvers, MA, USA) in 4 °C. Then, membranes were washed 3 times in TBST buffer 5 min and incubated for 1 h in horseradish peroxidase (HRP)-linked secondary antibodies (1:5000, Cell Signaling Technology), then visualized with Pierce™ ECL Substrate (ThermoFisher Scientific) with LAS-4000 Luminescent Image Analyzer (FujiFilm, Tokyo, Japan). The intensity of the bands was calculated with ImageJ software (NIH). The experiment was conducted with three biological replicates.

Table 1
Sequences of primers designed and used in the study. *CDH1*- *E*-cadherin, *VIM*-vimentin, *MMP-2*- metalloproteinase 2, *MMP-9*- metalloproteinase 9, *ZEB1*- zinc finger E-Box binding homeobox 1, *SNAIL1*- Snail family transcriptional repressor 1, *GAPDH*- glyceraldehyde-3-phosphate dehydrogenase, bp- base pair.

Gene	Sequence 5′ → 3’	Product size [bp]
CDH1	Rev-AGTTCAGGGAGCTCAGACTAGCAGCTTCGG	168
	For- TCCCCCGGTATCTTCCCCGCCCTG	
VIM	Rev- ACCGTCTTAATCAGAAGTGTCC	127
	For- AAGGCGAGGAGAGCAGGAT	
MMP-2	Rev- TGTCCTTCAGCACAAACAGGTTGC	184
	For- ACCAGCTGGCCTAGTGATGATGTT	
MMP-9	Rev- CTGGCAGGGTTTCCCATCAG	101
	For- GCAGTACCACGGCCAACTAC	
ZEB1	Rev- GCGGTGTAGAATCAGAGTCATT	105
	For- CCTTGAAAGTGATCCAGCCAAA	
SNAIL1	Rev- GCTGCTGGAAGGTAAACTCTG	115
	For- AGTGCCTCGACCACTATGC	
GAPDH	Rev- GATTTGGTCGTATTGGGCGC	169
	For- TTCCCGTTCTCAGCCTTGAC	

2.9. Transepithelial electrical resistance (TEER)

For TEER measurements, a triple co-culture model was used (CCD-18Co/Caco-2/HT-29-MTX-E12). Ten thousand of CCD-18Co cells were suspended in Geltrex™ hESC-Qualified, Ready-To-Use, Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific) and seeded onto 12-well 0.4 μm PET insert for 1 h. Then the growth medium was added to the apical and basolateral side and cells were left overnight in standard cell culture conditions. Next day, Caco-2: HT-29-MTX-E12 cells in the mixture of 9:1 in the number of 8.5×10^4 cells/cm^2 were seeded onto inserts and left to adhere overnight. Two different experiments were conducted:

1. The next day, the medium was exchanged for high glucose or normal glucose for both sides of insert and cells were differentiated for 14 days. The medium was exchanged every second day. Before the medium exchange, TEER was measured with EVOM2 epithelial voltmeter (World Precision Instruments, Sarasota, FL, USA). After 14 days, the medium from the apical side was exchanged for experimental medium containing mycotoxins: AOH (0.1–20 μmol/L) and DON (0.1–1 μmol/L), and basolateral medium was exchanged for experimental one and incubated for additional 48 h. TEER measurement was conducted at 0 and 48 h after mycotoxins exposition.

2. The medium was exchanged for high glucose/ normal glucose for both sides of the insert, and the to the apical side, the mycotoxin treatment solution was added: AOH (10 and 20 μmol/L) and DON (0.5 and 1 μmol/L). The medium was exchanged every second day for fresh one containing mycotoxins. TEER was measured before medium exchange. The cells were differentiated for 7 days.

TEER values were calculated as Ω × cm^2 and blank (PET insert with medium) was subtracted from each value. The experiment was conducted with three biological replicates.

2.10. Paracellular permeability

Lucifer Yellow (LY) assay was used to measure permeability of the monolayers in triple co-culture model (CCD-18Co/Caco-2/HT-29-MTX-E12). At the end of the co-culture model incubation (7 or 14 days), the medium from the apical and basolateral side of the insert was removed. To the apical side of the insert 0.1 mg/mL solution of LY in Hanks Balanced Salt solution (HBSS) was added, whereas to the basolateral side HBSS was added. Inserts were incubated for additional 1 h in standard cell culture conditions. Then, 150 μL of the basolateral medium was aspirated and measured on 96-well plate with Synergy plate reader at 485 nm_{ex}/535 nm_{em} (Biotek) The fluorescence was used to calculate the % of LY solution passing the monolayer of cells and expressed as permeability. The experiment was conducted with three biological replicates.

2.11. LC-MS/MS measurements

The co-culture model of CCD-18Co/Caco-2/HT-29-MTX-E12 cells on inserts was used as described above. The medium was exchanged for high glucose/ normal glucose on both sides of the insert, and mycotoxins treatment: AOH (10 and 20 μmol/L) and DON (0.5 and 1 μmol/L) was added to the apical side of the insert. The medium was exchanged every second day for a fresh one containing mycotoxins. The experiment was conducted for 7 days. The mycotoxin-containing incubation medium, basolateral and apical samples were collected after co-culture experiments and mixed with ice-cold extraction solvent (ACN/MeOH, 1:1) in the proportion of 1:4. Then samples were frozen overnight. Next day, the samples were centrifuged for 15 min at 18.400 x g, 4 °C. After centrifugation, 500 μL of the supernatant was mixed with equal amount of LC-MS grade water and stored in −20 °C until analysis.

LC-MS/MS measurements were conducted using an Agilent 1290 Infinity II high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) coupled to a Sciex QTrap 6500+ mass spectrometer, equipped with an electrospray ionization interface. MS data were acquired in multiple reaction monitoring (MRM) mode, applying negative electrospray ionization.

Quantitative analysis of AOH and the semi-quantitative determination of its selected metabolites was based on a previously published method. A Supelco Ascentis Express column (C18, 100 mm × 2.1 mm; 2.7 μm, Merck kGaA, Darmstadt, Germany) equipped with a Phenomenex SecurityGuard precolumn (C18, 2 mm; Phenomenex Inc., Torrance, USA) was used for the chromatographic separation at 30 °C. Eluent B was methanol, while eluent A consisted of an aqueous 5 mmol/L ammonium acetate solution with a pH of 8.6 adjusted using a 25% ammonia solution. Gradient elution was applied at a flow rate of 0.4 mL/min, starting with 10% eluent B for the first minute. The MeOH content was then raised linearly to 38% and 60% until minutes 5.5 and 7, respectively. Subsequently, a steep gradient was employed, reaching 100% eluent B within half a minute. This condition was then held for two additional minutes. Lastly, the column was re-equilibrated at the initial conditions, reaching an overall run time of 11.5 min.

The analysis of DON and its metabolites was performed according to the method reported by Beisl, Varga, Braun, et al., 2021. Briefly, a Kinetex Biphenyl 100 Å column (150 × 3.0 mm, 2.6 μm, Phenomenex) equipped with a compatible precolumn was operated at 40 °C. An aqueous solution of 10% MeOH with 0.05% acetic acid served as eluent A and MeOH supplemented with 0.05% acetic acid was used as eluent B at a flow rate of 0.4 mL/min. The 19-min method started with a one-minute period of pure eluent A, followed by a linear increase in the percentage of eluent B to 16% within 9 min, further to 95% B within 2 min. This condition was held constant for 4 min, after which the proportion of eluent A was raised to 100% over 0.1 min and held for 3 min.

The parent toxins DON and AOH were quantified *via* solvent-based external calibration whereas their metabolites could merely be semi-quantified due to the lack of available reference materials. The limit of detection (LOD) and quantitation (LOQ) in the samples were estimated based on a signal-to noise ratio of 3:1 and 10:1, respectively. The LOD was *ca.* 0.29 μg/L (1.2 nmol/L) for AOH and 4.9 μg/L (16 nmol/L) for DON while the LOQ were 0.95 μg/L (AOH, 3.7 nmol/L) and 16 μg/L (DON, 54 nmol/L). For AOH metabolites identification was based on theoretical exact masses and assumed fragmentations yielding the parent compound ion.

If the measured concentration results exceeded the provided calibration range (1–300 μg/L for DON and 0.3–100 μg/L for AOH), respective samples were diluted using ACN/water (50/50, *v/v*) and re-measured. The standard injection volume was 5 μL but samples were additionally injected with 1 or 10 μL in case the toxin levels were outside the calibration range. Data analysis was performed using the Skyline software (version 25.1., MacCoss Lab, Department of Genome Sciences, University of Washington). The mass spectrometric parameters of the analytes quantified were presented in Supplementary file.

2.12. Statistical analysis

Statistical analysis was performed with GraphPad Prism software. One-way ANOVA with Bonferroni correction was used for calculations. $p < 0.05$ was considered significant. All the data are presented as mean ± SE.

3. Results

3.1. AOH and DON modulate the viability of CRC cells

Firstly, we evaluated how both tested mycotoxins affect the viability of CRC cells, as well as whether hyperglycemic condition changes the response of the cells to mycotoxin-induced cytotoxicity. We observed that DON significantly reduced the viability of both tested cell lines in a dose- and time-dependent manner (Fig. 1A-B). In the case of DON, doses higher than 0.5 μmol/L caused a significant decrease in cell viability, in

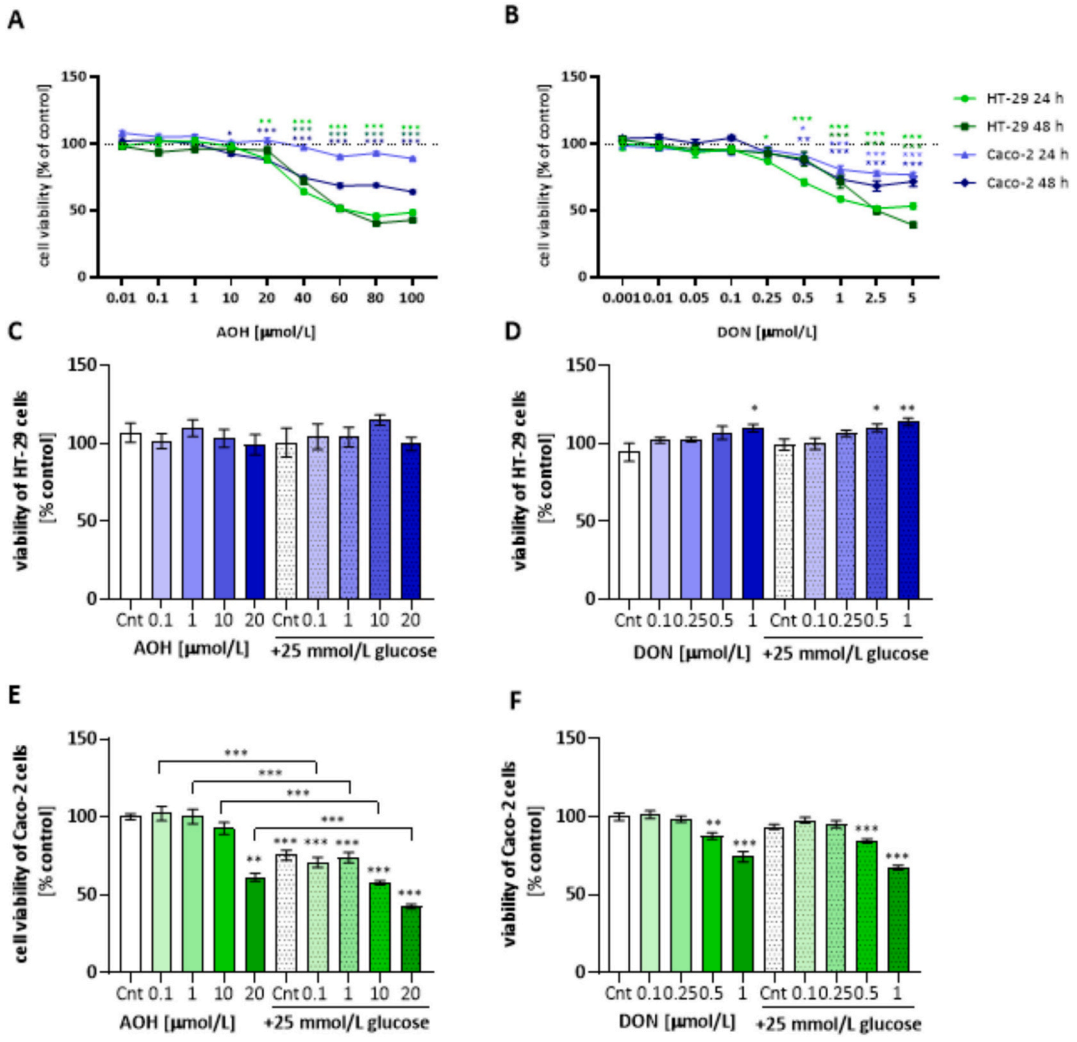

Fig. 1. AOH (A, C, E) and DON (B, D, F) modulate the viability of CRC cells. Cells were pretreated with normal (25 mmol/L D-glucose) or high glucose (50 mmol/L D-glucose) for 120 h and then treated with AOH/DON for additional 48 h. The viability of cells was evaluated with CellTiter-Blue® assay. All data were normalized to control (non-treated cells) and expressed as a % of control cells. The results are expressed as mean ± SE as three biological replicates. One way ANOVA with Bonferroni correction was used for statistical analysis. $p < 0.05$ was considered as statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Cnt - control; AOH - alternariol; DON - deoxynivalenol; CRC - colon cancer cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both tested time points. When the cells were pretreated with high glucose concentration, an additional 25 mmol/L D-glucose for 120 h, statistically significant (***$p < 0.001$) differences were observed only in the case of Caco-2 cells and treatment with AOH (Fig. 1 C—F). In other cases, high glucose treatment conditions did not change the response of the cells to mycotoxin treatment.

3.2. AOH and DON affect the expression of glucose receptors in CRC cell lines

Many environmental factors might modulate the expression of sugar receptors, which are responsible for the glucose/fructose uptake in the cells (Hajiaghaalipour, Khalilpourfarshbafi, & Arya, 2015). Thus, in the next part we evaluated the modulation of the expression of glucose receptors: sodium/glucose cotransporter 1 (SGLT1), glucose transporter 2 (GLUT2), and fructose receptor glucose transporter 5 (GLUT5) in the presence of mycotoxins in monoculture of CRC lines: Caco-2 and HT-29 cells. The concentration range of mycotoxins chosen (1–20 µmol/L) was based on the previous research studies (Kowalska, Habrowska-Górczyńska, et al., 2021; Gufler, Heffeter, Kowol, et al., 2025).

The response of cells to both AOH and DON was different. HT-29 cells treated with AOH for 24 h presented a statistically significant increase in the expression of *GLUT2* and *GLUT5* only for the lowest tested dose 0.1 µmol/L (**$p < 0.01$). Some modulation of the *SGLT1* expression was also observed, but not significant (Fig. 1A-C). In the case of Caco-2, a dose-dependent significant decrease was observed in the expression of *GLUT2*, not dose dependent decrease in the case of *SGLT1* (***$p < 0.001$). No significant modulation of the *GLUT5* expression was observed (Fig. 2D-F).

The second tested mycotoxin DON showed a more potent effect after 24 h of incubation. In HT-29 cells, DON dose-dependently and significantly modulated the expression of *SGLT1* (***$p < 0.001$), whereas a significant decrease (*$p < 0.05$) in the expression of *GLUT2* and *GLUT5* was observed only for 0.5 µmol/L of DON (Fig. 2 G-I). In Caco-2, 1 µmol/L of DON significantly increased the expression of *GLUT2* (***$p < 0.001$) and *GLUT5* (***$p < 0.001$), whereas a significant, dose-dependent decrease (***$p < 0.001$) was observed in the expression of *SGLT1,* similarly to HT-29 cells (Fig. 2J-L).

The expression of SGLT1 and GLUT2 was also verified on the protein level. We observed that AOH caused no significant decrease of GLUT2 expression in both tested cell lines, as well as no significant modulation of the SGLT1 expression (Fig. 3A-E), which is in line with mRNA

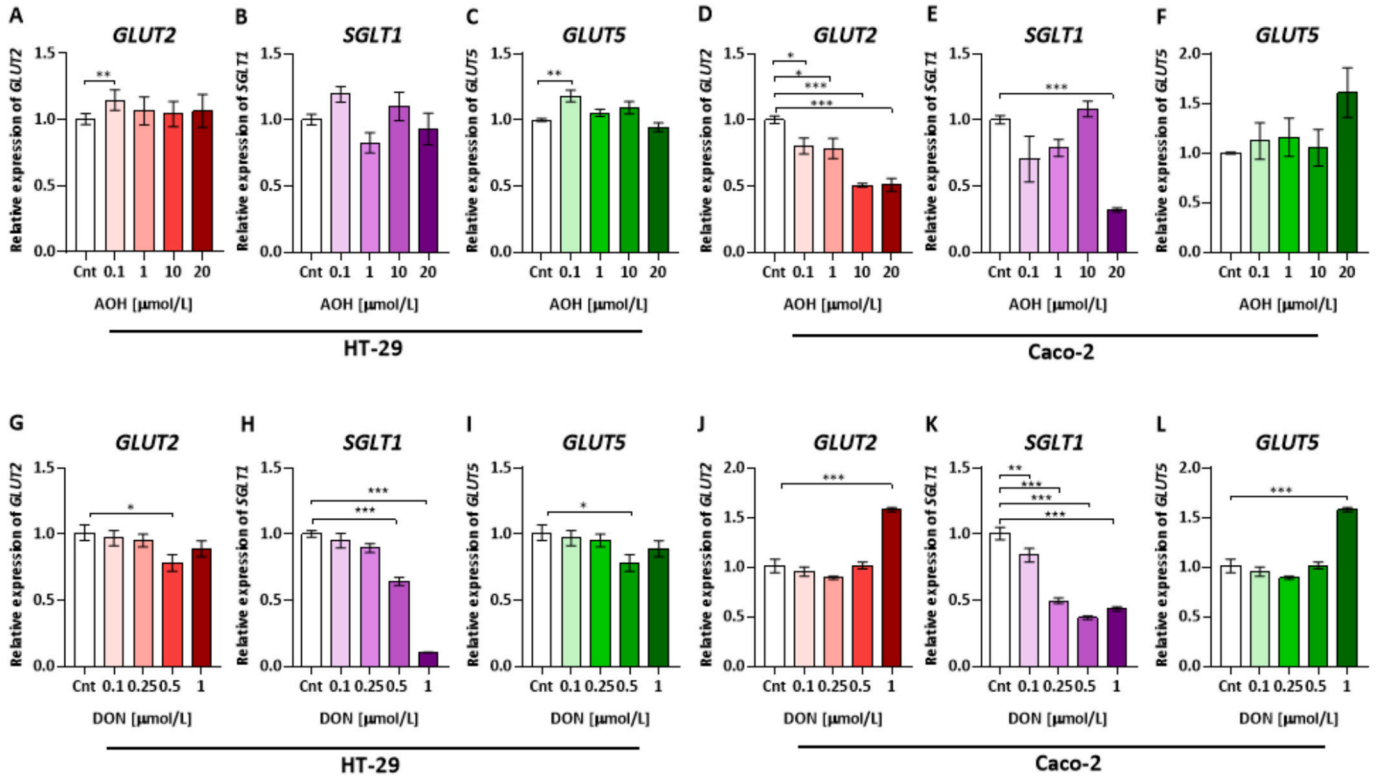

Fig. 2. AOH (A-F) and DON (G-L) modulate the expression of glucose and fructose receptors on mRNA level in CRC cells. The relative expression of *GLUT2* (A, D, G, J), *SGLT1* (B, E, H, K) and *GLTU5* (C, F, I, L) after mycotoxin treatment was evaluated with a RT-qPCR method in HT-29 and Caco-2 cells after 24 h treatment with mycotoxins. *GAPDH* was used as reference gene. The results are expressed as mean ± SE as three biological replicates. One-way ANOVA with Bonferroni correction was used for statistical analysis. $p < 0.05$ was considered as statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Cnt - control; AOH - alternariol; DON - deoxynivalenol; CRC - colon cancer cells; *GLUT2 - SLC2A2,* solute carrier family 2 member 2; *SGLT1 - SLC5A1*, solute carrier family 5 member 1; *GLUT5 - SLC2A5,* solute carrier family 2 member 5; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase.

expression. In the case of DON, a different expression pattern was observed between protein and mRNA expression. A non-significant dose-dependent decrease in the expression of GLUT2 was observed in HT-29 cells, significant dose-dependent for two of the highest tested doses in Caco-2 cells (*$p < 0.05$, **$p < 0.01$). In both tested cell lines, a significant dose-dependent decrease was observed in the expression of SGLT1 upon DON treatment (*$p < 0.05$) (Fig. 3F-J). In both tested cell lines as well as mycotoxins the protein expression modulation seems to be less potent than mRNA expression, what might be explained both by the time point of measurement as well as in-cell mechanisms.

3.3. AOH and DON modify the migration and invasiveness of CRC cells differently in normal and hyperglycemic conditions

Next, we focused on the properties of cancer cells: the ability to migrate and invade with modulation of epithelial to mesenchymal transition (EMT), due to the previous reports showing that high glucose affects these processes in CRC cells (Lin et al., 2015). To evaluate the potential modulation of the migration of cells, the scratch assay was used. Firstly, we observed that tested mycotoxins slightly modulated the migration of cells after 48 h of exposure (Fig. 4A-F). The statistically significant decrease in cell migration was observed in Caco-2 cells after exposure to 1 µmol/L DON (***$p < 0.001$). A modulation in the migration of HT-29 cells after AOH and DON exposure was observed, however not statistically significant. In a hyperglycemic condition, an increase in the migration of cells was observed in HT-29 cells in the control and all treatment conditions; however, treatment with AOH resulted in a decrease in cell migration only for the highest tested dose of AOH (20 µmol/L). The statistically significant differences were observed between 1 and 10 µmol/L of AOH as compared between normal and high glucose conditions (*$p < 0.05$, **$p < 0.01$). A similar dose-dependent effect of DON was observed in the case of Caco-2 cells treated under high glucose conditions, with significant differences between 0.25 and 0.5 µmol/L DON in normal and high glucose conditions (*$p < 0.05$, **$p < 0.01$). A statistically significant decrease in cell migration after 1 µmol/L of DON was observed as compared to the high glucose control condition (**$p < 0.01$). The decrease in cell migration observed for 1 µmol/L of DON was present both in normal as well as high glucose condition, although the one in high glucose condition was not significantly different when compared to normal glucose control.

Then, we evaluated whether AOH and DON modulate the invasion of CRC cells in both normal and high glucose conditions. We observed that in normal glucose conditions in both tested cell lines, mycotoxins caused no significant change in cell invasion at the tested concentrations (Fig. 4G-K), whereas pre-treatment with high glucose conditions modulated the response of cells to mycotoxins. First of all, we observed that in case of HT-29 cells, AOH caused a significant increase after 1 µmol/L AOH exposure, as compared to control cells (*$p < 0.05$). In the case of DON, a dose-dependent increase in cell invasion was observed as compared to control cells (**$p < 0.01$, ***$p < 0.001$). The observed effect was also statistically different when compared to the normal glucose condition for 0.25–1 µmol/L of DON (*$p < 0.05$, **$p < 0.01$). In the second cell line tested, Caco-2, an increase in cell invasion was observed after 0.1–10 µmol/L AOH treatment, as compared to control under high glucose conditions, as well as significantly higher than the same AOH treatment in normal glucose conditions (***$p < 0.001$). In the case of DON treatment, no significant change was observed neither for the treatment conditions nor after pre-treatment with high glucose before mycotoxin exposure.

Then, we evaluated the clonogenicity of cells and the activity of

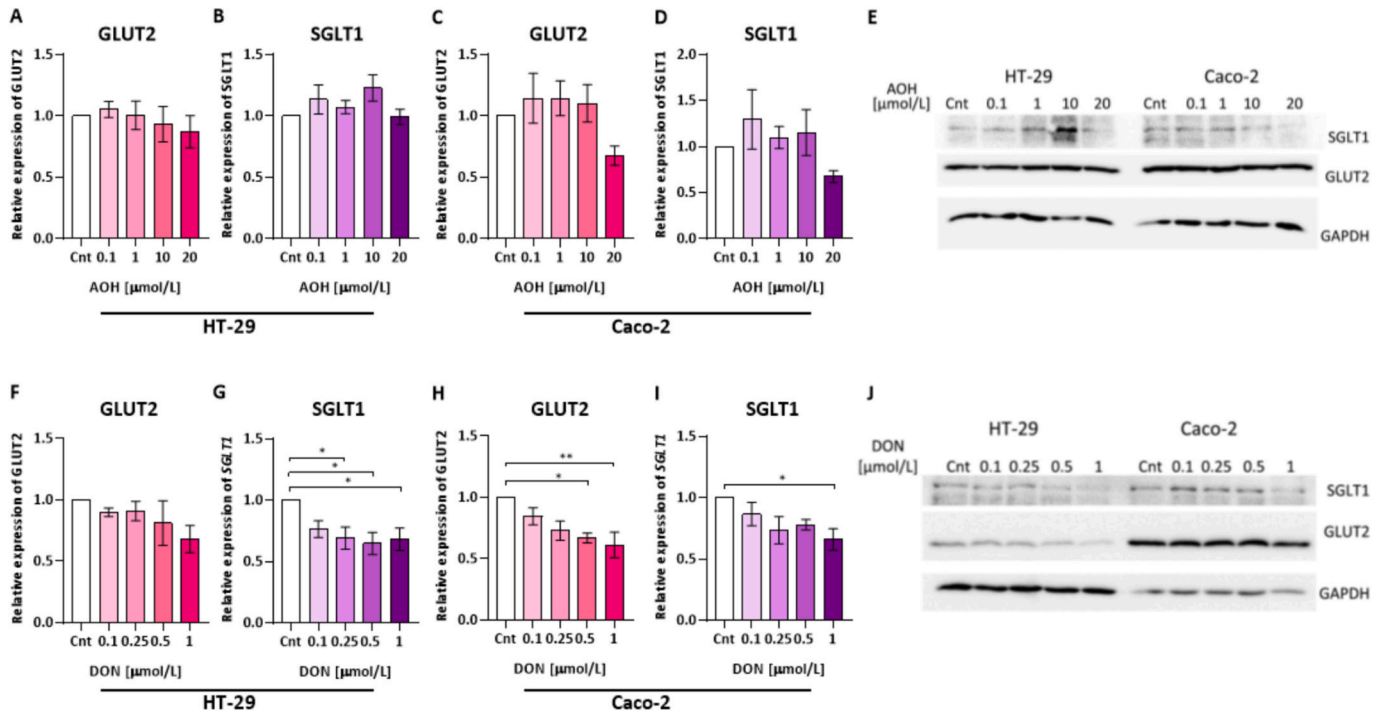

Fig. 3. AOH (A-E) and DON (F-J) modulate the expression of glucose receptors on protein level in CRC cells. E and J present the representative results of Western blot. The relative expression of GLUT2 (A, C, F, H) and SGLT1 (B, D, G, I) after mycotoxin treatment was evaluated with Western blot in HT-29 and Caco-2 cells after 24 h treatment with mycotoxins. The results are expressed as mean ± SE of fold change of control (Cnt) of at least three biological replicates. GAPDH was used as reference gene. One-way ANOVA with Bonferroni correction was used for statistical analysis. $p < 0.05$ was considered as statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Cnt - control; AOH - alternariol; DON - deoxynivalenol; CRC - colon cancer cells; *GLUT2 - SLC2A2,* solute carrier family 2 member 2; *SGLT1 - SLC5A1*, solute carrier family 5 member 1; GAPDH - glyceraldehyde-3-phosphate dehydrogenase.

MMPs. We observed that AOH dose-dependently and significantly decreased the clonogenicity of HT-29 cells starting from the dose of 1 μmol/L of AOH (Fig. 5 A and E). A similar effect was observed in Caco-2 cells for the doses of 10 and 20 μmol/L of AOH. DON caused an increase in the clonogenic potential in HT-29 cells in the dose of 0.5 μmol/L, whereas in Caco-2, a significant decrease was observed for the highest tested dose, 1 μmol/L ($^{**}p < 0.01$). In a hyperglycemic condition we observed that the overall clonogenic potential of both cell lines was higher than in a normal glucose condition ($^{***}p < 0.001$). In HT-29 cells AOH in all tested doses significantly decreased the clonogenic potential, and the effect was dose-dependent. A contradictory effect was observed for DON in HT-29, where 0.5 and 1 μmol/L caused an increase in clonogenic potential as compared to both normal as well as high glucose control. In Caco-2 cells, both tested mycotoxins caused dose-dependent decrease ($^{*}p < 0.05$) in clonogenicity, as compared to both normal and high glucose control.

We also evaluated the activity and expression of matrix metalloproteinases (MMPs). We observed no modulation in the activity of MMPs in Caco-2:HT-29-MTX-E12 co-culture model (Fig. 5F-H). However, when the expression in single cell lines was evaluated, the modulation was present. The highest tested concentration of AOH decreased the expression of *MMP-9* and *MMP-2* (Fig. 5I-J), and the effect was potentiated by high glucose conditions. In Caco-2 cells, *MMP-2* expression was not detectable on the mRNA level; however, in the case of *MMP-9*, an increased expression was observed with no significant changes in the modulation of the expression in high glucose conditions. DON also modulated the expression of *MMPs* in HT-29 cells. The increase in the expression was observed for both *MMP-9* and *MMP-2* in normal glucose conditions, whereas high glucose caused a decrease in *MMP-9* and an increase in *MMP-2* expression.

Moreover, we evaluated the expression of EMT genes to compare it with the clonogenicity assay results. We observed decreased expression of *ZEB1*, *SNAIL1*, and *CDH1* after AOH treatment in HT-29 cells, and high glucose conditions increased the expression of *SNAIL1*, *VIM*, and *CDH1* as compared to normal glucose conditions. In the case of Caco-2, no significant change was observed in a high glucose condition; in both cases, AOH increased the expression of *ZEB1*, *SNAIL1*, *VIM,* and *CDH1*. DON modulated the expression of EMT genes in a different way than AOH. We observed an increased expression of *ZEB1* and *SNAIL1* and decreased expression of *VIM* and *CDH1* in HT-29 cells in normal glucose conditions, whereas high glucose conditions potentiated the increase in *ZEB1*, *SNAIL1*, and *CDH1*, whereas the expression of *VIM* was not changed, as compared to normal glucose conditions. In Caco-2 cells, an increased expression of *ZEB1*, *SNAIL1*, *VIM*, and *CDH1* was observed in normal glucose conditions after DON treatment, whereas high glucose decreased the expression of *ZEB1*, *VIM,* and *CDH1* as compared to normal glucose treatment.

3.4. AOH and DON differently act on the intestinal barrier integrity and permeability in the normal and high glucose condition

The intestine is considered the first barrier to natural contaminants *e.g.*, mycotoxins (Gao, Meng, Liu, et al., 2020) and both mycotoxins and high glucose might affect its function (Dubois et al., 2023). In that case, we decided to enrich the classical *in vitro* model of intestinal barrier (Caco-2:HT-29-MTX-E12) with colon fibroblasts (CCD-18Co) in a 3D structure to better mimic the complex intestinal function. We evaluated the long-term as well as short-term changes in the intestinal integrity and permeability after mycotoxin exposure.

Firstly, we evaluated 48 h of exposure to mycotoxins after 14 days of differentiation of the co-culture intestinal barrier model. We observed that neither mycotoxin significantly modulated the integrity of the monolayer of cells measured with TEER (Fig. 6 A-B), and a similar effect was observed in the permeability of the monolayer (Fig. 6 C—D). Furthermore, we decided to change the tested model and evaluate the effect during the formation of a monolayer of cells, simultaneously with

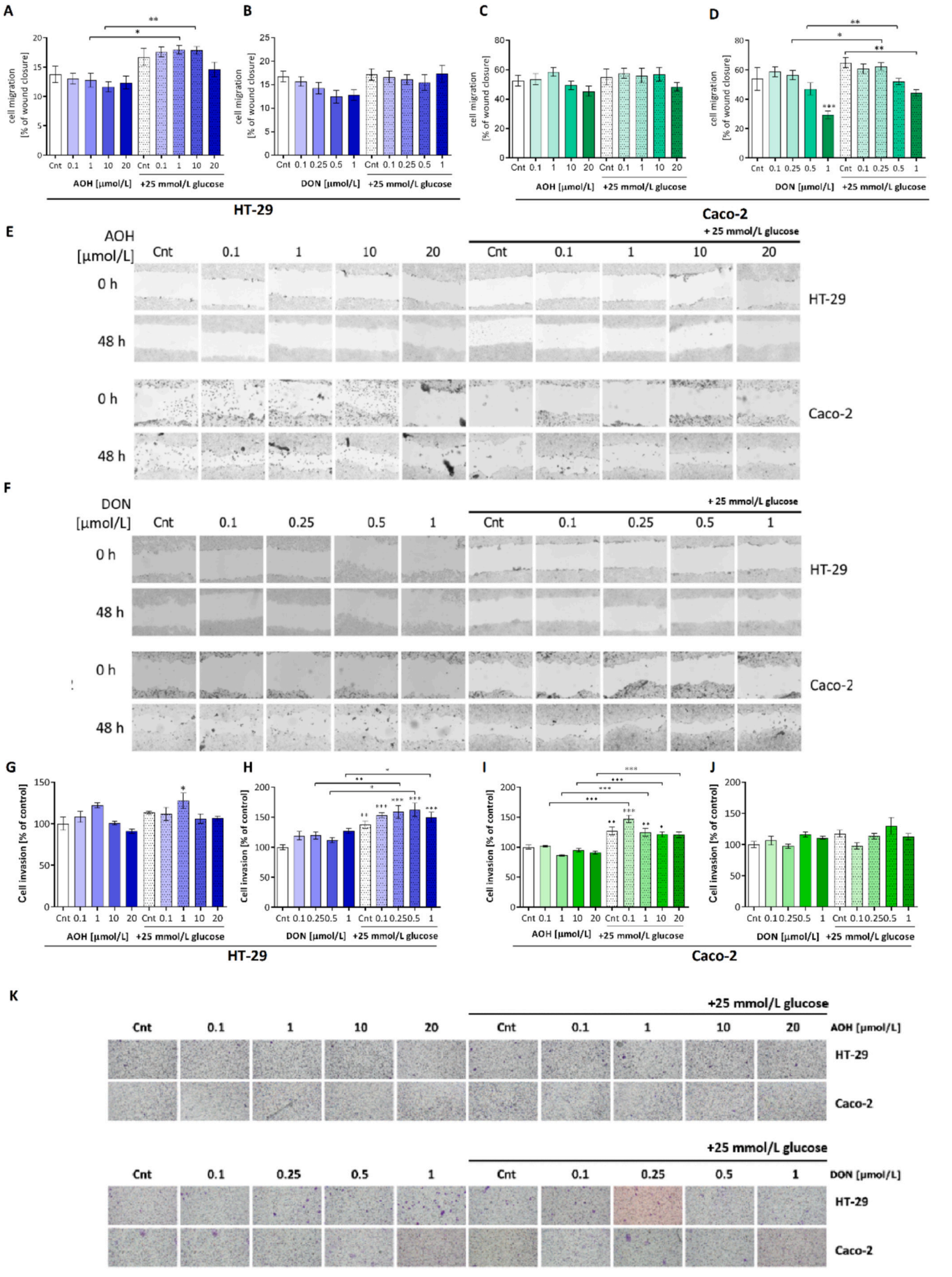

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Fig. 4. Modulation of the migration and invasion of CRC cells by mycotoxins in normal and high glucose conditions. A-D the results are expressed as mean ± SE of % of migration of cells between 0 h and 48 h of treatment. For high glucose condition the cells were pretreated with addition of 25 mmol/L D-glucose for 120 h and then treated with AOH or DON for additional 48 h. E and F- the results of migration assay. G-J- the results of invasion assay (modified Boyden chamber assay). The cells were treated with normal glucose or pretreated for 120 h with high glucose (+25 mmol/L D-glucose), then the cells were seeded on the cell insert (PET, 8 μm) in experimental medium containing mycotoxin. The invasion of cells was stimulated by experimental medium with FBS in lower compartment for 48 h. The cells for both assays were stained with crystal violet (Sigma Aldrich), dissolved in 10% acetic acid, and the absorbance was measured at 550 nm. K- representative images of migrated cells. One-way ANOVA with Bonferroni correction was used for statistical analysis, $n = 3$. $p < 0.05$ was considered significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to control. Cnt- control; AOH- alternariol; DON- deoxynivalenol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

different glucose conditions. We observed that on the second day, AOH significantly increased the integrity of cells ($^{*}p < 0.05$), and the effect was potentiated by high glucose (Fig. 6E). A similar, but lower effect was observed after 4 days of incubation ($^{*}p < 0.05$) and was lost with the longer incubation. This fact indicates that longer exposure to AOH has no effect on epithelial barrier integrity. In the case of DON, a contradictory effect was observed (Fig. 6F). From the 4th day of incubation on, a statistically significant ($^{*}p < 0.05$) decrease in the intestinal integrity was observed up to 7 days (with the exception of day 6) and also in this case, high glucose potentiated the observed change in TEER value. The modulation of permeability of the intestinal barrier was evaluated at day 7 and for AOH showed no changes (Fig. 6G), whereas for DON presented statistically significant ($^{**}p < 0.01$) increase in the permeability of the monolayer of cells (Fig. 6H), corresponding with its decreased integrity.

3.5. High glucose condition affects the concentration and metabolism of AOH and DON

Lastly, to understand the effect of the high glucose condition on the concentration and metabolism of toxins, LC-MS/MS analysis was conducted both on the apical and basolateral side. First of all, none of the DON metabolites incorporated in our method was detected in any sides of the insert, which might indicate minor or no metabolization of DON in the tested co-culture model and chosen doses. In the case of AOH, metabolites were present; however, due to a lack of standards, the total peak area was calculated and compared. The concentrations of AOH and DON were measured in the apical and basolateral sides of the co-culture model. We observed a statistically significant ($^{***}p < 0.01$) difference in the concentration of AOH in the higher tested dose (20 μmol/L) between normal and high glucose conditions on the basolateral side (Fig. 7A). In the case of DON, a significant decrease in the concentration of mycotoxins was present in the high glucose condition as compared to the normal glucose condition ($^{***}p < 0.001$). In the case of the basolateral side of the insert, we measured a higher concentration of DON than was used in the experiments. This fact might be associated with the multiple additions of mycotoxin as well as the lack of its metabolization, which, in consequence, might result in an accumulation of DON.

The high glucose condition modulated the metabolism of AOH (Fig. 3C, D). We observed a significant decrease in the peak area of AOH sulfates (AOH-S1 and AOH-S2) in the apical side for both concentrations tested ($^{***}p < 0.001$). In the case of AOH-glucuronides (AOH-GlcA1 and AOH-GLcA2) and hydroxy-AOH (OH-AOH), the decrease in concentration was also present, but not significant. In the basolateral side, a similar effect was observed for AOH-S1 (20 and 10 μmol/L) and AOH-S2 (20 μmol/L) as on the apical side. In the case of OH-AOH, the peak was detected only in 10 μmol/L of AOH in normal glucose conditions.

4. Discussion

High glucose condition associated with obesity and diabetes mellitus is one of the risk factors for CRC development and metastases (Luo, Luo, Zhang, et al., 2024). Being overweight and obese are considered a civilization diseases and concern about 30 and 18% of the global human population, respectively (GBD 2015 Obesity Collaborators et al. 2017). On the molecular level, high glucose conditions stimulate the proliferation, migration and invasion of cells and promote the epithelial to mesenchymal transition (EMT) process. It modulates the broad scope of cell signaling molecules (Stefano, Challenger, & Kream, 2016) and changes the sensitivity and response of cells to different stimuli (Zhang, Lu, Abudukeyoumu, et al., 2022). However, to the best of our knowledge, this is the first study in which we observed that high glucose conditions might modulate the response of CRC cells to known mycotoxins, and in which we showed that DON and AOH themselves might modulate the migration and invasion of CRC cells.

The effect of glucose in CRC cells is mediated by both active and facilitated transporters of glucose: SGLT1 and GLUT2 (Helmke, Reisser, Idzkoe, et al., 2004). DON itself was reported to decrease the expression of SGLT1 and GLUT2 in CRC cells (Maresca et al., 2002), and this effect was mainly linked with malnutrition associated with DON. Our results are in line with previous observations and showed that in both tested cell lines DON in a relatively non-toxic concentration of 1 μmol/L caused a significant decrease in protein expression, and the modulation was also present for lower concentrations tested. We also observed that AOH reduced the expression of glucose receptors, and to the best of our knowledge, this is the first study reporting this effect. A similar effect was observed for T-2 toxin, zearalenone (ZEN) (Cheng, Jiang, Huang, et al., 2022), and ochratoxin A (OTA) (Liew & Mohd-Redzwan, 2018), which in consequence indicates that this modulation might be common for mycotoxins and not dependent on the mechanism of their toxicity.

Previous studies showed that DON inhibits the migration and invasion of the CRC cell line HTC116 in a dose of 2.7 μmol/L (800 ng/mL) (Mu, Mu, Zhu, et al., 2020) and induces oxidative stress in HT-29 cells in doses higher than 1 μmol/L (Del Favero, Woelflingseder, Janker, et al., 2018). In our study, we observed that under normal glucose condition, DON decreased the migration and clonogenicity of Caco-2 cells in a dose of 1 μmol/L, and in HT-29, the effect was not observed, indicating that different CRC cells might present different sensitivity to DON. DON was also reported to impair the response of epidermoid carcinoma A-431 cells to physical stress in sub-toxic doses (<1 μmol/L) as well as modulate the adhesion and cytoskeleton protein expression (Del Favero, Woelflingseder, Braun, et al., 2018). Similar to other mycotoxins, DON can induce oxidative stress and apoptosis in different cell lines, however, usually in doses higher than 5 μmol/L (Kowalska, Kozieł, Habrowska-Górczyńska, et al., 2022). However, the intestinal and colon cell lines seem to present different sensitivity as compared to other cell lines. The significant toxicity in porcine intestinal IPEC-J2 cells was reported even after 4 h and the dose of 1 μmol/L (Gu, Guo, Zhao, et al., 2019) and correlated with TEER measurements (Pinton et al., 2009), and in Caco-2 cells the significant decrease was observed for 0.5 μmol/L DON (He, Yin, Dong, et al., 2021). This observation is in line with the results obtained in this study; however, the modulation of the invasiveness potential of cells was much higher than the observed decrease in cell viability. AOH similarly decreased the migration and invasion of both tested cell lines, and in the clonogenicity assay, the effect of AOH was more potent in HT-29 cells. This effect seems to be in line with the known effect of AOH; however, the potential to impact the invasiveness of CRC cells has not been reported yet. AOH was shown to reduce the migration of prostate normal PNT1A cells with down-regulation of MMPs activity (Kowalska, Kozieł, et al., 2021), observed also in CRC cells in this study. A similar effect was observed in mammary breast epithelial cells in which 10 μmol/L of AOH decreased cell migration as well as MMPs activity and expression (Kowalska, Habrowska-Górczyńska, et al., 2021). The

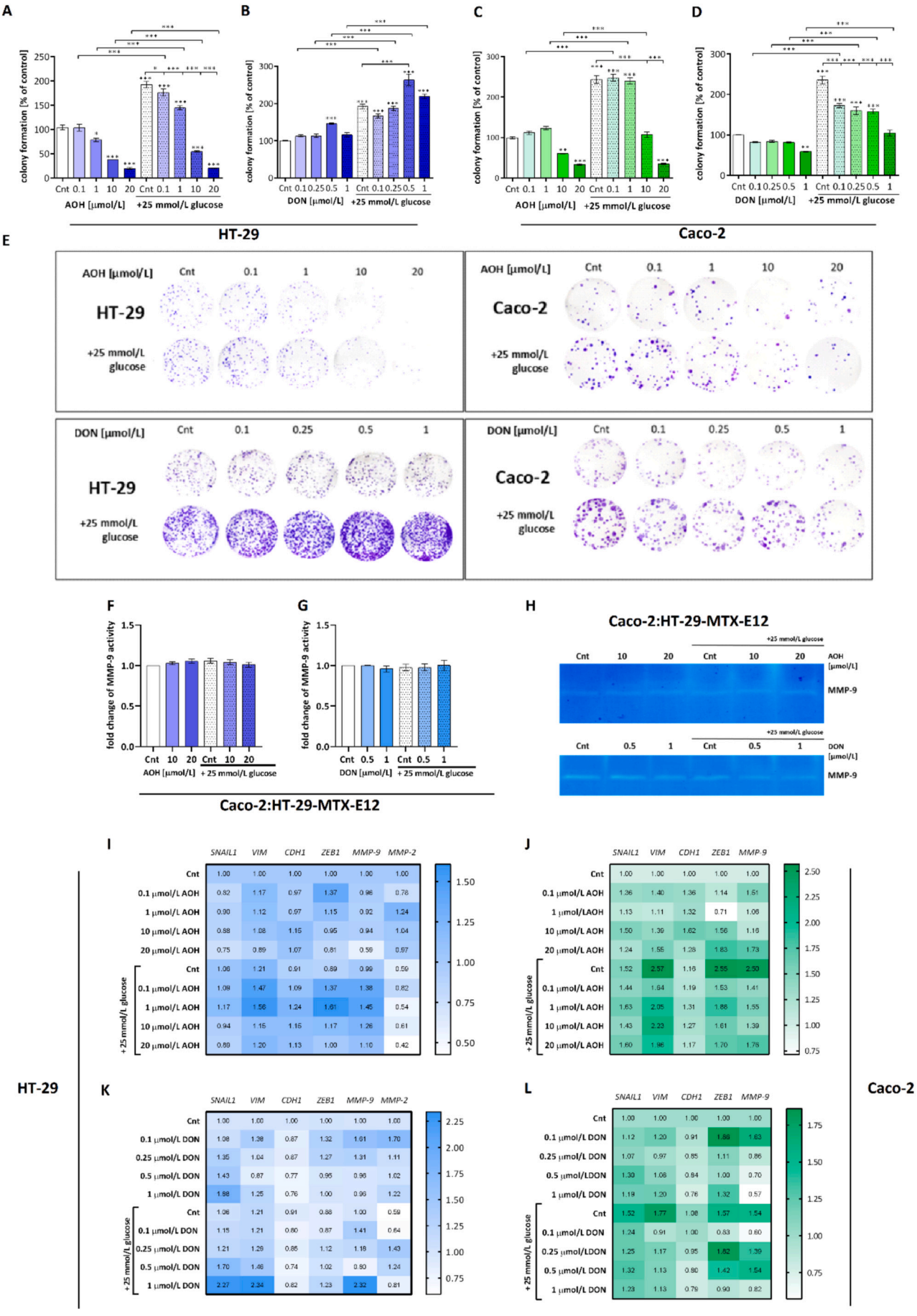

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Fig. 5. AOH and DON modulate the clonogenicity and MMPs activity in CRC cells. The clonogenicity was evaluated with colony formation assay (A-D). Cells were pre-treated with normal or high glucose for 120 h, then cells were seeded in the low number (500 or 1000/ well) and treated with mycotoxins for 48 h. Then the medium was exchanged for normal one and colonies were grown for additional 14 days. Then stained with crystal violet. The representative results of the assay are shown (E). The zymography assay was conducted after 7 days of co-culture experiments (F-G). Representative zymograms (H. The results of the RT-qPCR assay of expression of EMT genes in HT-29 (I, K) and Caco-2 (J, L) cell lines after exposure to 48 h of AOH and DON are presented as a heat map of a fold change of control. *GAPDH* was used as reference gene. The results are expressed as mean ± SE. One-way ANOVA was used for statistical analysis, $p < 0.05$ was considered significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to control (Cnt). In case of significant differences between control high glucose and other high glucose treatments, short brackets were used to present. Cnt- control; AOH- alternariol; DON-deoxynivalenol, *CDH1- E*-cadherin, *VIM*- vimentin, *MMP-2*- metalloproteinase 2, *MMP-9*- metalloproteinase 9, *ZEB1*- zinc finger E-Box binding homeobox 1, *SNAIL1*- Snail family transcriptional repressor 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

modulation of the invasiveness of ovarian cancer cells in a similar way was also observed (Kozieł, Habrowska-Górczyńska, Urbanek, et al., 2023). Thus, it might be stated that AOH is a potential modulator of cancer cells' invasiveness.

In colon HCT116 cells, AOH was reported to decrease the cell viability and induce apoptosis (Bensassi, Gallerne, Sharaf El Dein, et al., 2012) *via* a mitochondria-dependent pathway in concentration higher than 50 μmol/L, indicating relatively low sensitivity. In porcine epithelial cells the induction of oxidative stress as a cause of cytotoxicity was observed in much lower concentration of AOH (5 μmol/L) (Marin, Grosu, Pistol, et al., 2024). The Caco-2 cell line was reported to be quite insensitive to AOH-induced cytotoxicity (above 50 μmol/L) (Fernández-Blanco, Font, & Ruiz, 2014), however, in our study, we observed that 10 and 20 μmol/L of AOH were enough to modulate significantly the migration and clonogenicity of Caco-2 cells. This observation might be explained by the fact that the viability tests measure the metabolic activity of the cells, which might be not changed even with growth inhibition and loss of the proliferative capacity by the cells, thus they are not recommended as standalone evaluation of the cytotoxic effects (Rai, Pathak, Kumari, et al., 2018). The by us and others chosen CRC lines seem to present different sensitivity to tested mycotoxins, which might be explained by a different morphology, gene expression profile, and metabolic ratio, as well as the origin of the samples used to establish the cell line (Berg, Eide, Eilertsen, et al., 2017). One of the possible mechanism explaining the differences in the response of Caco-2 and HT-29 cells might be the fact that as previously reported, the undifferentiated cell lines in comparison to colon-like or differentiated, possess higher EMT signature score and increased expression of tumor growth factor β (TGF-β) induced genes (Berg et al., 2017). HT-29 represents poorly differentiated cell line, whereas Caco-2 are a spontaneously differentiating cell line. This observation might be also explained by a different tumor protein 53 (p53) expression, which HT-29 cells are missing, resulting in the stronger growth arrest and cell death in response to stimulants in Caco-2 cells as compared to HT-29 (Barberi-Heyob, Védrine, Merlin, et al., 2004). HT-29 are also forming more defined colonies, as compared to Caco-2, possibly linked with stronger tight junctions in Caco-2 (Gan, Yanni, & Thakker, 1998). However, the obtained results showed that even low doses of mycotoxins might modulate the basic processes in cancer cells, which play a crucial role in metastasis.

High glucose condition, beside its direct link with promotion of tumor growth, is also reported as a significant modulator of the response of CRC patients to cancer therapies. The studies showed that high glucose decreases the effectiveness of 5-fluorouracil (5-FU) in the CRC *in vitro* model (Ma et al., 2023) and enhances oxaliplatin chemoresistance in CRC stage III patients (Yang, Miao, Huang, et al., 2019). On the other hand, hyperglycemia is one of the side effects in solid tumor therapy, which in turn correlates with infections and non-malignancy-related mortality (Yang, Jia, Qiao, et al., 2016). In this study, we observed that high glucose condition modulates the response of CRC cells to known mycotoxins. It might both potentiate the effect of mycotoxin as observed in the case of AOH, or completely change it, as observed in the case of the effect of DON on the clonogenicity potential of CRC cells. The effect of DON on the obese mice was studied before. Firstly, it was shown that obese mice treated with DON do not gain weight and present a reduced food intake. Moreover, the obesity *per se* did not make mice more sensitive to DON induced weight loss (Amuzie, Flannery, Ulrich, & Pestka, 2011). Flannery et al. observed that obese mice treated with DON did not gain the weight due to reduced food intake and known anorexic effect of DON. Furthermore, this effect is reversible when DON is removed from the diet, and not associated with double-stranded RNA-activated protein kinase (PKR), suggested to be responsible for ribotoxic stress caused by DON (Flannery, He, & Pestka, 2013). This work suggested a different mechanism responsible, which might be the modulation of the glucose receptors, as observed by us. However, this study considered only the weight loss of the animals as an outcome of the exposure to DON, and suggested that the observed effect might be regulated on the hormonal balance (growth hormone axis), which in turn is also a known endocrine disrupting effect of DON (Voss, 2010). Kobayashi-Hattori et al. observed that obese mice treated with DON presented reduced leptin production, which corresponded with reduced body fat in DON-treated mice (Kobayashi-Hattori, Amuzie, Flannery, & Pestka, 2011). These studies did not consider and observe the different toxicity of DON in obese mice, the molecular effect associated with the structure, permeability and motility of the colon was also not taken into consideration so far. However, a similar effect was observed for other mycotoxin in the study conducted by Dopavogui, Régnier, Polizzi, et al. (2023), where obese mice exposed to fumonisin B_1 (FB1) presented lower body mass and glucose levels, hepatic steatosis, and induced inflammation as compared to respective control (Dopavogui et al., 2023). On the other hand, some studies suggested that exposure to mycotoxin aflatoxin M_1 (AFM1) might correlate with diabetes mellitus, based on the correlation of the higher level of detection of AFM1 in diabetes mellitus patients (Akash, Haq, Qader, & Rehman, 2021). However, this observation might be associated with a different aspect: the increased permeability and inflammation in the colon induced by high glucose. Hyperglycemic condition is linked with higher permeability of the colon barrier, modulation of the microbiome, as well as NF-κB-associated inflammation (Zhang, Monnoye, Mariadassou, et al., 2021). High glucose modulates tight junctions in epithelial colon cells and modulates mucus production (Dubois et al., 2023). These results are also in line with previous ones, in which higher levels of FB1 in serum were detected in obese mice (Dopavogui et al., 2023).

In this study, we also observed increased TEER measurements after 14 days of expose of the co-culture model to high glucose, which is in line with previous results; however, we did not observe increased permeability of the colon barrier. This fact might be explained by the complexity of the model used (triple co-culture), which needed an additional matrix, what in turn might interact with the chemicals used. Nevertheless, high glucose potentiated the effect of mycotoxins, especially in an early onset of barrier formation for AOH (4 days) and DON (7 days). Unfortunately, the *in vitro* models of the colon barrier do not exactly mimic the natural conditions in colon due to the lack of the complex interaction between different cell type as well as lack of the physical characteristics; however, they seem to be in line with previous observations. It was reported that hyperglycemia increases trans-epithelial flux in mice, what in turn might increase the susceptibility to bacterial infections (Chávez-Reyes, Escárcega-González, Chavira-Suárez, et al., 2021). However, our results showed that a hyperglycemic condition might also change the susceptibility to mycotoxins-induced

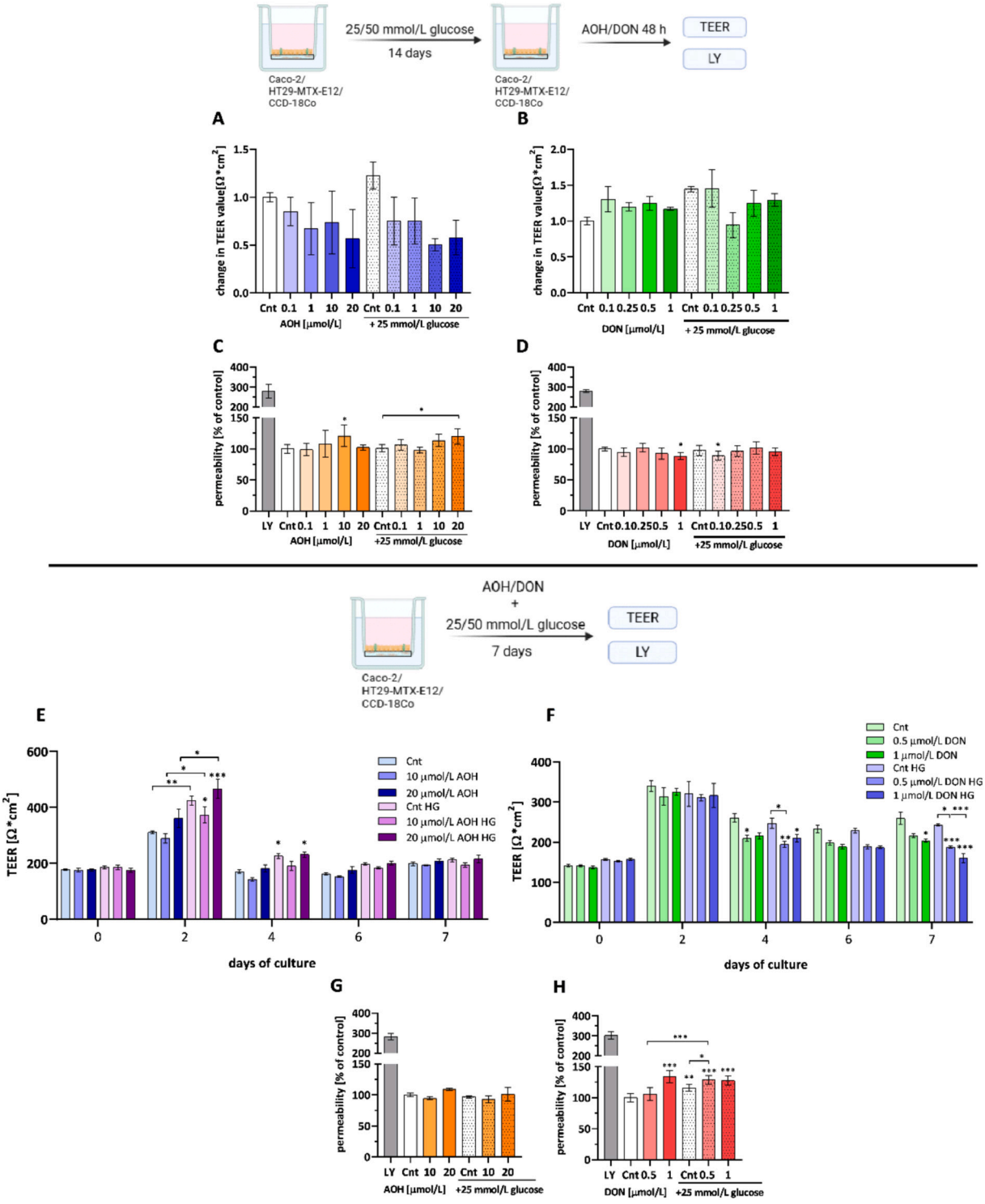

Fig. 6. Modulation of the integrity and permeability of intestinal barrier by AOH and DON in normal and high glucose conditions. TEER measurement results (A, B, E, F) are presented as TEER value (E, F) or change in TEER value (A, B) between day 14th and 16th of treatment. Co-culture model of cell inserts of CCD-18Co/Caco-2:HT-29-MTX-E12 (9:1) cells was used. C, D, G and H present the permeability of monolayer measured with luciferase yellow assay and expressed as % of control sample. The results are expressed as mean ± SE, one way ANOVA was used for statistical analysis, $p < 0.05$ was considered significant, $^{*}p < 0.05$, $p < 0.01$, $^{***}p < 0.001$ as compared to control (Cnt). Cnt - control; AOH - alternariol; DON -deoxynivalenol. The schematic representation of the workflow of the experiments was done in Biorender.com (https://biorender.com/5ragvqt). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

changes in colon cells.

Previous studies have shown that high glucose can modulate the cytotoxicity of xenobiotics (Ma et al., 2023). Less is known about how hyperglycemic conditions might affect the metabolism of environmental pollutants, particularly mycotoxins. To address this, we incorporated the LC-MS/MS method to evaluate the concentrations and metabolic fate of the mycotoxins tested. No metabolites of DON could be detected in our *in vitro* model at the tested doses. In contrast, the phase II metabolite

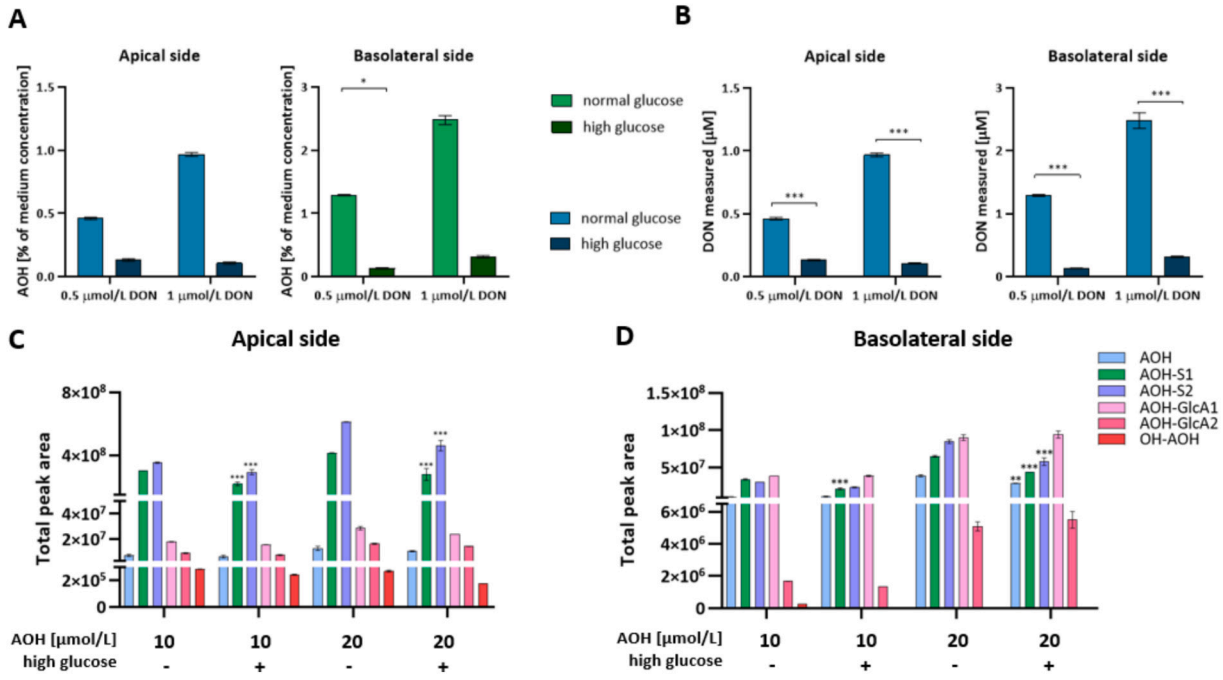

Fig. 7. LC-MS/MS analyses of AOH and DON concentrations and metabolism in normal and high glucose condition. The concentration of AOH (A) and DON (B) measured in the apical and basolateral compartment. The total peak area of AOH metabolites measured in apical and basolateral side (C, D). The co-culture model of CCD-18Co/Caco-2/HT-29-MTX-E1 was used. The high glucose (+25 mmol/L) as well as the mycotoxins were administered for 7 days. The medium was exchanged every second day. The results are expressed and mean ± SE of three biological replicates. One-way ANOVA was used for statistical analysis, $p < 0.05$ was considered significant. ***$p < 0.001$ as compared to corresponding concentration in normal glucose condition. AOH - alternariol, DON - deoxynivalenol, AOH-S1, AOH-S2 - alternariol sulfates, AOH-GlcA1, AOH-GlcA2 - alternariol glucuronides, OH-AOH- hydroxy- alternariol.

DON-3-sulfate (DON-3-S) was previously described in differentiated Caco-2 (Beisl et al., 2021) and HepG2 cells (Flasch, Bueschl, Woelflingseder, et al., 2020); however, at higher DON concentrations (10 µmol/L), which might explain the absence of this metabolite in our assay setup. Previous observations also confirm that more sophisticated intestine models better mimic the exposure of DON, because DON has a greater effect *via* basolateral exposure than the apical (Hanyu, Yokoi, Nakamura, et al., 2020). Moreover, the lack of DON metabolites in our model, as well as the efficient metabolization of AOH is consistent with a previous study in the Caco-2 cell culture model (Groestlinger et al., 2022).

To the best of our knowledge, this is the first study that showed that high glucose conditions might affect the concentration of DON in *in vitro* intestinal cell culture model. A hyperglycemic condition is reported to disrupt the cytochrome P450 (CYP450) enzymes (Davidson, Ballinger, & Khetani, 2016), which play a pivotal role in the metabolism of mycotoxins (Fliszár-Nyúl, Ungvári, Dombi, et al., 2022). The observed effect confirms the hypothesis that a high glucose condition might modulate the absorption and metabolism of mycotoxins in the intestine. The detected higher effect on the concentration of DON measured might also be connected with the fact that high glucose mostly affects the CYP3A4 enzyme (Davidson et al., 2016), which is mostly involved in DON metabolism (Liu, Yang, Guo, et al., 2022), whereas in the case of AOH, CYP1A1 is mainly involved (Pfeiffer, Burkhardt, Altemöller, et al., 2008). However, to confirm this hypothesis, further studies are needed.

In contrast to DON, AOH was efficiently metabolized in our model, yielding hydroxylated, sulfated, and glucuronide-conjugated metabolites. The extent metabolism and the observed products are consistent with previous findings in Caco-2 (Groestlinger et al., 2022) and HT-29 cells (Pfeiffer et al., 2008). In our data, we specifically observed a difference in the concentration of AOH-sulfates between high and low glucose conditions, while glucuronide conjugates and hydroxy-AOH were less affected.

There are indications in the literature that suggest sulfotransferase enzymes (SULTs) to be downregulated in diabetic or high-glucose states. For instance, streptozotocin-induced diabetes leads to dose-dependent suppression (up to ~65%) of hepatic sulfotransferase mRNA (*e.g.*, HST-a) and protein levels in female Sprague-Dawley rats (Runge-Morris & Vento, 1995). These findings support that high glucose-induced suppression of SULT enzymes may have caused the altered levels of AOH-sulfates observed in this study. However, while hyperglycemia-induced downregulation of SULTs is well-documented at the systemic level, translating these findings to intestinal *in vitro* models – particularly CRC cell lines – requires careful interpretation. Additionally, the presence of multiple cell lines expressing different sets of metabolic enzymes in our co-culture introduces additional complexity and may contribute to the differences observed under high glucose state. Therefore, further investigations are needed to elucidate the effect high glucose exerts on the metabolism of AOH, with particular attention on the expression and activity of different metabolic enzymes under varying glucose conditions.

5. Conclusions

To conclude, this is the first study that showed that there are differences in the response of CRC cells to AOH and DON in high and normal glucose conditions, when considering both the migration and invasion of CRC cells, intestinal barrier function, as well as mycotoxin concentrations and metabolism. These results need further studies, especially in more complex *in vitro* and *in vivo* models, to better mimic the intestinal complexity and confirm the hypothesis that high glucose might be a significant player in mycotoxin toxicity.

CRediT authorship contribution statement

Karolina Kowalska: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Eszter Borsos:** Writing – review & editing, Writing – original draft,

Methodology, Investigation. **Elisabeth Varga:** Writing – review & editing, Methodology, Investigation. **Doris Marko:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Karolina Kowalska reports financial support was provided by Polish National Agency for Academic Exchange (NAWA). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.118350.

Data availability

Data will be made available on request.

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