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Original Research Article

Traditional and emerging *Fusarium* mycotoxins disrupt homeostasis of bovine mammary cells by altering cell permeability and innate immune function

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ABSTRACT

High incidence of traditional and emerging *Fusarium* mycotoxins in cereal grains and silages can be a potential threat to feed safety and ruminants. Inadequate biodegradation of *Fusarium* mycotoxins by rumen microflora following ingestion of mycotoxin-contaminated feeds can lead to their circulatory transport to target tissues such as mammary gland. The bovine udder plays a pivotal role in maintaining milk yield and composition, thus, human health. However, toxic effects of *Fusarium* mycotoxins on bovine mammary gland are rarely studied. In this study, the bovine mammary epithelial cell line was used as an in-vitro model of bovine mammary epithelium to investigate effects of deoxynivalenol (DON), enniatin B (ENB) and beauvericin (BEA) on bovine mammary gland homeostasis. Results indicated that exposure to DON, ENB and BEA for 48 h significantly decreased cell viability in a concentration-dependent manner ($P < 0.001$). Exposure to DON at 0.39 μmol/L and BEA at 2.5 μmol/L for 48 h also decreased paracellular flux of FITC-40 kDa dextran ($P < 0.05$), whereas none of the mycotoxins affected transepithelial electrical resistance after 48 h exposure. The qPCR was performed for assessment of expression of gene coding tight junction (TJ) proteins, toll-like receptor 4 (*TLR4*) and cytokines after 4, 24 and 48 h of exposure. DON, ENB and BEA significantly upregulated the TJ protein zonula occludens-1, whereas markedly downregulated claudin 3 ($P < 0.05$). Exposure to DON at 1.35 μmol/L for 4 h significantly increased expression of occludin ($P < 0.01$). DON, ENB and BEA significant downregulated *TLR4* ($P < 0.05$). In contrast, ENB markedly increased expression of cytokines interleukin-6 (*IL-6*) ($P < 0.001$), tumor necrosis factor α (*TNF-a*) ($P < 0.05$) and transforming growth factor-β (*TGF-β*) ($P < 0.01$). BEA significantly upregulated *IL-6* ($P < 0.001$) and *TGF-β* ($P = 0.01$), but downregulated *TNF-α* ($P < 0.001$). These results suggest that DON, ENB and BEA can disrupt mammary gland homeostasis by inducing cell death as well as altering its paracellular permeability and expression of genes involved in innate immune function.
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1. Introduction

Feed supply and safety is crucial to farm animal production systems and any limiting factors of feed security could constrain the production outcome (Bryden, 2012; Pinotti et al., 2016). Mycotoxins have become one of the highest risk factors for feed security worldwide and represent a significant issue to livestock and feed industries (Pinotti et al., 2016; Santos Pereira et al., 2019). They commonly contaminate various commodities of plant origin, especially cereal grains, and therefore are often detected in silage and animal feeds (Bryden, 2012; Jajić et al., 2019; Mannaa and Kim,

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2017; Reisinger et al., 2019; Xu et al., 2020). Consumption of mycotoxin-contaminated feed can lead to adverse effects on animal production, including reduced performance, impaired immunity leading to increased disease susceptibility, and reduced fertility, all contributing to enormous economic losses worldwide (Antonissen et al., 2014; Chilaka et al., 2017; Fouad et al., 2019; Gallo et al., 2020; Pierron et al., 2016; Pinton and Oswald, 2014; Rodrigues, 2014).

Fusarium spp. is one of the predominant mycotoxin-producing genera of filamentous fungi. Deoxynivalenol (DON) is amongst the most frequently detected *Fusarium* mycotoxins in silage (Ogunade et al., 2018; Panasiuk et al., 2019; Reisinger et al., 2019), and adverse weather conditions associated with climate change are expected to increase the occurrence of *Fusarium* mycotoxin contamination in feedstuff (Ji et al., 2019; Liu and Van der Fels-Klerx, 2021). High incidence of the emerging *Fusarium* mycotoxins enniatins, and beauvericin (BEA) in silage has also been reported (Jajić et al., 2019; Khoshal et al., 2019; Křížová et al., 2021; Panasiuk et al., 2019; Reisinger et al., 2019), and evidence of their occurrence is rapidly accumulating (Agriopoulou et al., 2020). Relevant data on the toxicity of these emerging mycotoxins, however, is currently lacking (European Food Safety Authority, 2014). Moreover, the frequent co-occurrence of *Fusarium* mycotoxins in grains and animal feeds (European Food Safety Authority, 2014; Khoshal et al., 2019) makes the understanding of their interactive toxicity challenging.

In general, ruminants have been considered less susceptible than monogastric animals to mycotoxins due to ability of rumen to degrade mycotoxins into less toxic metabolites by rumen microbes (Bertero et al., 2018; Fink-Gremmels, 2008; Rodrigues, 2014; Xu et al., 2020). However, ruminal biodegradation of mycotoxins could be influenced by antimicrobial properties of certain mycotoxins (Bertero et al., 2020; Křížová et al., 2021; Wu et al., 2018), feed composition (Upadhaya et al., 2010), and the consequent changes in the rumen environment characteristics (subacute ruminal acidosis, rumen dysbiosis and redox potential) (Billenkamp et al., 2021; Huang et al., 2018), diseases (Chen et al., 2021; Debevere et al., 2020; Nagaraja and Titgemeyer, 2007), and production stages (Valgaeren et al., 2019). Collectively, these influences suggest that in some circumstances, mycotoxins could by-pass ruminal degradation and be distributed to different tissues via systemic circulation (Debevere et al., 2020; Escrivá et al., 2017), and may therefore exert their toxic effects in different tissues. The mammary gland for example, is likely to encounter with mycotoxins, which can be evidenced by the reported carry-over of *Fusarium* mycotoxins into ruminant milk (Becker-Algeri et al., 2016; Piątkowska et al., 2018; Völkel et al., 2011).

The bovine mammary gland is a highly specialized organ that synthesizes and secrets colostrum and milk. These mammary products are not only critical to calf health and growth, but also serve as an important source of nutrients and bioactives for humans. Maintaining homeostasis of the mammary gland is crucial for its functionality. Since the teat canal is susceptible to microbial invasion, mammary epithelial cells (MECs) act as a barrier against invading environmental pathogens and participate in the innate immune response (Brenaut et al., 2014; Gray et al., 2005; Kessler et al., 2019), which is the first line of defense against intramammary infection (Ezzat Alnakip et al., 2014). Mammary epithelial cells have 2 critical roles in the innate immune response during intramammary infection: (1) they recognize the pathogen-associated molecular pattern (PAMPs) of invading pathogens via pattern-recognition receptors (PRRs), and (2) secrete cytokines (IL-6 and TNF-α) and chemokines that initiate the onset of inflammation, which includes the influx of professional immune cells to clear the invading pathogens (Newton and Dixit, 2012; Rainard and Riollet, 2006; Zhuang et al., 2020). Among the PRRs, Toll-like receptor 4 (TLR4) is of particular importance to mammary gland defense due to its capability to recognize lipopolysaccharide, the PAMP derived from Gram-negative mastitis-causing pathogens (Sordillo, 2018). Although inflammation is a critical component of the innate immune response against infections, an exaggerated or protracted dysfunctional mammary innate immune response could have deleterious effects resulting in uncontrolled acute or chronic mastitis (Sordillo, 2018). Production of anti-inflammatory soluble mediators such as transforming growth factor-β (TGF-β) is one of the mechanisms regulating the innate immune response to maintain immune homeostasis (Bannerman, 2009; Ezzat Alnakip et al., 2014).

Mammary epithelial cells also play a critical role in sustaining optimal milk composition by controlling the exchange of components between blood and milk via paracellular transport (Kessler et al., 2019; Wellnitz and Bruckmaier, 2021; Zhao et al., 2019). Disruption of this homeostatic balance of the mammary gland could result in reduced milk production and quality, which could therefore lead to economic losses for dairy farmers. Effects of *Fusarium* mycotoxins on the bovine mammary gland are rarely documented. Yet, there is evidence indicating MECs play a critical role in initiating immune response in the mammary gland during intramammary infection (Brenaut et al., 2014; Gray et al., 2005).

A wealth of studies using various cell models have previously shown that DON, enniatin B (ENB), and BEA could affect epithelial permeability and immune function (Bertero et al., 2020; Gao et al., 2020; Xu et al., 2020). The aim of this present study was to investigate the effects of DON, ENB and BEA on permeability and innate immune function of MECs by using bovine mammary epithelial (MAC-T) cells.

2. Materials and methods

2.1. Chemicals

DON, ENB and BEA (Sigma-Aldrich) were dissolved in dimethylsulfoxide (DMSO) to stock solutions of 5 mg/mL. All stock solutions were stored at - 20 °C. Mycotoxin working solutions at designated concentrations were prepared in serial dilutions in cell culture medium described below.

2.2. Cell culture

The MAC-T cells were maintained according to the previous protocol (Huynh et al., 1991) in T75 flasks in the medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.0 mmol/L L-glutamine, 10% heat inactivated fetal bovine serum, 25 mmol/L HEPES buffer (Invitrogen), 1% Penicillin/Streptomycin (100 units/mL of Penicillin and 100 μg/mL Streptomycin; Invitrogen) and 1 mmol/L Sodium Pyruvate (Invitrogen). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2, and the culture medium was changed every other day.

2.3. Cytotoxicity assay

MAC-T cells were seeded in 96-well microplates at a density of 2×10^4 cells per well and reached confluence within 24 h. The cells were then treated with DON (0, 0.67, 1.35, 3.4 13.4 μmol/L), ENB (0, 2.5, 5, 20, 100 μmol/L) and BEA (0, 5, 10, 20, 80 μmol/L) for 48 h. The range of exposure concentrations were selected based on previous studies (Diesing et al., 2011; Kolf-Clauw et al., 2013; Olleik et al., 2019) and our preliminary studies (data not shown). According to the references (Li et al., 2016; Oh et al., 2015) with slight modifications, at the end of the exposure, Calcein AM (Invitrogen, CA, USA), a cell-permeant fluorescent dye was added to each well at a

concentration of 2 μmol/L and the plates were incubated at room temperature for 45 min. The fluorescence intensity (FI) of Calcein AM was measured by a microplate reader (BioTek Instruments, VT, USA) at 498/528 nm. The percentage of viable cells was calculated using the following formula:

$$\text{Cell viability (\%)} = \text{Mean}\,(FI_{498\ \text{treated cells}} - FI_{\text{Blank}})/\text{Mean}\,(FI_{498\ \text{untreated cells}} - FI_{\text{Blank}}) \times 100$$

where $FI_{498\ \text{treated cells}}$ is the FI obtained from mycotoxin-treated groups, $FI_{498\ \text{untreated cells}}$ is FI obtained from groups without any mycotoxin treatment, and FI_{Blank} is the background signal resulted from Calcein AM treated wells with no cells. Mean is the average FI of 3 replicates.

The 50% inhibitory concentration (IC_{50}) was also calculated by fitting the data to non-linear Hill equation using GraphPad Prism version 8.2.1 for Windows (GraphPad Software, San Diego, California USA). According to these cell viability data, non-cytotoxic concentrations were chosen for subsequent experiments in an attempt to exclude any changes in experiment endpoints caused by the damage of cell monolayer (Gao et al., 2017).

2.4. Measurement of transepithelial electrical resistance (TEER)

A two-dimension (2-D) MAC-T monolayer model was established according to the reference (Wang et al., 2019a) with slight modifications. MAC-T cells were seeded at the density of 2.5×10^4 cells per Transwell insert (Corning Transwell #3470, 6.5 mm, 0.4 μm pore size) coated with Type I collagen at 10 μg/cm^2 (C3867, Sigma-Aldrich) according to the manufacture's instruction and cultured in the same medium describe above for 33 d based on our preliminary studies to ensure cell monolayer yielding stable TEER readings. The cells were then ready for mycotoxin treatments. Medium was refreshed for both apical and basal compartments of the Transwell inserts every other day (Wang et al., 2019a). The cells were challenged by adding DON (0, 0.39, 0.78 and 1.35 μmol/L), ENB (0, 0.48, 1.25 and 2.5 μmol/L) or BEA (0, 2.5, 5 and 10 μmol/L) at non-cytotoxic concentrations to the apical side. The TEER of MAC-T cell monolayers was measured before the addition of mycotoxins ($TEER_0$) and 48 h after mycotoxin exposure ($TEER_{48}$) (Majima et al., 2017) using Millicell ERS-2 Voltohmmeter (EMD Millipore Corporation, MA, USA) according to the manufacturer's instruction. The change in TEER was expressed as the ratio of $TEER_{48}$ to $TEER_0$, which was calculated according to the following formula (Majima et al., 2017):

$$\text{TEER (\%)} = TEER_{48}\,(\Omega \bullet \text{cm}^2) \,/\, TEER_0\,(\Omega \bullet \text{cm}^2) \times 100.$$

2.5. Permeability tracer flux assay

Fluorescein isothiocyanate (FITC)-dextran (40 kDa molecular weight) (FD-40, Sigma-Aldrich) was dissolved in DMEM/Nutrient Ham's Mixture F-12 (F12) to a final concentration of 1 mg/mL to create the tracer working solution (Solhaug et al., 2015; Sun et al., 2017; Wang et al., 2019a). The 2-D MAC-T monolayer model was established as described above. On d 33 post confluency when stable tight junction was formed, the cells were treated with DON, ENB or BEA for 48 h as described above in Section 2.4. The tracer solution was added to the apical compartment with DMEM/F12 added to the basal compartment. Based on the incubation periods previously described (Chopyk et al., 2017; Gao et al., 2017; Wang et al., 2018; Wang et al., 2019a) and our preliminary studies, after 3 h incubation, the fluorescence intensity of FITC-40 kDa in the basal compartment was measured by a microplate reader (Agilent Technologies formerly BioTek Instruments, VT, USA) at 498/528 nm.

2.6. RNA extraction and quantitative real time-qPCR (qPCR)

MAC-T cells were seeded in 24-well plates in triplicate at a density of 1×10^5 cells per well. After 24 h, cells were treated with DON (0, 1.35 μmol/L), ENB (0, 2.5 μmol/L) and BEA (0, 10 μmol/L) at the highest non-cytotoxic concentrations for 4, 24 and 48 h. Cells were lysed with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions and stored at −80 °C until further RNA extraction. Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen). The quantity and purity (A_{260}/A_{280}) of isolated RNA in the present study were determined using Agilent BioTek Take 3 microvolume plate (Agilent Technologies formally BioTek Instruments, VT, USA). RNA samples with A_{260}:A_{280} ratio between 1.9 and 2.1 were considered useable purified samples and used for further experiments according to the manufacturer's instructions. The integrity of RNA in the present study was determined using agarose gel electrophoresis. Sharp, clear 28S and 18S rRNA bands with their ratio about 2.0 were obtained on a denaturing gel indicated intact total RNA. As previously described (Shandilya et al., 2021), the purified RNA samples were reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Then qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a total reaction volume of 10 μL in a PCR 96-well plate (Applied Biosystems). The polymerase was activated at 95 °C for 10 min and the PCR was performed for 40 cycles (95 °C for 15 s and 60 °C for 1 min) using a StepOne Plus instrument (Applied Biosystems). The primers were either selected based on previous studies (Lee et al., 2019; Shandilya et al., 2021; Wu et al., 2022; Zhao et al., 2016) or designed using Primer3Plus software (Hung and Weng, 2016; Mallikarjunappa et al., 2020). The sequences of the selected primers are listed in Table 1. Primer specificity was checked by sequence alignment using Nucleotide BLAST at the NCBI genome browser gateway, and was further confirmed in a standard PCR reaction followed by ethidium-bromide staining on 2% agarose gel as previously described (Shandilya et al., 2021). The reference genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Sharma et al., 2019) and ubiquitously expressed prefoldin like chaperone (*UXT*) (Wang et al., 2022) were used as the internal control to normalize the expression of the target gene transcript levels. A total of 4 reference genes (*GAPDH, UXT,* β-actin and β2-microglobulin) were tested as internal control genes. *GAPDH* and *UXT* were selected over the other reference genes because they were more stable across all treatment groups. The relative levels of genes were determined using ΔΔCt method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The results were analyzed by ANOVA followed by Dunnett's post-hoc test for multiple mean comparisons using GraphPad Prism version 8.2.1 for Windows (GraphPad Software, San Diego, California USA). Data are presented as mean ± SEM of 3 independent experiments conducted in triplicate, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity of Fusarium mycotoxins

To assess the cytotoxicity of mycotoxins, cell viability of MAC-T cells was determined upon exposure to DON, ENB and BEA for

Table 1
Details of primer sequences, PCR efficiency, amplicon length, accession number of the target and reference genes.

Gene	Primer sequence 5′−3′[1]	PCR efficiency, %	Amplicon length[2], bp	Accession no.
GAPDH	F: GATGGTGAAGGTCGGAGTGAAC	101.711	100	NM_001034034.2
GAPDH	R: GTCATTGATGGCGACGATGT			
UXT	F: TTGACACAGTGGTCCCAGAC	96.603	143	NM_001037471.2
UXT	R: CTTGGTGAGGTTGTCGCTGA			
ZO-1	F: GCGAAATGAGAAACAAGCACC	97.78	121	XM_024982012.1
ZO-1	R: ATGAGTTGAGTTGGGCAGGAC			
Claudin 3	F: AGGGACTGTGGATGAACTGC	108.701	128	NM_205801.2
Claudin 3	R: CAGTAGGATGGCGATGACG			
Occludin	F: GCCAGCATATTCCTTCTACCC	103.487	139	NM_001082433.2
Occludin	R: AAGAGTGGAGGCAACACAGG			
IL-6	F: GGCTCCCATGATTGTGGTAGTT	105.691	523	NM_173923.2
IL-6	R: GCCCAGTGGACAGGTTTCTG			
TNF-α	F: CGGTGGTGGGACTCGTATG	103.751	352	NM_173966.3
TNF-α	R: CTGGTTGTCTTCCAGCTTCACA			
TGF-β	F: CCTGAGCCAGAGGCGGACTAC	99.181	130	NM_001166068.1
TGF-β	R: GCTCGGACGTGTTGAAGAAC			
TLR4	F: GAACAGGTAGCCCAGACAGC	99.35	151	NM_174198.6
TLR4	R: AGGCCATGATACGGTTGAAG			

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; *UXT* = ubiquitously expressed prefoldin like chaperone; *ZO-1* = zonula occludens-1; *TNF-α* = tumor necrosis factor α; *TGF-β* = transforming growth factor-β; *TLR4* = toll-like receptor 4.
[1] F = forward; R = reverse.
[2] bp = base pair.

48 h (Fig. 1). DON, ENB and BEA reduced viability of MAC-T cells in a concentration-dependent manner within the concentration range used in the present study. DON, ENB and BEA significantly decreased cell viability at concentrations greater than 1.35, 2.5 and 10 μmol/L, respectively ($P < 0.001$). The calculated IC_{50} for DON and BEA were 12.87 ± 1.418 μmol/L ($R^2 = 0.93$) and 12.93 ± 0.939 μmol/L ($R^2 = 0.99$), respectively. No IC_{50} was obtained for ENB from the present study. The calculated 25% inhibitory concentrations (IC_{25}) of DON, BEA and ENB were 4.517 ± 0.50, 11.6 ± 0.56 and 62.2 ± 15.45 μmol/L, respectively.

3.2. Transepithelial electrical resistance and paracellular tracer flux

Transepithelial electrical resistance (TEER) and then paracellular flux of 40 kDa dextran of the MAC-T monolayer were measured to assess the effects of mycotoxins on paracellular permeability of mammary epithelium. Based on the cytotoxicity results, the concentrations that did not exhibit cytotoxicity to MAC-T cells were used for each mycotoxin to carry out TEER measurement and the paracellular flux of 40 kDa dextran. As shown in Table 2, exposure to tested mycotoxins for 48 h at the selected concentrations did not alter TEER of MAC-T cell monolayer. With regards to paracellular flux assay (Table 3), after 48 h exposure, DON ($P = 0.02$) and BEA ($P = 0.013$) at their lower concentrations decreased FITC-40 kDa dextran flux across the MAC-T cell monolayer, whereas at higher concentrations, DON and BEA did not have any impact on dextran flux. In contrast, ENB did not alter dextran flux at any concentrations used in the experiment.

3.3. Gene expression analysis

The effects of mycotoxins on the relative mRNA expression of selected tight junction (TJ) proteins (zonula occludens-1 [*ZO-1*], occludin and claudin 3) and immune-related genes (*TLR4, IL- 6,*

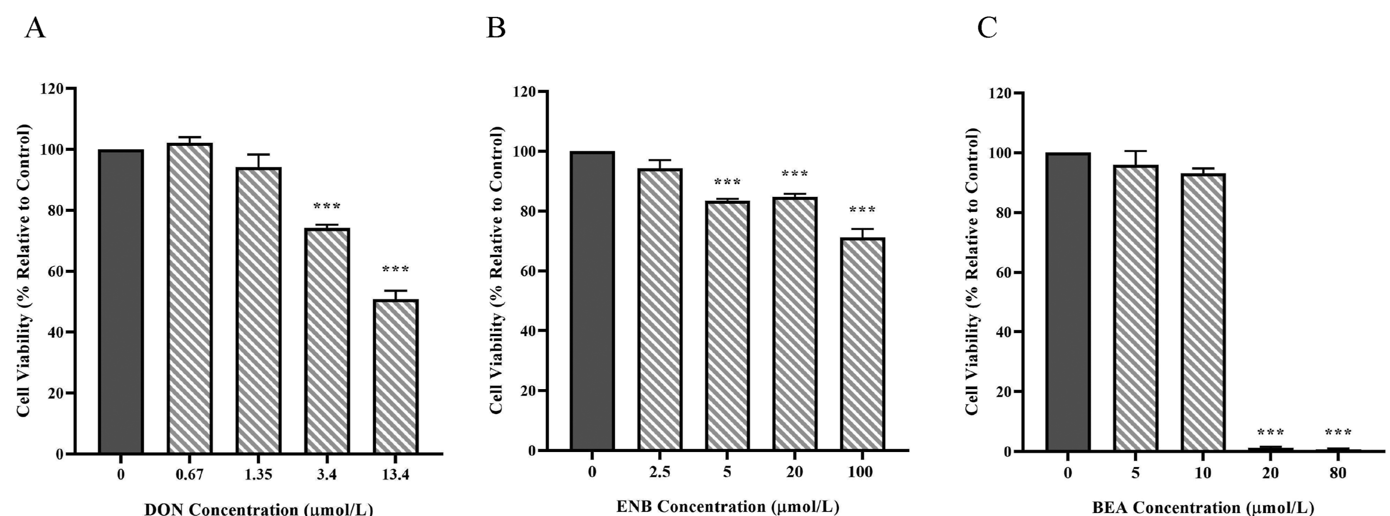

Fig. 1. Effects of (A) deoxynivalenol (DON), (B) enniatin B (ENB) and (C) beauvericin (BEA) on MAC-T cell viability after 48 h exposure, respectively. Results are presented as the percentage of viable cells compared to the untreated control (0 μmol/L); values are the mean ± SEM of 3 independent experiments. Significant differences compared to control are indicated at $P < 0.001$ (***). MAC-T cells = bovine mammary epithelial cells.

Table 2
Effects of *Fusarium* mycotoxins on transepithelial electrical resistance (TEER) of MAC-T cells after 48 h exposure.

Mycotoxin	Treatment, μmol/L	TEER[1], % of initial value	SEM	*P*-value
DON	Untreated	98.47	13.01	0.6609
	0.39	112		
	0.78	105		
	1.35	106.5		
ENB	Untreated	100	5.45	0.2579
	0.48	109.8		
	1.25	104.2		
	2.5	104.9		
BEA	Untreated	98.1	5.70	0.1205
	2.5	99.1		
	5	87.4		
	10	93.27		

DON = deoxynivalenol; ENB = enniatin B; BEA = beauvericin; MAC-T = bovine mammary epithelial cell line.
[1] Results are presented as mean values of 3 independent experiments.

TNF-α, and *TGF-β*) were analyzed in MAC-T cells at 3 time points of mycotoxin exposure (4, 24 and 48 h) by qPCR. The highest concentration for each mycotoxin that did not exhibit cytotoxicity to MAC-T cells (1.35 μmol/L for DON, 2.5 μmol/L for ENB and 10 μmol/L for BEA) were used in this experiment. Results demonstrated that DON, ENB and BEA significantly affected mRNA expression of the studied genes. As shown in Fig. 2 for example, mRNA expression of *ZO-1* was significantly upregulated after exposure to DON at 1.35 μmol/L for 4 h, 24 h and 48 h ($P < 0.05$) compared to control groups without DON treatment. Similarly, mRNA expression of *ZO-1* was upregulated by ENB at 2.5 μmol/L and by BEA at 10 μmol/L but only at 24 h ($P < 0.05$). DON also significantly upregulated mRNA expression of occludin at 4 h ($P = 0.003$). Messenger RNA expression of occludin exhibited a trend towards increasing ($P = 0.052$) after exposure to DON for 48 h. In contrast to *ZO-1* and occludin, claudin 3 mRNA expression was significantly downregulated by DON at 1.35 μmol/L at 24 h and 48 h ($P < 0.001$), respectively. At 48 h, claudin 3 mRNA expression was also significantly downregulated by 2.5 μmol/L ENB ($P < 0.001$) and 10 μmol/L BEA ($P = 0.02$).

We also analyzed mRNA expression of pro- and anti-inflammatory cytokines as well as *TLR4* to assess the potential effects of DON, ENB and BEA on the innate immune function of MECs. Results showed that expression of immune-related genes was differentially altered by tested mycotoxins at different timepoints (Fig. 3). *IL-6* expression was significantly upregulated by 10 μmol/L BEA at 4 h ($P < 0.001$), and by 2.5 μmol/L ENB at 24 h and 48 h ($P < 0.001$). Whereas DON at 1.35 μmol/L did not affect *IL-6* mRNA expression at any timepoints. We also observed a significant upregulation of *TNF-α* by 2.5 μmol/L ENB at all 3 timepoints ($P < 0.05$). In contrast, significant downregulation of *TNF-α* expression was observed after exposure to BEA at 10 μmol/L for 48 h ($P < 0.001$). The expression of *TGF-β* was significantly upregulated by ENB at 24 and 48 h ($P < 0.01$) as well as by BEA at 24 h ($P = 0.01$). With regards to *TLR4*, expression was significantly downregulated by DON and BEA at 4 h ($P < 0.01$) and by ENB ($P = 0.04$) and BEA ($P = 0.008$) at 48 h. There were no significant changes on mRNA expressions of *TLR4* caused by any tested mycotoxins at 24 h.

Table 3
Effects of *Fusarium* mycotoxins on paracellular flux of FITC-40 kDa dextran in MAC-T cells after 48 h exposure.

Mycotoxin	Treatment, μmol/L	FITC-dextran flux[1], % relative to untreated control	SEM	*P*-value
DON	Untreated	100	5.29	0.0122
	0.39	84.8*		
	0.78	92.8		
	1.35	103		
ENB	Untreated	100	5.04	0.5439
	0.48	90.17		
	1.25	93.3		
	2.5	110		
BEA	Untreated	100	5.85	0.0285
	2.5	81.73*		
	5	94.4		
	10	93.1		

DON = deoxynivalenol; ENB = enniatin B; BEA = beauvericin; MAC-T = bovine mammary epithelial cell line.
[1] Results are presented as mean values of 3 independent experiments. Significant differences compared to control (untreated) are indicated at $P < 0.05$ (*).

4. Discussion

Maintaining udder health is of utmost importance for production of quality milk, and therefore food security and human well-being. This present study was designed to investigate the effects of frequently detected *Fusarium* mycotoxins, specifically DON, ENB and BEA on paracellular permeability and innate immune function of bovine mammary epithelium using the MAC-T cell line as in vitro model. Epithelial cell lines have been widely accepted as in vitro models for mycotoxin toxicity testing (Bertero et al., 2020; Xu et al., 2020), and MAC-T cells are widely used cell line to help address basic questions related to mammary gland physiology (Mitz and Viloria-Petit, 2019; Silva et al., 2017; Wang et al., 2019b; Zhang et al., 2018). To our best knowledge, this study is the first in vitro study to simultaneously investigate 2 important functions of MECs, barrier and immunological functions, in response to mycotoxins. Our study enhances the understanding of the effects of traditional and emerging *Fusarium* mycotoxins on the bovine mammary gland epithelium.

Alveolar MECs are the fundamental milk secretory units of mammary gland (Fu et al., 2020; Montalbetti et al., 2014). The number of alveolar MECs predetermines the amount of milk produced by lactating animals (Akers and Nickerson, 2011; Capuco and Ellis, 2013). These MECs also form an epithelial barrier between blood and milk during lactation, and damage to this barrier resulting in cell loss can lead to sub-optimal milk composition and production (Wellnitz and Bruckmaier, 2021). This barrier also plays a critical defensive role against microbial invasion and is actively involved in the innate immune response during intramammary infection (Newton and Dixit, 2012; Rainard et al., 2008).

To assess the effects of DON, ENB and BEA on MAC-T cell viability, we performed cell viability assays using Calcein AM, a cell-permeant dye that is frequently used to determine cell viability in eukaryotic cells (Li et al., 2016, 2018; Oh et al., 2015). In the present study, we observed a concentration-dependent decrease in cell viability after 48 h exposure to DON, ENB and BEA. These observed cytotoxic effects were expected and in line with other epithelial studies (Lei et al., 2017; Olleik et al., 2019; Wang et al., 2019b). Based on the calculated IC_{50} and IC_{25} values, the rank of toxicity of tested mycotoxins to MAC-T cells was DON > BEA > ENB, which was consistent with previous in vitro studies using other epithelial cell models (Fraeyman et al., 2018; Olleik et al., 2019; Ruiz et al., 2011). Our cell viability results suggested that exposure to DON, ENB and BEA at certain concentrations could potentially lead to perturbation of mammary gland function by inducing the loss of milk secreting cells.

Maintaining the mammary gland tissue barrier between the alveolar lumen and interstitium is essential to maternal lactogenesis, subsequent galactopoiesis and milk secretion (Markov

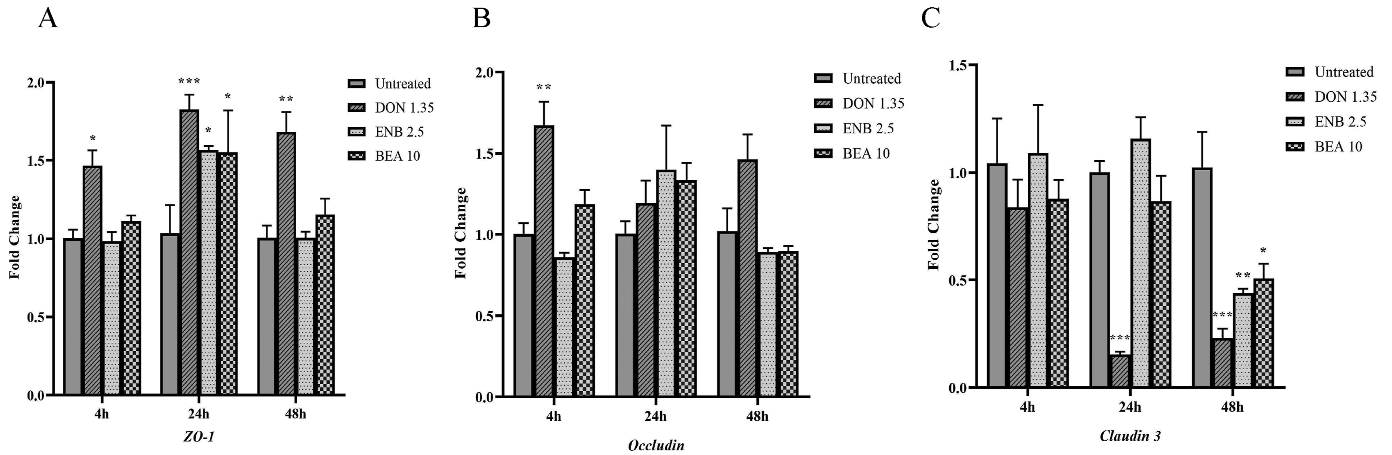

Fig. 2. Effects of DON at 1.35 μmol/L (DON 1.35), ENB at 2.5 μmol/L (ENB 2.5) and BEA at 10 μmol/L (BEA 10) on mRNA expression of tight junction proteins (A) *ZO-1*, (B) occludin and (C) claudin 3 in MAC-T cells after 4, 24 and 48 h exposure, respectively. Results are presented as fold change and control groups (untreated) of each timepoint was used as calibrators; values are the mean ± SEM of 3 independent experiments. Significant differences compared to control (untreated) are indicated at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), respectively. DON = deoxynivalenol; ENB = enniatin B; BEA = beauvericin; MAC-T = bovine mammary epithelial cell line.

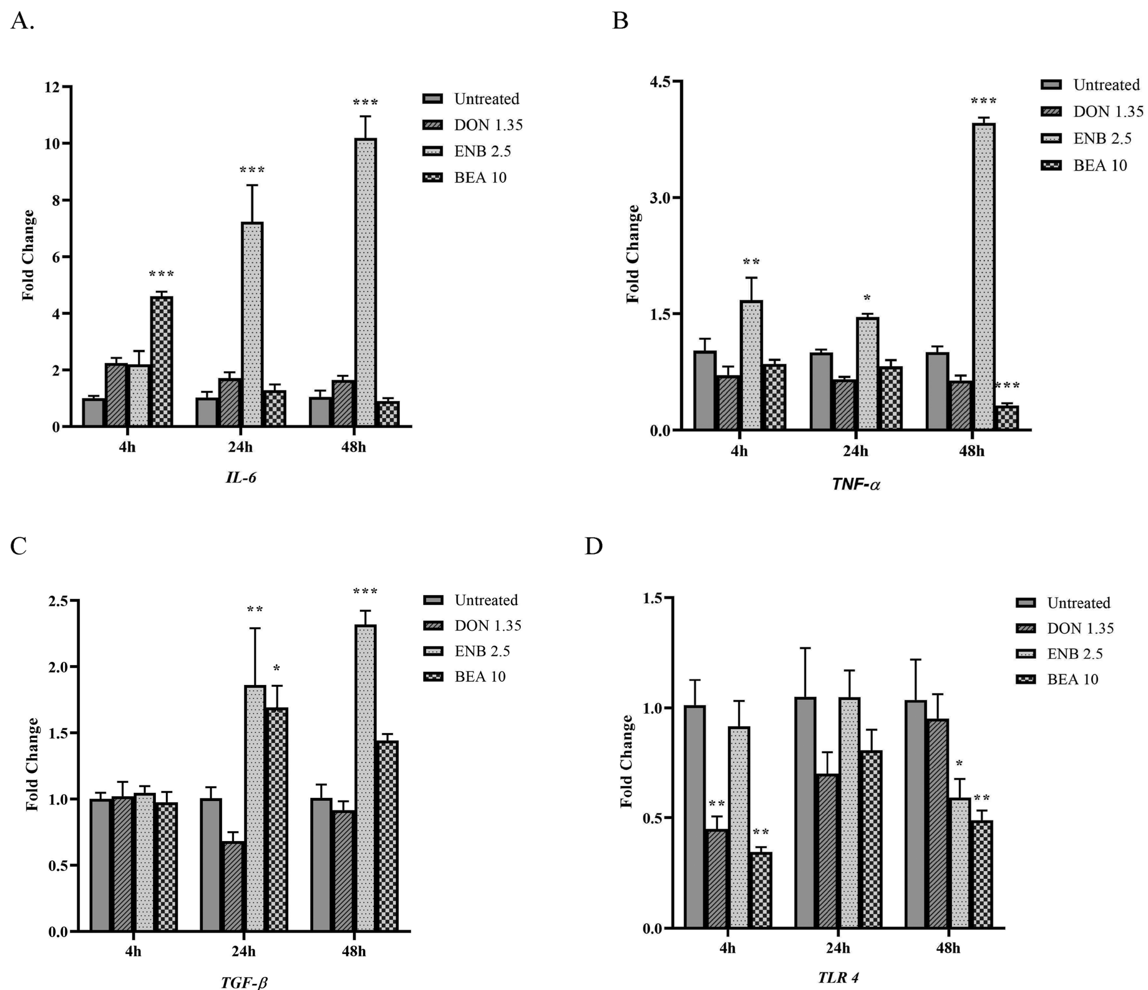

Fig. 3. Effects of DON at 1.35 μmol/L (DON 1.35), ENB at 2.5 μmol/L (ENB 2.5) and BEA at 10 μmol/L (BEA 10) on mRNA expression of immune-related genes (A) *IL-6*, (B) *TNF-α*, (C) *TGF-β* and (D) *TLR4* in MAC-T cells after 4, 24 and 48 h exposure, respectively. Results are presented as fold change; control groups (untreated) of each timepoint were used as calibrators; values are the mean ± SEM of 3 independent experiments. Significant differences compared to control (untreated) are indicated at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), respectively. DON = deoxynivalenol; ENB = enniatin B; BEA = beauvericin; MAC-T = bovine mammary epithelial cell line.

et al., 2017; Montalbetti et al., 2014), and for preventing microbial invasion (Ezzat Alnakip et al., 2014). The integrity of this barrier during lactation is primarily determined by TJ proteins, which connect adjacent secretory epithelial cells. During lactation, TJs become highly impermeable via the paracellular route to prevent paracellular transport of ion and small molecules (Markov et al., 2017; Stelwagen and Singh, 2014), and closure of TJs plays a pivotal role in maintaining bovine milk composition and yield. In this study, TEER measurement and paracellular tracer flux using FITC-40 kDa dextran were performed to investigate effects of tested mycotoxins on paracellular permeability of MAC-T cells. Although both measurements are the indicators of paracellular permeability, they reflect different mechanisms of paracellular route and may have differing sensitivities to mycotoxin exposure. TEER manifests ionic conductance of the paracellular pathway, whereas measurement of flux FITC-dextran reflects paracellular water flow and the pore size of the TJs (Zucco et al., 2005). Based on the previous results, we selected non-toxic concentrations for each mycotoxin in an attempt to exclude any change in paracellular permeability caused by the damage of cell monolayer. To this end, any observed alteration in paracellular permeability will be elucidated as the result of mycotoxin exposure. Decreased TEER values, or increased paracellular flux, is suggestive of increased MEC paracellular permeability. It is well documented that effects of different mycotoxins on TEER of various epithelial cell models were commonly dependent on concentration and exposure duration (Alizadeh et al., 2019; Luo et al., 2019; Springler et al., 2016a; Wang et al., 2019a; Weidner et al., 2013). In the present study, we did not observe any significant TEER modulation by DON, ENB or BEA within the concentrations and exposure duration used. Springler et al. (2016b) previously reported DON did not significantly decrease TEER values of differentiated porcine intestinal epithelial cells (IPEC-J2) at concentrations below 5 μmol/L, which was comparable to the low DON concentrations used in the present study. ENB and BEA were previously found to decrease IPEC-J2 cell TEER reading after 48 h exposure at comparable concentrations to the ones used in the present study (Springler et al., 2016a). Variation in the reported results could be attributed to different cell types, which was documented in previous work (Pinton et al., 2009).

In contrast, a significant decrease in FITC-40 kDa dextran flux was observed in the paracellular tracer flux assay at the lowest concentrations of DON and BEA, and there was a trend towards decreasing at the lowest concentration of ENB. Consistently, DON has been reported to decrease paracellular flux of human intestinal epithelial cells (Caco-2) (Wang et al., 2019a). This phenomenon could be explained as "hormesis", which is defined as a phenomenon in which exposure to a low dose of a chemical agent or environmental factor that is damaging at higher doses gives adaptive beneficial effects to cells or living organisms, and it is indeed considered an adaptive compensatory process following an initial disruption in homeostasis (Mattson, 2008). Detection of such low-dose stimulatory effects is highly dependent on many study design features (Calabrese, 1999). Hormesis has been established in several research fields (Bhakta-Guha and Efferth, 2015; Calabrese and Blain, 2005; Calabrese and Mattson, 2017; Li et al., 2019), and such hormesic effects have also been observed in other experimental endpoints after exposure to DON (Diesing et al., 2011; Liu et al., 2020; Maresca et al., 2008; Pietsch et al., 2011; Razafimanjato et al., 2011) and the *Fusarium* mycotoxin zearalenone (ZEN) (Ranzenigo et al., 2008). Yet, toxicity data on BEA and ENB are limited in the literature as mentioned earlier. Consequently, our study demonstrated that exposure to DON, ENB and BEA at lower concentrations could potentially disrupt the homeostasis of MECs.

We next investigated the effects of mycotoxins on membrane integrity at the gene level by performing qPCR for assessing the expression of the TJ proteins, *ZO-1*, occludin and claudin 3. Our results showed that gene expression of *ZO-1*, occludin and claudin 3 was differentially modulated in response to mycotoxin exposure: *ZO-1* being upregulated, whereas claudin 3 was downregulated and occludin expression was unaffected overall. This suggests TJ gene networks could be affected by tested mycotoxins. It could also be suggestive of different response mechanism of MAC-T cells to cope with the presence of different mycotoxins. Such mycotoxin-induced differential expression of TJs have also been previously reported for other epithelial cell models (Liao et al., 2017; Ling et al., 2016). By correlating the results between TEER and paracellular flux, as well as TJ protein expression, our study indicated that DON at 1.35 μmol/L altered the gene expression of selected TJ proteins without altering MAC-T cell permeability. Although we did not assess their protein expression levels, these changes in gene expression suggested that other TJ proteins could play a role in MAC-T cell paracellular permeability upon DON exposure. For example, Wang et al. (2019a) found that Claudin 4 was the core TJ protein to Caco-2 cell permeability upon DON exposure. In contrast, in the present study, the unaltered permeability upon ENB and BEA exposure was in accordance with unaffected mRNA expression of *ZO-1* and occludin. Unchanged permeability, however, might not be attributed exclusively to these 2 TJ proteins, as TJ complexes are also composed of different co-localizing proteins (Stelwagen and Singh, 2014). Our results indicated that DON, ENB and BEA differentially altered TJ networks of MECs.

Immunocompetence in mammary gland determines the ability of mammals to resist the establishment of intramammary infection, which is one of the leading causes of economic losses for dairy farmers. The qPCR for analysis of *TLR4, IL-6, TNF-α and TGF-β* was performed to investigate the effects of DON, ENB and BEA on the competence of innate immune response capacity of the mammary gland. We observed that exposure to DON, ENB and BEA at non-cytotoxic concentrations decreased mRNA expression of *TLR4* at selective timepoints (4 and 48 h), suggesting possible immunoregulatory effects of these mycotoxins, and raising the possibility of reduced ability to detect Gram-negative bacteria during intramammary infection. Studies investigating the effects of the tested mycotoxins on *TLR4* in epithelial cells are limited. However, a downregulation of *TLR4* in porcine alveolar macrophage cells was reported after DON exposure (Liu et al., 2020). Previously, a study using IPEC-J1 cells also reported that exposure to 25 μmol/L ZEN decreased mRNA expression of *TLR4* (Taranu et al., 2015).

After PAMPs are recognized by PRRs expressed on MECs, PRR-PAMP ligation initiates a signaling cascade that leads to the secretion of soluble mediators such as cytokines that trigger the onset of inflammation. Therefore, we next assessed the effects of DON, ENB and BEA on mRNA expression of *IL-6*, *TNF-α* and *TGF-β*. The pro-inflammatory cytokines IL-6 and TNF-α are the main effectors to initiate the innate immune response at both local and systemic levels (Bougarn et al., 2011; Rainard et al., 2008), whereas TGF-β functions as an anti-inflammatory cytokine to dampen the inflammation (Bannerman, 2009; Ezzat Alnakip et al., 2014). Elevated levels of IL-6, TNF-α and TGF-β have been found to be associated with experimentally-induced inflammation in the bovine mammary gland (Bannerman et al., 2006; Chockalingam et al., 2005; Kauf et al., 2007; Bannerman, 2009). In the present study, ENB was a potent inducer of inflammation in MAC-T cells compared to DON and BEA, as indicated by the marked upregulation of *IL-6* and *TNF-α* gene expression, in company with significantly increased expression of *TGF-β*. Previously, the induction of inflammation by ENB was also observed in a murine monocyte–macrophage cell line (Gammelsrud et al., 2012).

Interestingly, we found that BEA upregulated expression of *IL-6* at 4 h exposure, but decreased expression of *TNF-α* at 48 h exposure, which indicated different BEA-induced kinetics of *IL-6* and *TNF-α* gene expression. It also suggested that BEA could exert time-dependent immunomodulatory effects like the biphasic time-effect relationships defined recently (Li et al., 2019). An immunostimulatory effect of BEA was previously observed in female mice (Maranghi et al., 2018), whereas its immunosuppressive property has been previously reported in various cell lines (Wätjen et al., 2014; Yoo et al., 2017).

DON-induced changes in mRNA expression of *IL*-6 (Liu et al., 2020; Wan et al., 2013; Yu et al., 2021; Zhang et al., 2020), *TNF-α* (Liu et al., 2020; Wang et al., 2019b; Zhang et al., 2020) and *TGF-β* (Liu et al., 2020) have been previously observed in various cell models. However, in the present study, DON did not change mRNA expression of *IL-6*, *TNF-α* and *TGF-β*. Similarly, Zhang et al. also did not observe changes in IPEC-J2 cell *IL-6* and *TNF-α* mRNA expression by DON (Zhang et al., 2020). Such inconsistent findings could be attributed to concentration- and duration-dependent exposure to DON that have been reported in previous work (Pestka, 2008; Pietsch et al., 2015; Wang et al., 2019b).

5. Conclusion

High incidence of *Fusarium* mycotoxins occurs in cereal grains and silage, the common ingredients for ruminant feeds. Inadequate biodegradation of these mycotoxins by rumen microflora can lead to the interaction between bovine mammary gland and mycotoxins, and they may elicit their toxic effects and disrupt mammary gland homeostasis. In this study, DON, ENB and BEA at their lower concentrations were found to differentially disrupt paracellular permeability of MAC-T. The studied mycotoxins could also potentially lead to the disruption in immune homeostasis by reducing mammary gland ability to recognize pathogen and altering cytokine profiles in the mammary gland microenvironment.

Author contributions

Ran Xu: conceptualization, methodology, validation, formal analysis, writing—original draft preparation; **Umesh K. Shandilya:** methodology, validation, formal analysis, project administration, writing—review and editing; **Alexandros Yiannikouris:** validation, writing—review and editing; **Niel A. Karrow:** conceptualization, validation, funding acquisition, supervision, writing—review and editing.

Availability of data and materials

The datasets used and analyzed during the current study available from the corresponding author upon request.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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