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# 1 **A novel toxic effect of foodborne trichothecenes: the exacerbation of genotoxicity**

2

3 Marion Garofalo<sup>a,b</sup>, Delphine Payros<sup>a,b</sup>, Marie Penary<sup>b</sup>, Eric Oswald<sup>b,c</sup>, Jean-Philippe  
4 Nougayrède<sup>b,T</sup>, Isabelle P. Oswald<sup>a,T\*</sup>.

5

6 <sup>a</sup>Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-  
7 Purpan, UPS, Toulouse, France

8 <sup>b</sup>IRSD, Université de Toulouse, INSERM, INRAE, ENVT, UPS, Toulouse, France

9 <sup>c</sup>CHU Toulouse, Hôpital Purpan, Service de Bactériologie-Hygiène, Toulouse, France

10

11 <sup>T</sup>Co senior author

12 \*Corresponding authors: [isabelle.oswald@inrae.fr](mailto:isabelle.oswald@inrae.fr);

13 Keywords: trichothecenes; genotoxins; colibactin; DNA damage.

14

## 15 **ABSTRACT**

16 Trichothecenes (TCTs) are very common mycotoxins. While the effects of DON, the most  
17 prevalent TCT, have been extensively studied, less is known about the effect of other TCTs.  
18 DON has ribotoxic, pro-inflammatory, and cytotoxic potential and induces multiple toxic  
19 effects in humans and animals. Although DON is not genotoxic by itself, it has recently been  
20 shown that this toxin exacerbates the genotoxicity induced by model or bacterial genotoxins.  
21 Here, we show that five TCTs, namely T-2 toxin (T-2), diacetoxyscirpenol (DAS), nivalenol  
22 (NIV), fusarenon-X (FX), and the newly discovered NX toxin, also exacerbated the DNA  
23 damage inflicted by various genotoxins. The exacerbation was dose dependent and observed  
24 with phleomycin, a model genotoxin, captan, a pesticide with genotoxic potential, and  
25 colibactin, a bacterial genotoxin produced by the intestinal microbiota. For this newly described  
26 effect, the TCTs ranked in the following order: T-2>DAS>FX>NIV≥DON≥NX. The genotoxic  
27 exacerbating effect of TCTs correlated with their ribotoxic potential, as measured by inhibition  
28 of protein synthesis. In conclusion, our data demonstrate that TCTs, which are not genotoxic  
29 by themselves, exacerbate DNA damage induced by various genotoxins. Therefore, foodborne  
30 TCTs could enhance the carcinogenic potential of genotoxins present in the diet or produced  
31 by intestinal bacteria.

32

## 33 **KEY WORDS**

34 mycotoxins, pesticides, microbiota, colibactin, carcinogenic potential

35

36

## 37 INTRODUCTION

38 Mycotoxins are the most prevalent natural dietary toxins and contaminate up to 70% of  
39 global crop production (Eskola *et al.*, 2020). They represent a major issue for food safety  
40 (Payros *et al.*, 2021a). These secondary metabolites produced by microscopic fungi are resistant  
41 to industrial processes and cooking and contaminate finished processed food. Trichothecenes  
42 (TCTs) are one of the most prevalent classes of mycotoxins, comprising over 200 structurally  
43 related compounds with a common sesquiterpenoid skeleton (Polak-Śliwińska *et al.*, 2021). The  
44 differences in their substitution patterns allow TCTs to be classified into four subgroups. Type  
45 A TCTs (TCTs-A) and type B TCTs (TCTs-B) are major food contaminants whereas type C  
46 and D TCTs rarely occur in food matrices. Type D TCTs have attracted more attention as indoor  
47 pollutants (Gottschalk *et al.*, 2008). TCTs-A include T-2 toxin (T-2), diacetoxyscirpenol  
48 (DAS), and the newly discovered form NX (Varga *et al.*, 2015; Pierron *et al.*, 2022). TCTs-B,  
49 which are distinguished from TCTs-A by the presence of a ketonic oxygen at C-8, are mainly  
50 represented by deoxynivalenol (DON), nivalenol (NIV) or fusarenon-X (FX) (Figure 1). In  
51 Europe, almost 50% of cereals are contaminated with DON (Knutsen *et al.*, 2017a), 16% with  
52 NIV (Knutsen *et al.*, 2017b), and 10% with FX (Schothorst *et al.*, 2003). T-2 was detected in  
53 20% of European cereal samples (Knutsen *et al.*, 2017c) and DAS in 1.5% of European cereals  
54 and cereal-based food (Knutsen *et al.*, 2018).

55 The toxicity of DON, the most prevalent foodborne TCT, is well documented. Acute DON  
56 poisoning causes vomiting, nausea, and diarrhoea, while chronic exposure results in food  
57 refusal, anorexia, reduced body weight gain, and altered immune responses (Terciolo *et al.*,  
58 2018; Pinton and Oswald, 2014; Payros *et al.* 2016). At the cellular level, DON triggers  
59 ribotoxicity signalled by protein translation arrest and recruitment of MAP kinases, resulting in  
60 inflammation, cytotoxicity and apoptosis, depending on the dose and duration of exposure  
61 (Payros *et al.*, 2016; Alassane-Kpembi *et al.*, 2013; Payros *et al.*, 2021b). By contrast, other  
62 TCTs are largely overlooked in toxicology studies (Seeboth *et al.*, 2010; Alassane-Kpembi *et*  
63 *al.*, 2017a; Alassane-Kpembi *et al.*, 2017b; Pierron *et al.*, 2022). Additional studies are needed,  
64 both because these TCTs are widely distributed in food, and because they induce not only  
65 effects similar to DON, such as ribotoxicity, cytotoxicity, inflammation, vomiting and food  
66 refusal, but also specific effects. For example, T-2 induces a potent oral irritation effect with  
67 skin blistering (Wyatt *et al.*, 1973), FX exhibits potent antiviral properties (Tani *et al.*, 1995),  
68 DAS induces intestinal cell hyperplasia (Weaver, 1981), NIV triggers murine dendritic cells

69 necrosis not documented for other TCTs (Luongo *et al.*, 2010) and NX specifically targets the  
70 mitochondria (Soler *et al.*, 2022). The differences between TCTs are also at the molecular level,  
71 with differences in the translation step inhibited. Although all TCTs are thought to inhibit  
72 peptide elongation (Foroud *et al.*, 2019), T-2, DAS, NIV, and FX also inhibit the initiation step,  
73 and DON and FX also inhibit translation termination (Cundliffe *et al.*, 1977).

74 DON, which is not genotoxic on its own, has recently been described as capable of  
75 increasing the genotoxicity induced by model or bacterial genotoxins (Payros *et al.*, 2017,  
76 Garofalo *et al.*, 2022). This effect is observed with genotoxins with different modes of action,  
77 and a role for ribotoxicity has been proposed (Garofalo *et al.*, 2022). In this work, we show that  
78 the genotoxicity exacerbation is not only an effect of DON, but also of T-2, DAS, NIV, FX,  
79 and NX. Importantly, TCTs do not only exacerbate the genotoxicity induced by a model  
80 genotoxin, but also the genotoxicity induced by captan, a pesticide contaminating the food, and  
81 the genotoxicity induced by colibactin, a genotoxin produced by *Escherichia coli* bacteria in  
82 the gut. Thus, although TCTs are not genotoxic, they could enhance the carcinogenic potential  
83 of genotoxins present in the diet or in our microbiota.

84

## 85 **METHODS**

86 **Toxins and reagents.** DON, NIV, T-2, FX, and DAS were purchased from Sigma-Aldrich  
87 (Saint-Quentin Fallavier, France) and Captan by Dr. Ehrenstorfer (GmbH, Germany/CIL-  
88 Cluzeau). Phleomycin (PHM) (13.78 mM) was purchased from Invivogen (Toulouse, France).  
89 NX, obtained following the methods described by Aitken *et al.*, was a generous gift from D. J.  
90 Miller (Aitken *et al.*, 2019). Stock solutions were stored at -20°C. DON (5 mM), NIV (30 mM),  
91 T-2 (5 mM), FX (10 mM), DAS (3mM), and captan (50 mM) were dissolved in DMSO; NX  
92 (5mM) was dissolved in water.

93

94 **Cell treatments.** Non-transformed rat intestinal epithelial cells (IEC-6, ATCC CRL-1592)  
95 were cultured in complete DMEM medium supplemented with 10% foetal calf serum, 1% non-  
96 essential amino acids (Fisher scientific, Hampton, USA), and 0.1 U/mL bovine insulin (Sigma-  
97 Aldrich), at 37°C with 5% CO<sub>2</sub>. The cells were split regularly to maintain exponential growth.  
98 A fresh culture was started from a liquid nitrogen stock every 30 passages. The cells were  
99 confirmed free of mycoplasma contamination by 16S PCR. For viability assay, cells were  
100 seeded in white 96 well plates (Dutscher, Bruxelles, Belgium) and grown to reach ~80%  
101 confluence. Cells were then treated for 24 h with various doses of TCTs (or DMSO vehicle)  
102 before viability was measured. For ribotoxicity and genotoxicity measurement, cells were

103 seeded in black 96 well plates (Greiner bio-one, Les Ulis, France) and grown to reach ~80%  
104 confluence. For ribotoxicity measurement, cells were incubated for 4 h with various doses of  
105 TCTs or DMSO vehicle followed by a 30 min incubation with puromycin (Sigma-Aldrich) at  
106 a final concentration of 10  $\mu\text{g}/\text{mL}$ . For PHM and captan-induced genotoxicity measurement,  
107 cells were co-treated for 4 h with 5  $\mu\text{M}$  PHM or 10  $\mu\text{M}$  of captan and various doses of TCTs.

108 **Preparation of colibactin-producing bacteria.** The intestinal carcinogenic *Escherichia coli*  
109 strain NC101 that produces the genotoxin colibactin, and the isogenic mutant strain  
110 NC101 $\Delta\text{clbP}$  which do not produce the toxin (Yang *et al.*, 2020) were cultured in Lysogeny-  
111 broth (LB) Lennox medium overnight at 37°C with shaking. Epithelial cell-bacteria interaction  
112 medium DMEM 25 mM HEPES (Fisher scientific) was inoculated from overnight bacterial  
113 cultures and incubated at 37°C with shaking until the bacteria reached an optical density at 600  
114 nm of 0.5 before infection.

115

116 **Colibactin-induced genotoxicity measurement.** IEC-6 cells were infected with *E. coli*  
117 producing colibactin as described previously (Payros *et al.*, 2017). Briefly, cells were incubated  
118 with different doses of TCTs and infected for 4 h with wild type (WT) *E. coli* NC101 or the  
119 *clbP* isogenic mutant. Cells were then washed and incubated in complete DMEM medium  
120 supplemented with 200  $\mu\text{g}/\text{mL}$  gentamicin and maintained 4 h post-infection in the presence of  
121 TCTs or DMSO vehicle, and then fixed for DNA damage measurement.

122

123 **Viability assay.** Cell viability was assayed with the CellTiter-Glo Luminescent Cell Viability  
124 Assay (Promega, Charbonnières-les-Bains, France) as described (Khoshal *et al.*, 2019).  
125 Luminescence was measured with a spectrophotometer (TECAN Spark, Mannedorf,  
126 Switzerland).

127

128 **Ribotoxicity analysis by In-Cell-Western.** Ribotoxicity was measured using protein synthesis  
129 inhibition as a surrogate. The measurement of protein synthesis by puromycin labelling was  
130 performed as described (Henrich, 2016). Briefly, puromycin was immuno-detected by In-Cell-  
131 Western using an anti-puromycin antibody (clone 12D10 diluted 1:5000; Millipore, Molsheim,  
132 France). GAPDH, which is constitutively expressed and has a half-life of 8 h (Dani *et al.*, 1984),  
133 was used as a control. The anti GAPDH antibody was diluted 1:5000 (ABS16; Millipore).  
134 Secondary antibodies were diluted 1:5000 (IRDye 800CW; Rockland, and IRDye 680RD  
135 Licor). Puromycin signal was normalized with the average fluorescence of puromycin-labelled  
136 control cells (Henrich, 2016).

137

138 **Quantification of DNA damage by In-Cell-Western.** In-Cell-Western was performed as  
139 previously described (Martin *et al.*, 2013; Tronnet and Oswald, 2018; Theumer *et al.*, 2018).  
140 Briefly, fixed cells were permeabilized and stained with the primary antibody anti- $\gamma$ H2AX  
141 (20E3 diluted 1:200; Cell Signalling, Saint-Quentin en Yvelines, France). Secondary antibody  
142 (IRDye 800CW diluted 1:1000; Rockland) and RedDot2 DNA marker (Biotium) were  
143 measured at 680 and 800 nm with a Sapphire Biomolecular Imager (Azure Biosystems). The  
144 genotoxic index was calculated by dividing the  $\gamma$ H2AX signal by the corresponding DNA  
145 fluorescence and normalized with the average signal in control cells (Tronnet and Oswald,  
146 2018).

147

148 **Data analysis.** GraphPad Prism 8.0 was used to calculate concentrations that inhibited the cell  
149 viability by 20% (IC<sub>20</sub>), and the protein synthesis by 20% (20% PSI level), performing a four-  
150 parameter nonlinear regression model (sigmoidal dose-response analysis). Profile-likelihood  
151 confidence intervals were calculated from the nonlinear regressions. One-way analysis of  
152 variance (ANOVA) followed by Bonferroni's multiple comparison were performed. The data are  
153 expressed as mean  $\pm$  SEM.

154

## 155 **RESULTS**

156 **Trichothecenes induce a dose-dependent reduction in cell viability.** As the intestinal tract is  
157 the primary target of TCTs, the non-transformed intestinal epithelial cell line IEC-6 was used.  
158 Cytotoxicity was first evaluated 4 h and 8 h after TCTs treatment, and no cytotoxicity was  
159 detected (Table S1). The effect of TCTs on cell viability was then evaluated 24 h after treatment,  
160 and we observed a dose-dependent inhibition of viability induced by DON, NIV, T-2, FX, DAS  
161 and NX (Figure 2A). IC<sub>20</sub> values, which correspond to the dose inducing a 20% reduction of  
162 cell viability, were calculated for each TCT (Figure 2B). For cytotoxicity, TCTs were classified  
163 as follows: T-2>DAS>FX>NIV>NX>DON.

164

165 **Trichothecenes induce a dose-dependent ribotoxicity.** Ribotoxicity is the main mode of  
166 action of TCTs (Pestka, 2010). To quantify the ribotoxicity of the TCTs, we examined  
167 translational inhibition, assessed by the incorporation of the protein translation marker  
168 puromycin in newly synthesized peptides (Henrich, 2016). The incorporated puromycin was  
169 quantified by immunofluorescence. All the TCTs induced a dose-dependent ribotoxicity  
170 (Figure 3A). Treatment with TCTs did not induce a drop in GAPDH levels (which has an 8 h

171 half-life), confirming that puromycin specifically labelled newly synthesized peptides (Figure  
172 3A, Figure S2). The doses inducing a 20% reduction in protein synthesis were calculated  
173 (Figure 3B). For their ribotoxic effect, TCTs were classified as follows: T-  
174 2>DAS>FX>NIV>NX≥DON.

175

176 **Trichothecenes exacerbate DNA damage induced by the drug phleomycin.** The genotoxic  
177 exacerbation properties of TCTs were first evaluated with phleomycin (PHM), a genotoxin  
178 commonly used as a model for genotoxicity assessments (Chen and Stubbe, 2005). IEC-6 cells  
179 were treated for 4 h with PHM and/or TCTs. As expected, cells treated with PHM alone  
180 exhibited DNA damage signalled by the  $\gamma$ H2AX marker (Rogakou *et al.*, 1998). TCTs alone  
181 did not induce  $\gamma$ H2AX, indicating that they are not genotoxic by themselves. In contrast, all the  
182 TCTs induced exacerbation of PHM-induced DNA damage, in a dose-dependent manner  
183 (Figure 4A). A significant increase occurred from 1  $\mu$ M for DON, 3  $\mu$ M for NX, 10 nM for T-  
184 2, 0.3  $\mu$ M for FX, 30 nM for DAS, and 3  $\mu$ M for NX (Figure 4B). The exacerbation of  
185 genotoxicity did not induce cell detachment, indicating that this effect was not a consequence  
186 of massive cell death (Figure 4, Table S1).

187

188 **Trichothecenes exacerbate DNA damage induced by captan, a pesticide which**  
189 **contaminates food.** We then tested the ability of TCTs to exacerbate genotoxicity induced by  
190 a food-contaminating genotoxin, such as the pesticide captan, which can be found in fruits,  
191 vegetables, and cereals (Shinde *et al.*, 2019). For this purpose, IEC-6 cells were exposed to  
192 captan and/or TCTs for 4 h. Captan alone induced DNA damage, and TCTs induced an  
193 exacerbation of the genotoxicity of captan, in a dose-dependent manner. Significant increase  
194 occurred from 3  $\mu$ M for DON and NX, 1 nM for T-2, 1  $\mu$ M for FX, and 3 nM for DAS, and did  
195 not induce cell death (Figure 5, Table S1).

196

197 **Trichothecenes exacerbate genotoxicity induced by colibactin, a genotoxin produced by**  
198 **members of the intestinal microbiota.** We then determined whether TCTs also exacerbate  
199 genotoxicity induced by a bacterial genotoxin produced by the intestinal microbiota, colibactin.  
200 The genotoxicity of colibactin was measured by infecting IEC-6 cells with the live colibactin-  
201 producing bacterium *Escherichia coli* NC101. Since colibactin is unstable and is not purifiable,  
202 direct contact between live colibactin-producing *E. coli* strain and eukaryotic cells is required  
203 to induce DNA damage (Chagneau *et al.*, 2022; Nougayrède *et al.*, 2006; Bossuet-Greif *et al.*,  
204 2018). Cells were infected with strain NC101 and either co-treated with TCTs or not. Cells

205 infected with the WT strain showed an increase in their  $\gamma$ H2AX signal, resulting from colibactin  
206 damage, whereas cells infected with the NC101  $\Delta$ *clbP* strain, which is impaired for colibactin  
207 synthesis, did not show DNA damage (Figures 6, S1). By contrast, TCTs-treated and WT-  
208 infected cells exhibited exacerbation of colibactin-induced DNA damage, which increased with  
209 TCT dosage. A significant increase occurred at 3  $\mu$ M for DON, NX and NIV, 1 nM for T-2, 0.3  
210  $\mu$ M for FX, and 10 nM for DAS (Figure 6), and was not associated with cell death (Table S1).  
211 Exacerbation of DNA damage was not associated with increased colibactin production by TCT-  
212 treated bacteria, nor was it associated with an impact of TCTs on bacterial growth  
213 (Supplementary methods 1, Figure S1).

214

## 215 DISCUSSION

216 Food contamination by TCTs is a public health issue of the utmost importance. TCTs  
217 levels in foods are indeed high and may even increase in the future, in part due to climate change  
218 (Van Der Fels, 2016). DON, which is not genotoxic, was recently described as a genotoxicity  
219 enhancer (Garofalo *et al.*, 2022; Payros *et al.*, 2017). In this work, we show that this is not only  
220 an effect of DON, but also a novel effect attributable to at least five other TCTs: NIV, T-2, FX,  
221 DAS and the recently discovered NX toxin. These TCTs exacerbate not only the genotoxicity  
222 induced by the model genotoxin phleomycin, but also the genotoxicity induced by captan, a  
223 pesticide with genotoxic properties, and by colibactin, a bacterial genotoxin produced in the  
224 gut. For this newly identified effect, TCTs were classified as follows: T-  
225 2>DAS>FX>NIV $\geq$ DON $\geq$ NX.

226 The cytotoxicity of TCTs was compared in non-transformed IEC-6 intestinal cells. Our  
227 data show that TCTs, which were not cytotoxic after a treatment lasting 4 h or 8 h, induced a  
228 dose-dependent inhibition of cell viability after 24 h. These data allowed the classification of  
229 TCTs as follows: T-2>DAS>FX>NIV>NX>DON. Although, to the best of our knowledge, this  
230 is the first direct comparison and ranking of TCTs' cytotoxicity in IEC-6 cells, the classification  
231 is consistent with the literature. T-2 toxin is indeed known as the most toxic TCT, with IC<sub>20</sub>  
232 about 1000 times greater than others (Fernández-Blanco, 2018). DAS is slightly less cytotoxic  
233 than T-2, but more than FX (Moon, 2003), and FX is more cytotoxic than NIV and DON  
234 (Aupanun *et al.*, 2019; Alassane-Kpembé *et al.*, 2017a). We observed that IEC-6 cells exhibit a  
235 modest sensitivity to DON. As a matter of fact, after 24 h of treatment, we found an IC<sub>20</sub> of  
236 20.2  $\mu$ M when others found IC<sub>20</sub> of  $\approx$ 0.5  $\mu$ M in IPEC-1 cells, or  $\approx$ 3  $\mu$ M in Caco-2 cells  
237 (Alassane-Kpembé *et al.*, 2015; Pierron *et al.*, 2022). Our results are consistent with those  
238 obtained by Bianco *et al.*, who found an IC<sub>50</sub> for DON of 50.2  $\mu$ M in IEC-6 cells, confirming

239 the limited sensitivity of this cell line (Bianco *et al.*, 2012). In addition, our results highlight  
240 that NX is approximately twice as cytotoxic as DON in IEC-6 cells. This result is consistent  
241 with the results of Pierron *et al.* which showed a higher inflammatory potential for NX  
242 compared to DON in porcine intestinal explants (Pierron *et al.*, 2022). In contrast, the literature  
243 shows that NX-induced cytotoxicity is comparable to that of DON in HT-29 and Caco-2 cells  
244 (Varga *et al.*, 2018; Pierron *et al.*, 2022). Importantly, the doses of TCTs that were cytotoxic  
245 after 24 h of exposure were higher than the doses that exacerbated genotoxicity (Table 1). This  
246 indicates that non-cytotoxic doses of TCTs exacerbate genotoxicity. Because DNA damage is  
247 a source of genetic mutations through repair errors, this result raises questions about the fate of  
248 these cells (Basu, 2018). Cells co-exposed to genotoxins and TCTs survive and could therefore  
249 pursue their cell cycle and division following repair of DNA damage. Further studies are needed  
250 to examine whether cells co-exposed to TCTs and genotoxins could accumulate mutations,  
251 ultimately resulting in cellular transformation.

252 In this work, we also classified TCTs for their ribotoxicity. The ranking T-  
253 2>DAS>FX>NIV>NX≥DON parallels that obtained for cytotoxicity and is coherent with the  
254 structures of TCTs. The most ribotoxic TCTs carry substitutions thought to improve binding to  
255 the ribosome, such as the isovaleryl group at C<sub>8</sub> in T-2 or the acetyl groups at C<sub>4</sub> and C<sub>15</sub> in  
256 DAS (Wu *et al.*, 2013; Wang *et al.*, 2021). Differences in TCTs' ribotoxicity may also be related  
257 to divergences in structural rearrangements induced by ribosome binding (Garreau de  
258 Loubresse *et al.*, 2014). Depending on the structure of TCTs, structural rearrangements could  
259 differ and induce variations in the mode of protein synthesis inhibition. We have previously  
260 suggested that DON-induced ribotoxicity is involved in the genotoxicity exacerbation  
261 phenotype. Indeed, ribotoxic compounds reproduced the effect, while the non-ribotoxic DON  
262 derivative DOM-1, did not (Garofalo *et al.*, 2022). The correlation between TCTs' ribotoxic  
263 and genotoxicity exacerbating doses observed in this work (Table 1) support this hypothesis.  
264 Interestingly, TCTs with substitutions that increase affinity to the ribosome have a greater  
265 capacity to exacerbate genotoxicity. This suggests that there is a structure-function link between  
266 ribotoxicity and genotoxicity exacerbation. TCTs could be classified into 3 subgroups  
267 according to their capacity to exacerbate the genotoxicity, with regard to their affinity with the  
268 ribosome: T-2, DAS > FX > NIV, NX, DON.

269 Several mechanism could be involved the ribosome-dependent exacerbation of  
270 genotoxicity. In response to DNA damage, cells reprogram their gene expression to synthesize  
271 proteins of the DNA damage response (Spriggs, Bushell and Willis, 2010). TCTs-induced  
272 ribotoxicity could disrupt the production of these stress response proteins. Through their

273 ribotoxic effect, TCTs can induce inflammation (Pestka, 2010; Garcia et al 2018), which  
274 represses the DNA damage response (Jaiswal *et al.*, 2000). Finally, it has been documented that  
275 DON activates the protein kinase R (PKR), which triggers inhibition of the DNA damage repair  
276 and sensitizes cells to DNA damage (Zhou *et al.*, 2014). As they share structural similarities  
277 with DON, TCTs could also recruit PKR and induce sensitization to DNA damage. Further  
278 work is required to understand how ribotoxicity results in this novel effect of TCTs.

279 We observed that exacerbation of genotoxicity occurred at realistic doses of TCTs. The  
280 European Food Safety Authority (EFSA) established a no observable adverse effect level  
281 (NOAEL) for DON of 100 µg/kg body weight (bw)/day (Knutsen *et al.* 2017a), and 65 µg/kg  
282 bw/day for DAS (Knutsen *et al.*, 2018). The Benchmark Dose Limit (BMDL<sub>10</sub>) for NIV is 350  
283 µg/kg bw/day (Knutsen *et al.*, 2017b), and 3.3 µg/kg bw/day for T-2 (Knutsen *et al.*, 2017c).  
284 Estimating, as Maresca *et al.*, 2013, that for a human weighing 70 kg, the small intestine content  
285 is 1L, these doses can be converted to intestinal concentrations of 23.6 µM DON, 0.5 µM T-2,  
286 12.5 µM DAS, and 78.4 µM NIV. Our study shows no effect doses for genotoxicity  
287 exacerbation well below these reference values, with 1 µM, 3 nM, 10 nM, and 3 µM for DON,  
288 T-2, DAS and NIV, respectively. We observed that structurally related TCTs displayed  
289 comparable effects, and could therefore be classified into 3 subgroups: T-2, DAS > FX > NIV,  
290 NX, DON. As TCTs frequently co-occur in foodstuffs (Alassane-Kpembé *et al.*, 2017b), it  
291 would be appropriate to set group TDIs for TCTs, as it has been done for other groups of  
292 structurally related mycotoxins (Steinkellner *et al.* 2019).

293 The exacerbation effect occurred with captan, a pesticide which induces *in vitro* DNA  
294 damage (Fernandez-Vidal *et al.*, 2019). Captan can contaminate fruits and vegetables, but also  
295 cereals, which are the main source of TCTs (Shinde *et al.*, 2019). Captan has been associated  
296 with multiple myeloma in farmers (Presutti *et al.*, 2016), and is classified by the European  
297 Commission as “suspected of causing cancer” (European Commission, 2008). Notably, the  
298 exacerbation effect occurred at realistic doses of captan. The NOAEL for captan is indeed 25  
299 mg/kg bw per day (Anastassiadou *et al.*, 2020), which corresponds to an intestinal concentration  
300 of 5.8 mM. Here, the exacerbated genotoxic effect of captan was observed with a dose as low  
301 as 10 µM, well below the NOAEL. Interestingly, TCTs also exacerbate the effect of colibactin,  
302 an endogenous genotoxin produced by the intestinal microbiota throughout the host’s life.  
303 Indeed, approximately 15% of 3-day-old neonates are colonized by colibactin-producing *E. coli*  
304 (Payros *et al.*, 2014), and 25% of adults harbour these bacteria (Putze *et al.*, 2009; Tenaillon *et*  
305 *al.*, 2010; Johnson *et al.*, 2008). In addition, the prevalence of the B2 phylogenetic group of *E.*  
306 *coli*, which includes up to 50% of colibactin-producing strains, is increasing in developed

307 countries (Tenailon *et al.*, 2010). The intestinal microbiome also encodes other bacterial  
308 genotoxins such as cytolethal distending toxins (Taieb *et al.*, 2016). Thus, humans are  
309 potentially co-exposed to TCTs together with endogenous genotoxins produced by the  
310 microbiota as well as multiple exogenous diet-borne genotoxins, such as captan, and other  
311 genotoxic pesticides such as glyphosate (International Agency for Research on Cancer, 2017),  
312 alcohol-derivatives (Brooks *et al.*, 2014), and components of red meat (Bastide *et al.*, 2011).  
313 Given the high prevalence of TCTs in foods, it is conceivable that TCTs could exacerbate the  
314 effect of the multiple genotoxic agents to which we are exposed.

315

## 316 **CONCLUSION**

317 We report that even though TCTs are not genotoxic by themselves, these contaminants promote  
318 the genotoxicity of genotoxins which are in the diet, such as pesticides, or are produced by the  
319 intestinal microbiota. Considering the wide prevalence of both TCTs and genotoxins, a large  
320 part of the population could be impacted by this novel effect. This is alarming because DNA  
321 damage drives cancer development (Basu, 2018). A preliminary report on a large-scale  
322 epidemiological study in the European Union has suggested a link between long-term exposure  
323 to DON and an increased risk of colon cancer (Huybrechts *et al.*, 2019). If confirmed,  
324 exacerbation of DNA damage by DON could be a key to explaining this epidemiological link.  
325 Here, we show that DON is not the only dietary TCT to exhibit this genotoxicity-exacerbating  
326 property. There is an urgent need for additional studies on the impact of TCTs on carcinogenesis  
327 induced by other environmental toxicants.

328

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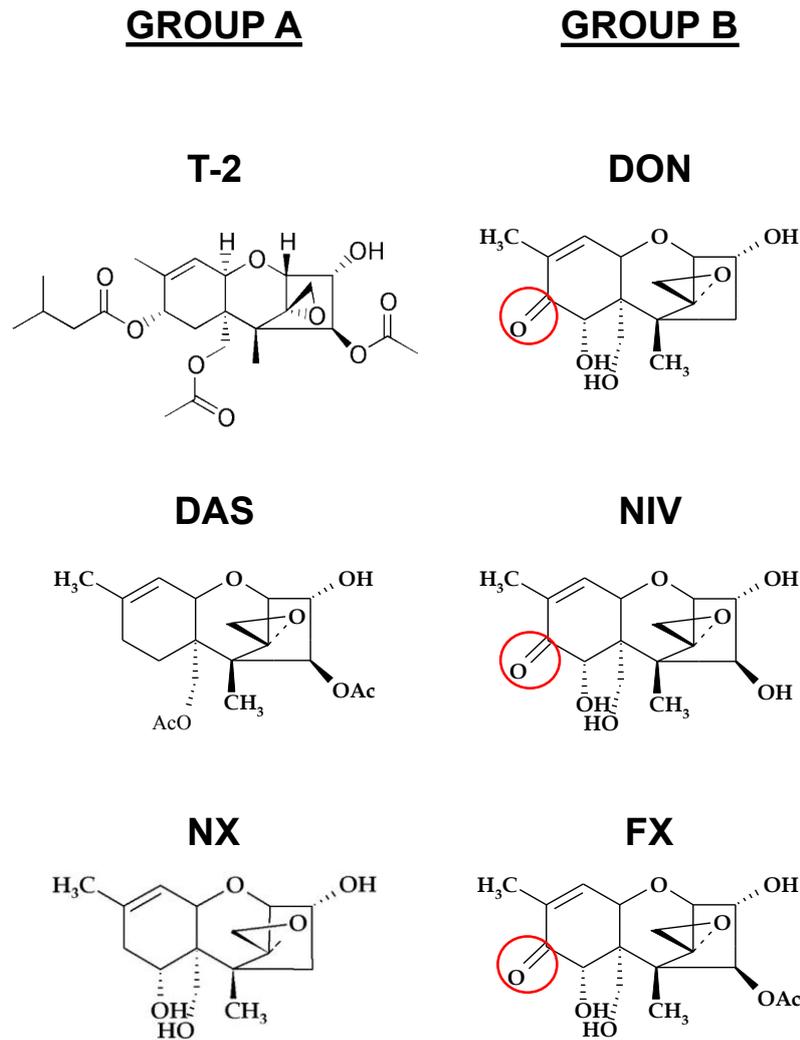
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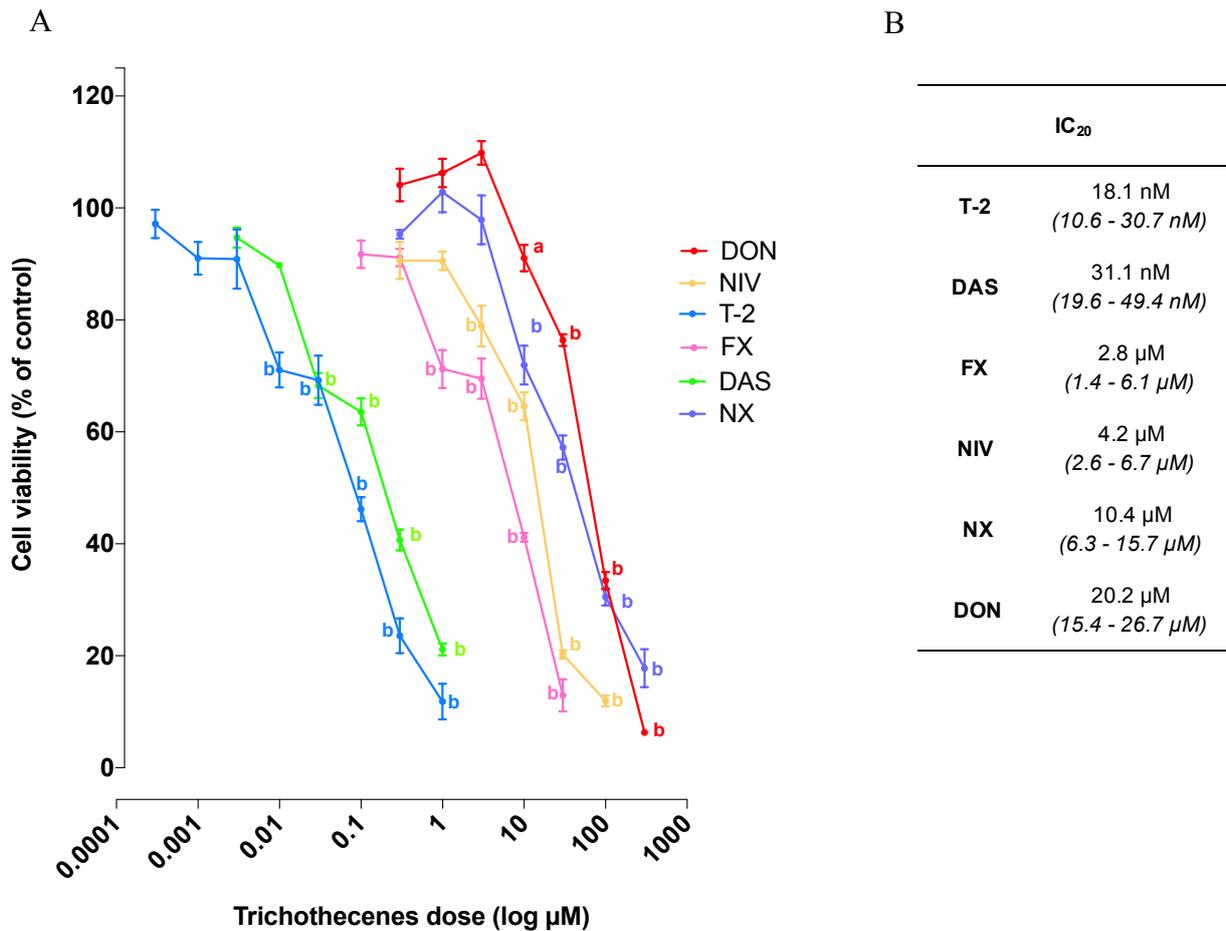
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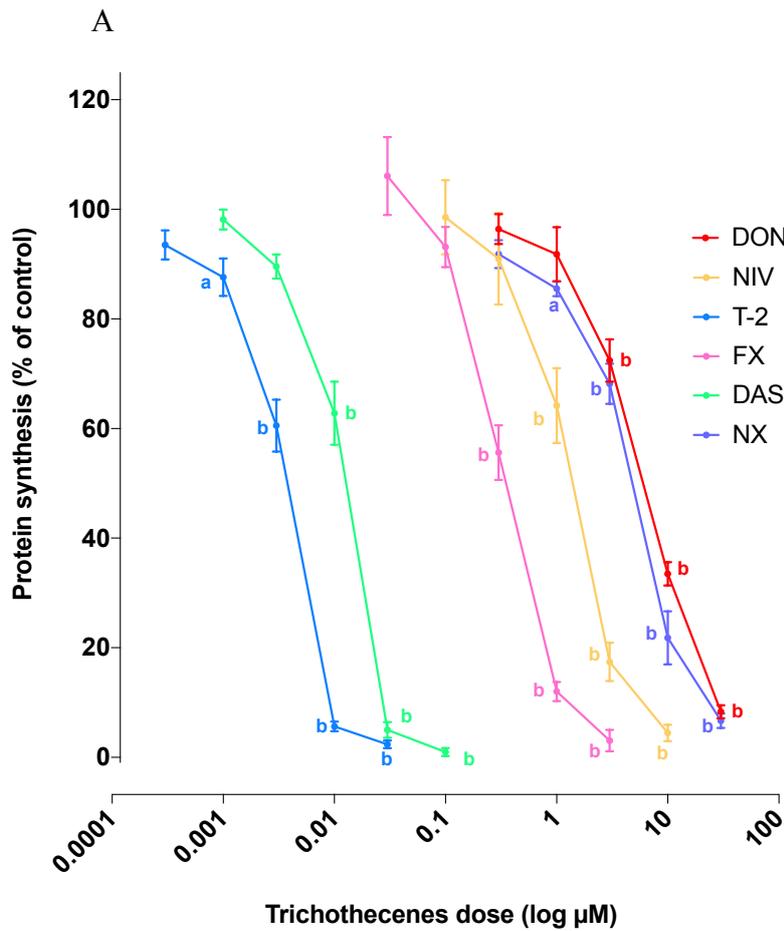
**Figure 1: Chemical structures of trichothecenes.** Dietary TCTs are classified in two main groups. Group A TCTs include T-2 toxin (T-2), diacetoxyscirpenol (DAS), and NX. Group B TCTs include deoxynivalenol (DON), nivalenol (NIV), and fusarenon-X (FX). Group A TCTs can be distinguished from group B TCTs by the presence of a C<sub>8</sub> keto-oxygen (circled in red).

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**Figure 2: Trichothecenes induce dose-dependent cytotoxicity in cultured intestinal epithelial cells.** A: Non-transformed rat intestinal epithelial IEC-6 cells were treated for 24 h with various concentrations of TCTs and then viability was assessed by measuring ATP levels. Data are expressed as mean ± SEM (3 to 6 independent experiments). P-values were calculated using one-way ANOVA with Bonferroni's multiple comparison, a: p<0.01; b: p<0.0001: B: Concentrations that inhibited cell viability by 20% (IC<sub>20</sub>) for each trichothecene were calculated using the data presented in panel A. 95% confidence intervals are shown in italics.

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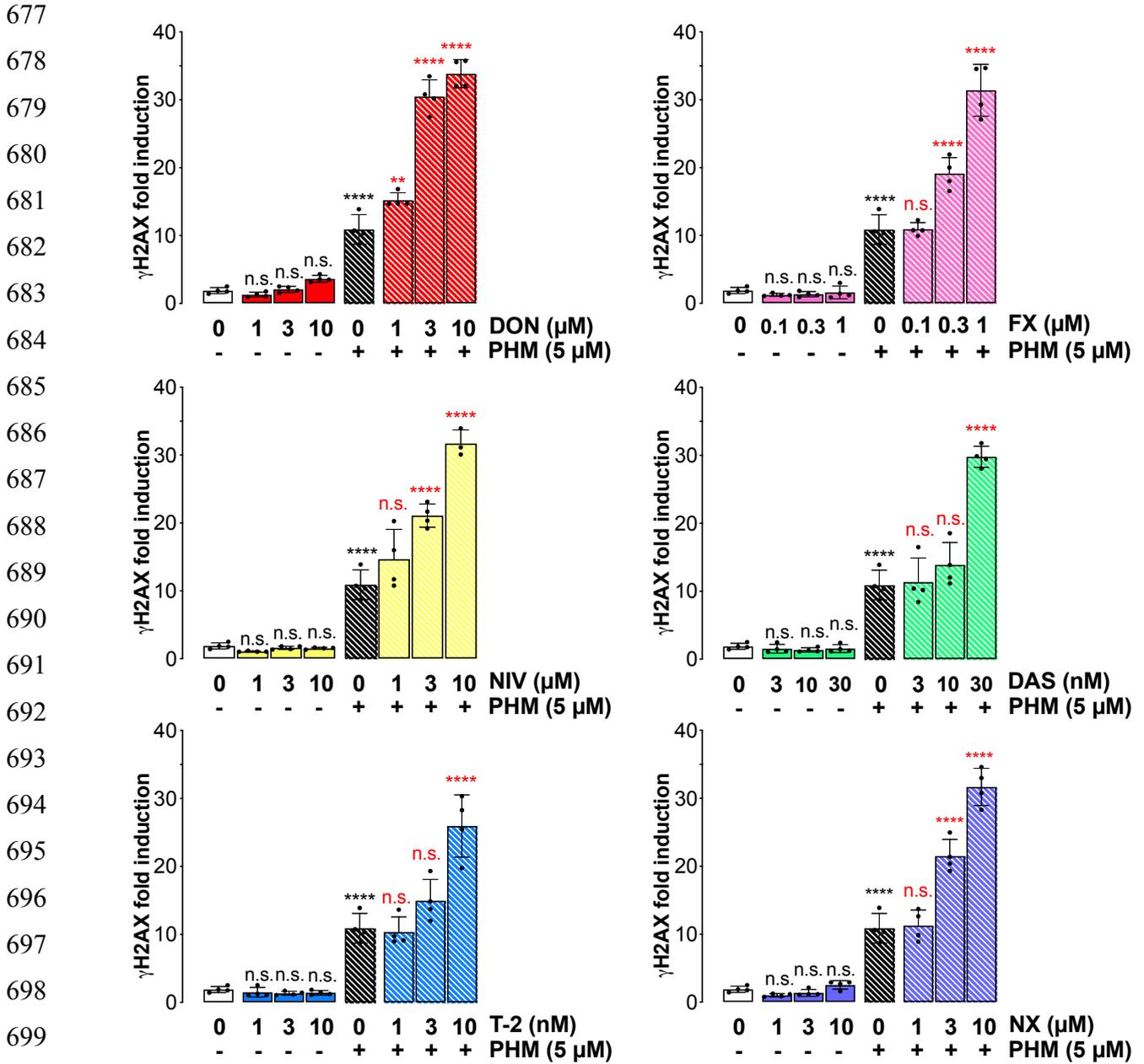


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20% PSI level	
T-2	1.9 nM <i>(1.6 - 2.3 nM)</i>
DAS	6 nM <i>(5 - 7 nM)</i>
FX	0.15 μM <i>(0.12 - 0.19 μM)</i>
NIV	0.6 μM <i>(0.4 - 0.9 μM)</i>
NX	2.5 μM <i>(2.0 - 3.1 μM)</i>
DON	2.9 μM <i>(2.2 - 3.9 μM)</i>

**Figure 3: Trichothecenes induce dose-dependent ribotoxicity in cultured intestinal epithelial cells.** A: IEC-6 cells were treated for 4 h with different concentrations of TCTs and then the cells were treated for 30 min with puromycin, a protein translation marker. Incorporation of puromycin into newly synthesized peptides was quantified with an anti-puromycin antibody by In-Cell-Western. All the data are expressed as mean ± SEM (4 independent experiments). Values that are significantly different compared to the vehicle control are indicated a: p<0.01; b: p<0.0001: B: Concentrations that inhibited the protein synthesis by 20% (20% PSI) were calculated using the data presented in panel A. 95% confidence intervals are shown in italics.

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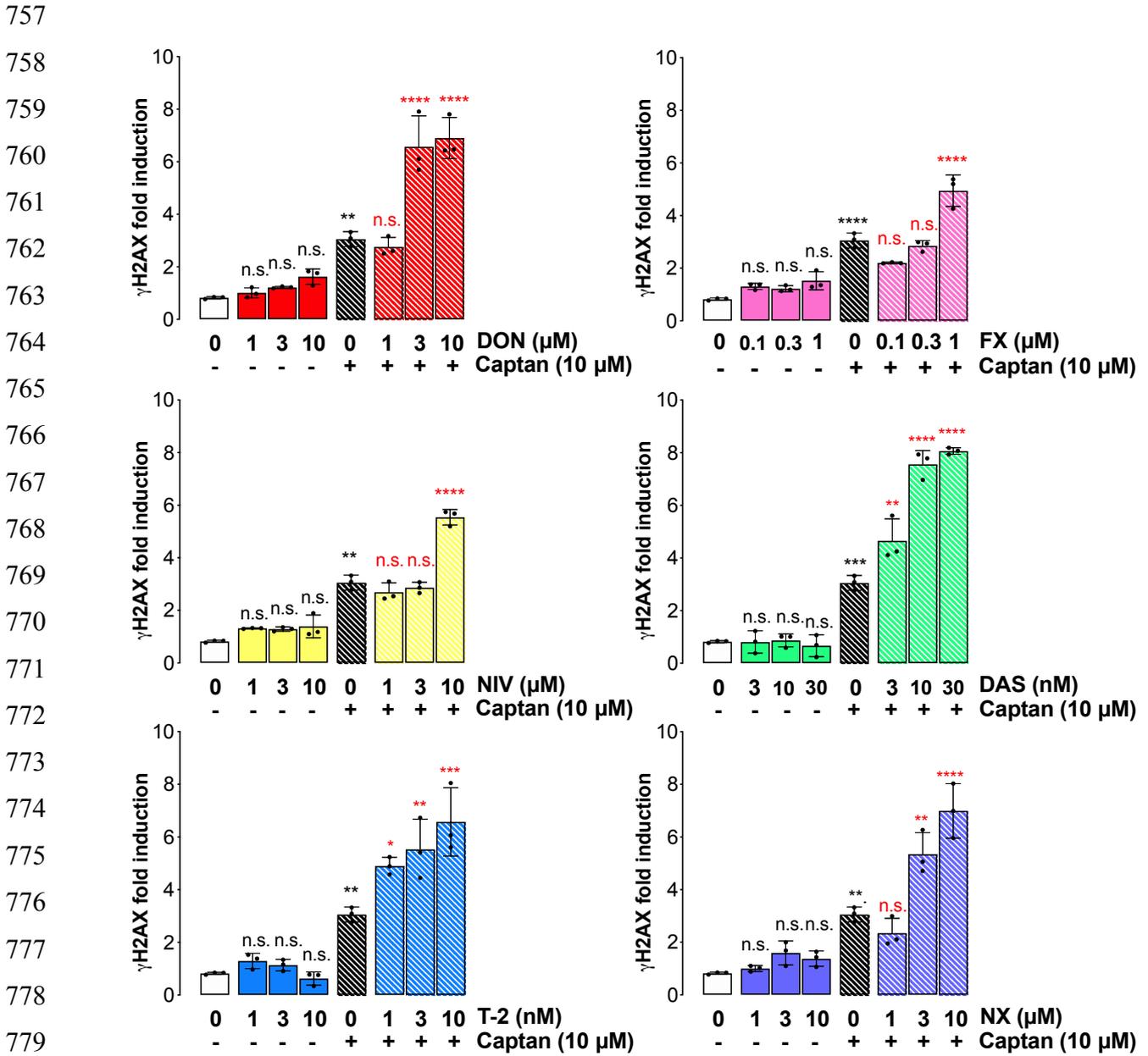
No effect dose

T-2	DAS	FX	NIV	DON	NX
3 nM	10 nM	0.1 $\mu$ M	1 $\mu$ M	< 1 $\mu$ M	1 $\mu$ M

711 **Figure 4: Trichothecenes exacerbate the genotoxicity induced by the DNA-damaging drug**  
712 **phleomycin in a dose-dependent manner.** A: IEC-6 cells were co-treated for 4 h with 5  $\mu$ M  
713 phleomycin (PHM) and increasing doses of DON (red), NIV (yellow), T-2 (blue), FX (pink), DAS  
714 (green), or NX (purple). Then, DNA damage was measured by quantification of H2AX  
715 phosphorylation by In-Cell-Western. All data are expressed as mean  $\pm$  SEM (4 independent  
716 experiments). P-values were calculated using a one-way ANOVA with Bonferroni's multiple  
717 comparison. Values that are significantly different compared to vehicle are indicated by black  
718 asterisks, and values that are significantly different from infected cells without TCT are indicated  
719 by red asterisks. \*:p<0.1; \*\*: p< 0.01; \*\*\*: p<0.001, \*\*\*\*: p< 0.0001, n.s. : not significant. B: No  
720 exacerbation doses for each TCT were defined from data in panel A.

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No effect dose

T-2	DAS	FX	NIV	DON	NX
< 1 nM	< 3 nM	0.3 $\mu$ M	3 $\mu$ M	1 $\mu$ M	1 $\mu$ M

789 **Figure 5: Trichothecenes exacerbate the genotoxicity induced by the fungicide captan in a**  
790 **dose-dependent manner.** A: IEC-6 cells were co-treated for 4 h with 10  $\mu$ M captan and increasing  
791 doses of DON (red), NIV (yellow), T-2 (blue), FX (pink), DAS (green), or NX (purple). Then, DNA  
792 damage was measured by quantification of H2AX phosphorylation by In-Cell-Western. All data are  
793 expressed as mean  $\pm$  SEM (3 independent experiments). P-values were calculated using one-way  
794 ANOVA with Bonferroni's multiple comparison. Values that are significantly different compared  
795 to vehicle are indicated by black asterisks, and values that are significantly different from infected  
796 cells without TCT are indicated by red asterisks. \*:p<0.1; \*\*: p< 0.01; \*\*\*: p<0.001, \*\*\*\*: p<  
797 0.0001, n.s.: not significant. B: No exacerbation dose for each TCT was defined from data in panel  
798 A.

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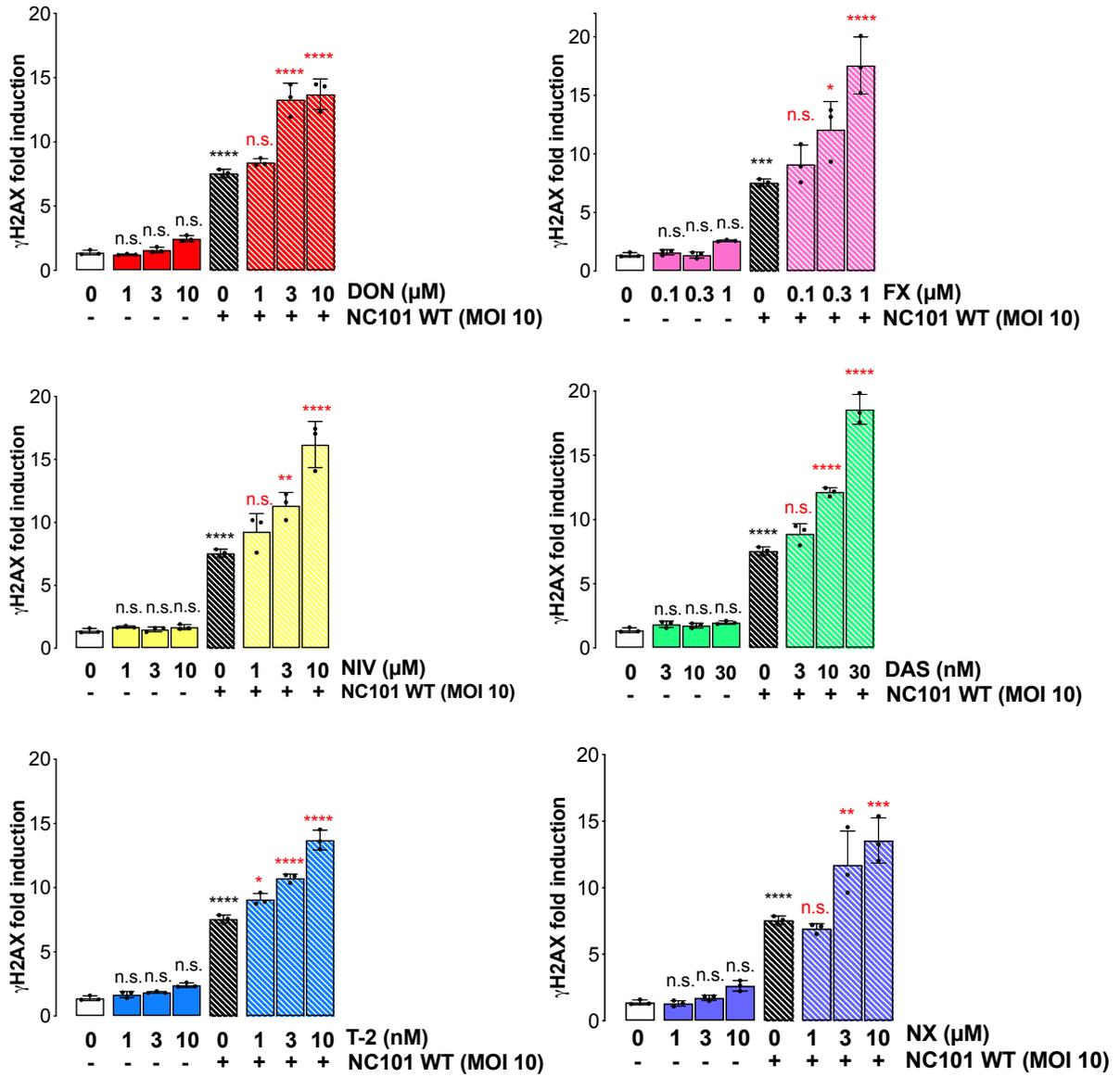
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849 B

850 No effect dose

852 T-2	852 DAS	852 FX	852 NIV	852 DON	852 NX
854 < 1 nM	854 3 nM	854 0.1 $\mu$ M	854 0.3 $\mu$ M	854 1 $\mu$ M	854 1 $\mu$ M

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857 **Figure 6: Trichothecenes exacerbate the genotoxicity induced by the bacterial genotoxin**  
858 **colibactin in a dose-dependent manner.** A: IEC-6 cells were infected 4 h with live colibactin-  
859 producing *E. coli* strain NC101 (multiplicity of infection of 10 bacteria per cell) with increasing  
860 doses of DON (red), NIV (yellow), T-2 (blue), FX (pink), DAS (green), or NX (purple). Cells were  
861 washed to remove bacteria and further incubated with the TCTs for 4 h. Then, DNA damage was  
862 measured by quantification of H2AX phosphorylation by In-Cell-Western. All data are expressed  
863 as mean  $\pm$  SEM (3 independent experiments). P-values were calculated using one-way ANOVA  
864 with Bonferroni's multiple comparison. Values that are significantly different compared to vehicle  
865 are indicated by black asterisks, and values that are significantly different from infected cells  
866 without TCT are indicated by red asterisks. \*:  $p < 0.1$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ , \*\*\*\*:  $p < 0.0001$ ,  
867 n.s.: not significant. B: No exacerbation doses for each TCT were defined from data shown in figure  
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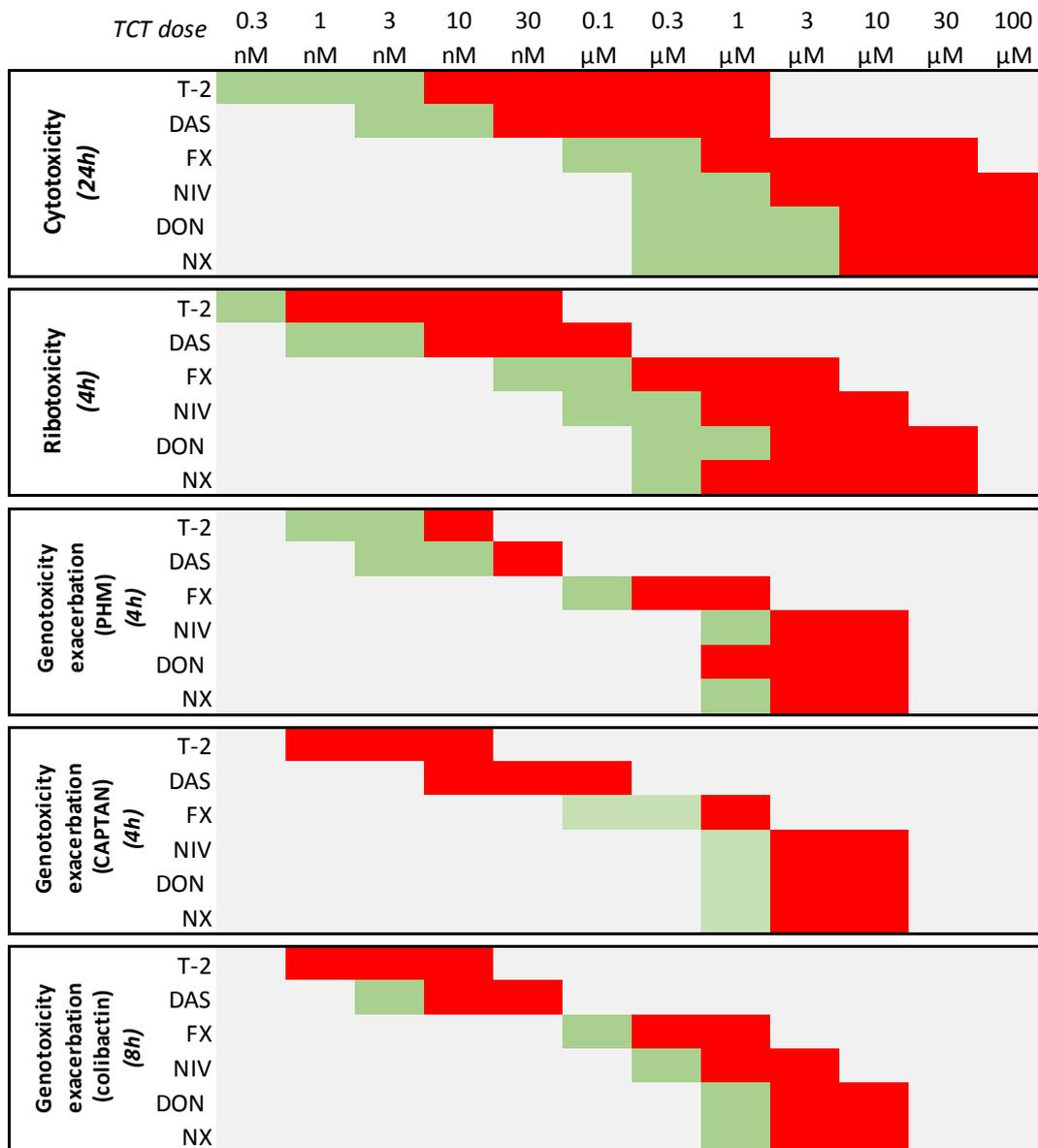
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894 **Table 1: Trichothecenes classification for cytotoxicity, ribotoxicity, and capacity to**  
 895 **exacerbate the genotoxicity.** Green boxes: doses inducing no significant cytotoxicity,  
 896 ribotoxicity or genotoxicity exacerbation. Red boxes: doses inducing significant cytotoxicity,  
 897 ribotoxicity or genotoxicity exacerbation.

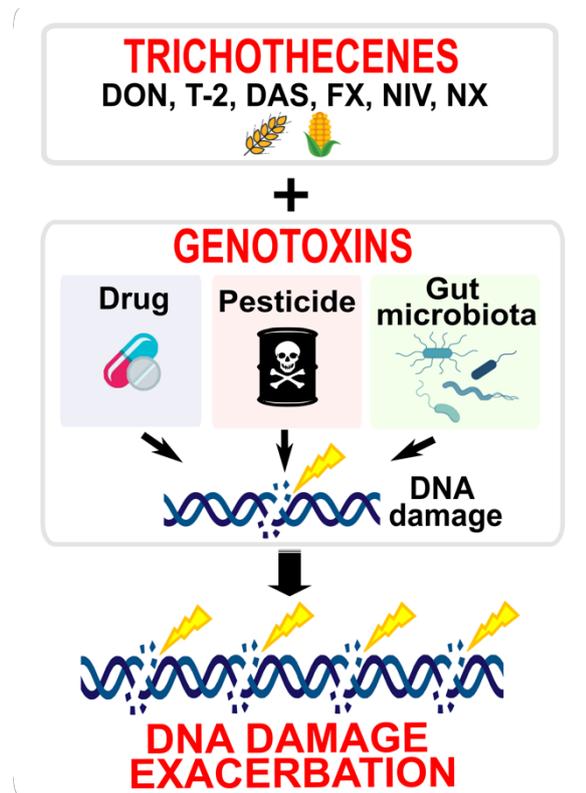
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## HIGHLIGHTS

- A novel effect of trichothecenes (TCTs) has been identified
- TCTs exacerbate genotoxicity
- Six type A or type B TCTs display this effect
- This effect is observed with a drug, a pesticide, and a toxin produced by microbiota
- For genotoxicity exacerbation, TCTs are classified: T-2>DAS>FX>NIV≥DON≥NX

GRAPHICAL ABSTRACT



1 **Supplementary material for**

2 **“A novel toxic effect of trichothecenes: the exacerbation of genotoxicity”**

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4 Marion Garofalo<sup>a,b</sup>, Delphine Payros<sup>a,b</sup>, Marie Penary<sup>b</sup>, Eric Oswald<sup>b,c</sup>, Jean-Philippe  
5 Nougayrède<sup>b\*</sup>, Isabelle P. Oswald<sup>a\*</sup>.

6  
7 <sup>a</sup>Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-  
8 Purpan, UPS, Toulouse, France

9 <sup>b</sup>IRSD, Université de Toulouse, INSERM, INRAE, ENVT, UPS, Toulouse, France

10 <sup>c</sup>CHU Toulouse, Hôpital Purpan, Service de Bactériologie-Hygiène, Toulouse, France

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12 \*Corresponding authors: isabelle.oswald@inrae.fr; jean-philippe.nougayrede@inrae.fr

13 Keywords: trichothecenes; genotoxins; colibactin; DNA damage.

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15 **Supplementary methods 1:**

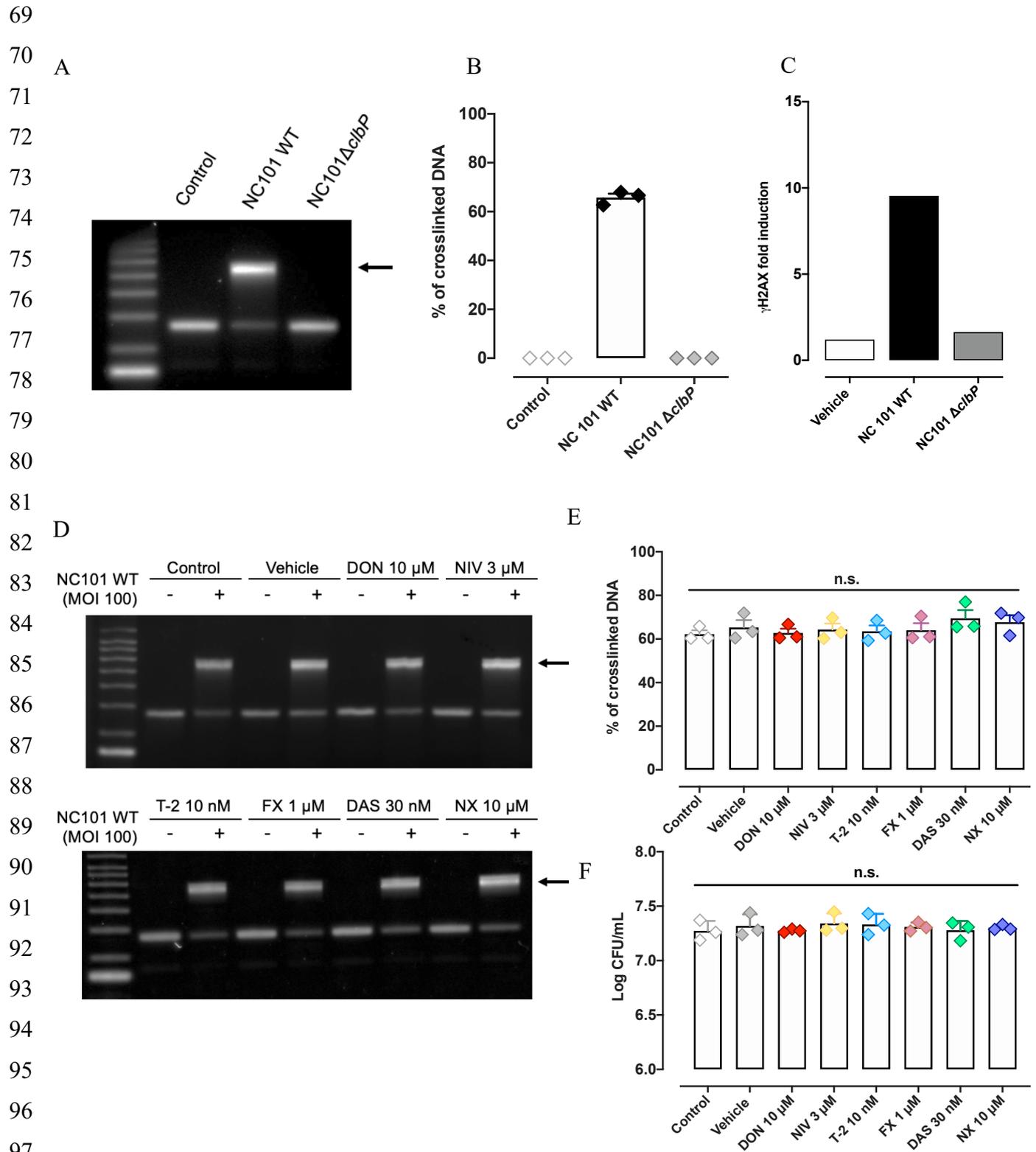
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17 ***In vitro* crosslinking assay and bacterial load analysis.** To examine colibactin production by  
18 the bacteria, a DNA crosslinking assay was performed as previously described (Bossuet-Greif  
19 *et al.*, 2018). Briefly, 400 ng linear DNA was exposed to  $1.5 \times 10^6$  bacteria in 100  $\mu$ l infection  
20 medium in the presence of TCTs or DMSO vehicle. After 4 h at 37°C, bacteria were pelleted,  
21 plated on LB agar plates, and enumerated. The DNA was purified from the culture supernatant  
22 using the Qiagen QIAquick PCR kit (Qiagen, Hilden, Germany). Purified DNA was loaded on  
23 a denaturing agarose gel (pH 8) and electrophoresis was carried for 45 min at 25V followed by  
24 2 h 30 min at 50V. After gel neutralization, DNA was stained with Gel Red (Biotium, San  
25 Francisco, USA) and visualized in a Bio-Rad Chemidoc XRS system. The percentage of  
26 crosslinked DNA was quantified by using the FIJI software (<https://imagej.net/Fiji>).

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DNA quantification (% of control cells)					
	No genotoxin (4h)	No genotoxin (8h)	Phleomycin (5 μM) (4h)	Captan (10 μM) (4h)	NC101 WT (MOI 10) (8h)
<b>Control (vehicle)</b>	100	100	98.8 ± 1.6	90.1 ± 1.3	105.1 ± 2.8
<b>DON (10 μM)</b>	83.7 ± 5.8	87.6 ± 6.9	82.4 ± 13.2	99.8 ± 12.5	91.6 ± 8.5
<b>T-2 (10 nM)</b>	86.2 ± 6.6	95.5 ± 2.0	97.7 ± 17.9	107.6 ± 7.8	112.8 ± 3.7
<b>DAS (30 nM)</b>	92.2 ± 12.8	94.2 ± 6.2	104.1 ± 11.6	108.8 ± 3.04	111.4 ± 3.7
<b>NIV (3 μM)</b>	100.6 ± 5.4	103.9 ± 8.4	100.1 ± 6.2	114.7 ± 4.3	109.8 ± 7.4
<b>FX (1 μM)</b>	101.8 ± 3.4	103.7 ± 1.0	90.5 ± 5.2	107.9 ± 12.0	107.0 ± 2.7
<b>NX (10 μM)</b>	85.9 ± 6.9	97.4 ± 2.4	77.4 ± 6.8	85.5 ± 4.1	79.1 ± 0.9

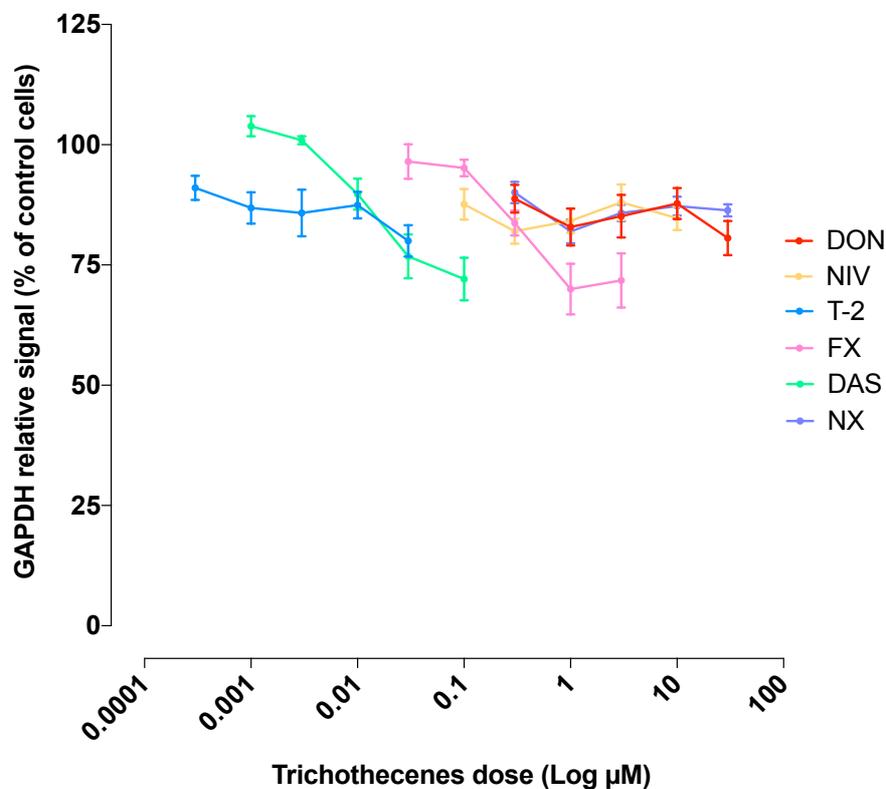
**Table S1: The genotoxicity exacerbation induced by trichothecenes is not associated with cytotoxicity.** DNA in the cells was calculated from RedDot2 DNA staining using the In-Cell-Western method. Values were obtained after normalization from untreated cells considered as 100%.



**Figure S1: Trichothecenes does not impact colibactin production and bacterial growth.**

A, B: *E. coli* NC101 WT or  $\Delta$ clbP (MOI 100) were grown for 3.5 h, and then linearized DNA was added and incubated for 30 min. DNA was purified and analysed by denaturing gel electrophoresis, and the percentage of crosslinked DNA (arrow) was quantified by image

103 analysis. C: Non-transformed rat intestinal epithelial IEC-6 cells were infected for 4 h with the  
 104 producing-colibactin *E. coli* strain NC101 WT or to the colibactin-deficient strain NC101  $\Delta clbP$   
 105 (MOI 10). Then, H2AX phosphorylation levels were quantified by In-Cell-Western. D, E:  
 106 colibactin production was measured as in panel A in presence of TCTs. F: after the interaction  
 107 between bacteria TCT and DNA, the bacteria were counted by serial dilution and plating. After  
 108 an overnight incubation, Colony Forming Units (CFU) were enumerated. All the data are  
 109 expressed as mean  $\pm$  SEM (3 independent experiments). All P-values are calculated using one-way  
 110 ANOVA with Bonferroni's multiple comparison. n.s: not significant.



129 **Figure S2:** Non-transformed rat intestinal epithelial IEC-6 cells were treated for 4 hours with  
 130 various concentrations of DON (red), NIV (yellow), T-2 (blue), FX (pink), DAS (green), or NX  
 131 (purple). GAPDH levels were measured with an anti-GAPDH antibody by In-Cell-Western. All the  
 132 data are expressed as mean  $\pm$  SEM (4 independent experiments).

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