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► To cite this version:

Su Luo, Chloé Terciolo, Manon Neves, Sylvie Puel, Claire Naylies, et al.. Comparative sensitivity of proliferative and differentiated intestinal epithelial cells to the food contaminant, deoxynivalenol. *Environmental Pollution*, 2021, 277, pp.116818. 10.1016/j.envpol.2021.116818 . hal-03180852

HAL Id: hal-03180852

<https://hal.inrae.fr/hal-03180852>

Submitted on 22 Mar 2023

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1 **Comparative sensitivity of proliferative and differentiated intestinal**
2 **epithelial cells to the food contaminant, deoxynivalenol**

3

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10

1 **Abstract:** The intestinal epithelium is a functional and physical barrier formed by a
2 cell monolayer that constantly differentiates from a stem cell in the crypt. This is the
3 first target for food contaminants, especially mycotoxins. Deoxynivalenol (DON) is
4 one of the most prevalent mycotoxins. This study compared the effects of DON (0-
5 100 μ M) on proliferative and differentiated intestinal epithelial cells. Three cell
6 viability assays (LDH release, ATP content and neutral red uptake) indicated that
7 proliferative Caco-2 cells are more sensitive to DON than differentiated ones. The
8 establishment of transepithelial electrical resistance (TEER), as a read out of the
9 differentiation process, was delayed in proliferative cells after exposure to 1 μ M DON.
10 Transcriptome analysis of proliferative and differentiated exposure to 0-3 μ M DON
11 for 24 hours revealed 4,862 differentially expressed genes (DEG) and indicated an
12 effect of both the differentiation status and the DON treatment. KEGG enrichment
13 analysis indicated involvement of metabolism, ECM receptors and tight junctions in
14 the differentiation process, while ribosome biogenesis, mRNA surveillance, and the
15 MAPK pathway were involved in the response to DON. The number of differentially
16 expressed genes and the amplitude of the effect were higher in proliferative cells
17 exposed to DON than that in differentiated cells. In conclusion, our study shows that
18 proliferative cells are more susceptible than differentiated ones to DON and that the
19 mycotoxin delays the differentiation process.

20

21 **Capsule:** Proliferative intestinal epithelial cells are more susceptible than
22 differentiated ones, to DON.

23

24 **Key words:** mycotoxin, cell toxicity, intestinal cell renewal, cell proliferation,
25 cytotoxicity, intestinal barrier function

1 **1. Introduction**

2 Food safety is a major issue worldwide. The intestine is the first barrier against
3 ingested toxicants, but also the first target organ for food contaminants. The intestinal
4 epithelium provides a selective semi-permeable membrane that allows the absorption
5 of nutrients, electrolytes and water while preventing the entrance of harmful
6 substances (Pinton and Oswald, 2014; Terciolo et al., 2019). The epithelium
7 undergoes rapid and continuous self-renewal and cell differentiation along the crypt-
8 villus axis. The stem cells at the bottom of the crypts generate proliferative cells and
9 deliver them to the top of villi. During their migration from the crypt to the villi,
10 proliferative cells mainly differentiate to absorptive enterocytes (Clevers, 2013). The
11 balance between proliferation and apoptosis of epithelial cells maintains the intestinal
12 barrier function and gut homeostasis. Proliferative cells in the crypt differ structurally
13 and functionally from differentiated enterocytes in the villi.

14 Mycotoxins are the most frequently occurring natural food contaminants in
15 human food and animal feed. Among them, Deoxynivalenol (DON), a widespread
16 trichothecene, is mainly produced by *Fusarium graminearum* and *F. culmorum*. It
17 frequently contaminates cereals and cereal products. Indeed, DON was detected in
18 almost half of 26,613 cereal samples collected from 21 European countries, with the
19 highest levels observed in wheat, maize and oat grains (Knutsen et al., 2017). Several
20 studies indicated that 40% to 98% of the adult population are exposed to this food
21 contaminant (De Santis et al., 2019; Turner et al., 2008). Moreover, European surveys
22 showed that the tolerable daily intake (TDI) of 1 µg/kg b.w./d established by EFSA is
23 exceeded in parts of the population, especially in children (Knutsen et al., 2017; Vin
24 et al., 2020).

25 At the molecular level, DON inhibits protein synthesis. It interacts with the
26 peptidyl transferase region of the 60S ribosomal subunit, impairing the initiation and
27 elongation of protein synthesis (Payros et al., 2016). This “ribotoxic stress” activates
28 mitogen-activated protein kinases and their downstream pathways (Lucioli et al., 2013;
29 Pestka, 2010). Acute exposure in human is associated with vomiting and bloody

1 diarrhea (Ruan et al., 2020). Chronic exposure to DON leads to reduced food
2 consumption and reduced weight gain, neuro-endocrine changes as well as alteration
3 of immune functions (Payros et al., 2016; Pestka, 2010; Robert et al., 2017).

4 DON also targets the intestine as described by our group and by others
5 (Maresca, 2013; Pinton and Oswald, 2014). This mycotoxin alters the intestinal
6 structure (García et al., 2018), impairs the expression of tight junction proteins
7 (Pinton et al., 2012), and reduces the production of mucus (Pinton et al., 2015),
8 leading to alteration of the barrier function (Pinton et al., 2009). DON also affects the
9 formation of the brush border and expression of intestinal enzymes (Kasuga et al.,
10 1998), reduces absorption of nutrients (Maresca et al., 2002), modulates intestinal
11 microbiota (Payros et al., 2017; Waché et al., 2009), and increases the
12 expression/secretion of pro-inflammatory cytokines in local immune responses
13 (Payros et al., 2016; Pestka, 2010; Pinton and Oswald, 2014).

14 Despite this damage, the mechanisms underlying the effects of food
15 contaminants on the proliferation and differentiation of intestinal cell have never been
16 studied. The aim of this study was to better understand the impact of deoxynivalenol
17 on intestinal disorders by analyzing its interaction with proliferative and differentiated
18 cells in the gut. We studied the influence of DON on the differentiation process of the
19 intestinal epithelium by measuring cytotoxicity and trans-epithelial electrical
20 resistance (TEER), and by identifying the gene expression profile of proliferative and
21 differentiated Caco-2 cells.

2. Materials and methods

2.1. Reagents

Purified DON (Sigma, St Quentin Fallavier, France) was dissolved in water stored at -20 °C. before being diluted in complete media DMEM-Glutamax medium (Life Technologies, Courtaboeuf, France) containing 5% fetal calf serum, 1% non-essential amino acid (Sigma) and 0.5% gentamycin (Eurobio, Courtaboeuf, France).

2.2. Cell culture

Caco-2 cells are able to undergo spontaneous and complete intestinal-like program differentiation (Hidalgo et al., 1989). They were cultured in complete medium, passaged by trypsinization at 80% confluence. Proliferative Caco-2 cells were analyzed at the sub-confluence stage; differentiated cells were allowed to differentiate for at least 14 days post-confluence.

2.3. Cytotoxicity assays

The cytotoxicity was measured by different indicators of cell viability or cell death: lactate dehydrogenase (LDH) release (Cytotox 96®, Promega, Charbonnières-les-Bains, France), amount of ATP (CellTiter-Glo®, Promega) and uptake of eurdodin dye (Neutral Red Cell Cytotoxicity, Clinisciences, Nanterre, France). Proliferative and differentiated cells were seeded in 96-well plates, grown for 24 hours or 21 days, exposed to DON (0-100 µM) for 48 hours then. Tests were performed according to the manufacturer's instructions.

2.4. Transepithelial electrical resistance (TEER) assay

Cells were seeded and cultured as previously described (Pinton et al., 2009), grown for 2 days or 14 days, then treated apically with DON. TEER was measured every 6 hours with a cellZscope (nanoAnalytics, Münster, Germany).

2.5 DNA labeling

At day 14 and 28, DNA labeling was performed on insert as surrogate marker of cell number. Cells were washed in PBS, directly fixed in the insert with 4%

1 paraformaldehyde in PBS (20 min, RT) and washed in PBS. Paraformaldehyde was
2 neutralized with 20 mM NH₄Cl. Cells were washed in PBS, permeabilized with 0.2%
3 Triton X-100 in PBS, washed in PST buffer (PBS, 2% fetal calf serum, 0.2% Triton
4 X-100) and incubated with RedDot (Interchim, Montluçon, France) 1/500 dilution in
5 PST. After 1 hour of incubation and washing in PST, DNA was quantified using an
6 Odyssey Infrared Imaging Scanner (Li-Cor ScienceTec, Les Ulis, France) with the
7 680 nm fluorophore.

8

9 2.6 Microarray data processing and functional analysis of differentially expressed 10 genes

11 Proliferative and differentiated cells were cultured in 6-well plates, grown for 2 or 21
12 days, and exposed to 3 μM DON for 24 hours. Total RNA was extracted as previously
13 described (Alassane-Kpembé et al., 2017b); all the samples exhibited an RNA
14 integrity number > 9.3.

15 Microarray experiments were conducted on the GeT-TriX platform using Agilent
16 Sureprint G3 Human GE V3 microarrays (8 × 60K, design 072363) according to the
17 manufacturer's instructions as already described (Alassane-Kpembé et al., 2017a).
18 Hierarchical clustering analysis of probes was carried out on the MATRiX APP
19 (<http://matrix.toulouse.inra.fr/>). Enrichment and functional analysis of DE genes in
20 each cluster was analyzed using DAVID 6.8 (<https://david.ncifcrf.gov/>) and KOBAS
21 3.0 (<http://kobas.cbi.pku.edu.cn/>) with the database KEGG pathway and Gene
22 Ontology. Only pathways with FDR p- value ≤ 0.05 were presented.

23

24 2.7 Quantitative real-time polymerase chain reaction (RT-qPCR)

25 Primers designed using PrimerQuest® software were purchased from Sigma (Table 1).
26 RT-PCR assays were performed as previously described (Maruo et al., 2018). The
27 expression values of the genes of interest were normalized against three housekeeping
28 genes PSMB6, ACTB and GAPDH validated with NormFinder software. Gene
29 expression is expressed relative to the control group.

30

31 2.8 Statistical analysis

1 Statistical analyses were performed using GraphPad Prism 4 (La Jolla, CA, USA).
2 One-way ANOVA (non-parametric) or two-way ANOVA with a Bonferroni test were
3 performed to analyze the differences between experimental groups. $p \leq 0.05$ was
4 considered significant.

5 The R Bioconductor packages (www.bioconductor.org, v 3.6) and the limma lmFit
6 function were used to analyze the microarray data as described previously (Alassane-
7 Kpembé et al., 2017a). Probes with adjusted p-value (FDR correction using
8 Benjamini-Hochberg method) ≤ 0.05 were considered to be differentially expressed
9 between conditions. Hierarchical clustering was applied to the samples and probes
10 using the 1-Pearson correlation coefficient as the distance and Ward's criterion for
11 agglomeration. The results are presented as a heatmap of gene expression profiles.
12

3 Results

3.1 Determination of toxicity in proliferative and differentiated cells in response to DON

The comparative effect of DON on proliferative and differentiated cells was first assessed through cytotoxicity assays. Three different tests were used, one measuring LDH release, the second, ATP content, and the third, neutral red uptake. As shown in figure 1A, DON at concentrations of up to 3 μM did not induce LDH release in either proliferative or differentiated cells. Concentrations of 10 and 30 μM DON caused the significant release of LDH in proliferative cells compared to untreated cells ($p < 0.001$). Similarly, concentrations of 30 and 100 μM DON caused the significant release of LDH in differentiated cells ($p < 0.001$). When exposed to 3 μM DON the LDH release in differentiated and proliferating cells were 99.7% and 181% of untreated control respectively. These values increased to 124% and 310% in differentiated and proliferating cells exposed to 10 μM DON and to 192% and 655% in cells exposed to 30 μM of toxins. To conclude, LDH release was significantly higher in proliferative cells exposed to DON than in differentiated cells ($p < 0.01$).

The cytotoxicity of DON was also analyzed using the CellTiter-Glo assay to quantify ATP content. Figure 1B shows that ATP content was not affected by DON at concentrations ranging from 0.01 to 0.3 μM in either proliferating or differentiated cells. By contrast, 1-30 μM DON markedly reduced ATP content in proliferative cells ($p < 0.001$). In differentiated cells, ATP content increased from 1 to 30 μM DON ($p < 0.001$). Only the highest concentration of DON (100 μM) reduced the ATP content ($p < 0.001$). The ATP content in proliferating and differentiated cells exposed to 10 μM DON were 55% and 134% of control; and 54% and 119% of control in proliferating and differentiated cells exposed to 30 μM DON. Thus, this cytotoxicity test also revealed higher sensitivity of proliferative Caco-2 to DON compared to that of differentiated cells.

The neutral red assay is based on selective uptake of eurythodine dye by viable cells and by the staining of their lysosomes. Eurythodine uptake was not affected by the concentrations of DON up to 0.3 μM in either proliferative or differentiated cells.

1 Conversely, uptake decreased significantly when proliferative and differentiated cells
2 were exposed to 1- 30 μM DON and to 30 and 100 μM DON ($p < 0.001$), respectively.
3 However, exposure to 1 to 100 μM DON led to a larger decrease in neutral red uptake
4 in proliferative cells than in differentiated cells ($p < 0.001$) (figure 1C). The neutral
5 red uptake were 95%, 93% and 85% of control in differentiated cells exposed to 3, 10
6 and 30 μM DON respectively; it decreased to 50%, 27% and 34% of control in
7 proliferating cells exposed to the same concentrations of toxins.

8 Using three different tests, we thus demonstrated that the proliferative Caco-2 cells
9 are more sensitive to DON toxicity than differentiated ones.

10

11 3.2 Effect of DON on the establishment and the persistence of the intestinal 12 barrier integrity

13 Using the establishment of TEER as a readout, we next investigated the effect of
14 DON on the differentiation processes. As shown in figure 2A, 10 days of culture were
15 required for the monolayer to start differentiating and for the TEER to increase; the
16 complete differentiation was assessed when the TEER reached a plateau after 16 days
17 of cell culture. Exposure of undifferentiated cells to 0.3 μM DON had no effect on the
18 differentiation process. Exposure to 1 μM slightly delayed the TEER at 16 days and
19 exposure to 3 μM DON delayed the differentiation process between the 14th day and
20 the 22nd day ($p < 0.001$). Nevertheless, after 24 days, the TEER of the monolayers
21 exposed to 0.3 to 3 μM DON was the same as that of untreated cells. Conversely, the
22 treatment of undifferentiated cells with 10 μM of DON did not allow the
23 establishment of TEER. This relates to the toxicity of this concentration of DON
24 observed on proliferation cells (figure 1). On the opposite, no effect on viability was
25 observed on cells treated with 1 or 3 μM DON. Indeed, using DNA staining as a
26 proxy of total cell number, we did not observed any changes throughout the
27 experiment. At day 14, the DNA content of trans-well devices treated with 1 μM and
28 3 μM were $76 \pm 7\%$ and $96 \pm 19\%$ ($p > 0.05$) of control wells respectively. At day 28,
29 the values were $83 \pm 8\%$ and $107 \pm 26\%$ ($p > 0.05$).

1 As shown in figure 2B, DON also modulated the TEER of differentiated cells. Indeed,
2 exposure of differentiated cells to 0.3 and 1 μ M of DON numerically decreased the
3 TEER values but the effect was not statistically significant. By contrast, 3 and 10 μ M
4 of DON reduced the TEER by 22% ($p < 0.05$) and by 98% ($p < 0.001$), respectively.
5 Of note, the decreased TEER observed at 10 μ M on differentiated cells as early as 48
6 hours was not associated with an increased cytotoxicity of differentiated cell to the
7 mycotoxin (figure 1).

8 Taken together, these data indicate that DON differentially influenced the intestinal
9 barrier function during and after differentiation.

10

11 3.3 Comparative gene expression profiles of proliferative and differentiated 12 intestinal epithelial cells in response to DON

13 To gain more insight into the comparative toxicity of DON for proliferative and
14 differentiated intestinal cells, we used a genome wide transcriptomic approach.
15 Microarray analysis identified 4,862 differentially expressed genes (DEG) with a fold
16 change over 2 (FDR p-value ≤ 0.05 , FC ≥ 2).

17 Principal component analysis (figure 3A) clearly separated four different groups and
18 showed that cell status (proliferative versus differentiated) contributed up to 59% of
19 the changes in gene expression, while DON treatment contributed 29%. The Venn
20 diagram (figure 3B) highlights the cell status-dependent changes in gene expression
21 upon exposure to DON. It shows that, among these DEG, 1,659 were specific to the
22 proliferation status, 1,117 were specific to the differentiation status, and 1,043 genes
23 overlap the two phenotypes. Figure 3B also shows that DON modulated 25% more
24 genes in proliferative cells than in differentiated ones (2,702 versus 2,160 DE genes,
25 respectively).

26 The 80 genes with the biggest changes caused by the DON treatment (down- and up-
27 regulated) are shown in figure 3C. The figure also shows that DON had a similar
28 effect on proliferative and differentiated cells but that the amplitude of the effect was
29 higher in proliferative than in differentiated cells. These results were confirmed by

1 RT-qPCR for the representative up-regulated gene ANKRD1 and the representative
2 down regulated gene MYBPH (figure 3D).

3 A supervised hierarchical clustering analysis was then performed using 5,843 probes
4 representing 4,862 differentially expressed genes. The results are shown in figure 4.
5 Four main clusters were identified; two clusters (clusters 1 and 3 containing 1,326 and
6 1,537 probes, respectively) illustrate a cell status effect; while a second set of clusters
7 (clusters 2 and 4 containing 1,348 and 1,632 probes, respectively) illustrates the effect
8 of exposure to DON. Clusters 1 and 3 comprise genes modulated by the
9 differentiation process independently of DON exposure. Clusters 2 and 4 comprise
10 genes whose expression is sensitive to DON whatever the phenotype of Caco-2 cells
11 (figure 4).

12 Functional enrichment via KEGG and GO revealed that genes in cluster 1 are mainly
13 involved in cell growth, including genes involved in the cell cycle in DNA replication,
14 and in cellular senescence. Cluster 3 indicates that the main functions modulated by
15 cell differentiation included genes involved in ECM-receptor interaction, tight
16 junction and metabolism (figure 5). Functional enrichment analysis revealed that
17 cluster 2 is composed of genes involved in several functions including ribosome
18 biogenesis, mRNA surveillance, and the MAPK signaling pathway (figure 5), all
19 linked with the ribotoxic stress. Analysis of cluster 4 revealed that the main pathways
20 down-regulated upon DON exposure are differentiation processes and intestinal
21 functions such as cholesterol metabolism, metabolic pathway, and protein digestion
22 and absorption. We examined 18 genes (8 regulated by cell status and 10 regulated by
23 DON) using qRT-PCR to confirm the changes observed in the microarray (figure 6).
24 For cluster 1, we chose genes involved in the progression of the cell cycle through
25 mitotic events to confirm the effect of differentiation. *PLK1* is a serine/threonine-
26 protein kinase involved in functional maturation of the centrosome in late G2 and in
27 the establishment of the bipolar spindle, whereas *CDC20* (cell-division cycle)
28 activates the anaphase promoting complex (APC/C) that initiates chromatid
29 separation and entrance into anaphase. The serine/threonine kinase *BUB1* plays the
30 role of mitotic checkpoint and cyclinA2 (*CCNA2*) is tightly linked to the progression

1 of the cell cycle. All these characteristic genes in cluster 1 (*PLK1*, *BUB1*, *CDC20*,
2 *CCNA2* genes) were down-regulated in differentiated cells.

3 Selected genes from cluster 3 (such as *CRYL*, *CEL*, *NPNT* and *LAMC2* genes) are
4 involved in cell adhesion and metabolic pathways. The Laminin Subunit Gamma 2
5 (*LAMC2*) is a major constituent of basement membrane, and is involved in cell
6 adhesion, migration, and differentiation processes as well as nephronectin (*NPNT*),
7 ligand belonging to the family of integrins involved in development, differentiation,
8 and adhesion processes. Genes involved in the digestion and absorption of lipid
9 nutrients were also selected: Lambda-crystallin 1 (*CRYL1*) and carboxyl ester lipase
10 (*CEL*). All these genes were up-regulated in differentiated cells.

11 Characteristic genes were selected in cluster 2 (ribosomal RNA small subunit
12 methyltransferase NEP1 (*EMG1*), NIN1 Binding Protein 1 Homolog (*NOB1*) and
13 Serine/threonine-protein kinase (*RIOK2*)) are involved in the maturation of ribosomal
14 subunits (40s and 60s), in ribosome assembly in nucleolus and protein synthesis
15 (RNA-binding protein 28, *RBM28* and RNA Polymerase II subunit A, *POLR2A*). The
16 expression of representative genes in cluster 2 (*EMG1*, *NOB1*, *RIOK2*, *RBM28*,
17 *POLR2A*), confirmed up-regulation of gene expression in response to DON.

18 Genes in cluster 4 are more involved in cell functions; Lipase A (*LIPA*), responsible
19 for cholesterol degradation for use by the cell; *KCNN4* (heterotetrameric voltage-
20 independent potassium channel) which leads to membrane hyperpolarization after its
21 activation and promotes calcium influx, whereas *XPNPEP2* (X-Propyl
22 Aminopeptidase P), involved in collagen, peptide and cytokine degradation, was
23 down-regulated upon DON exposure.

24

1 **4 Discussion**

2 The barrier function of the intestinal epithelium is regulated by the villus and crypt
3 compartments together. Proliferative cells in the crypt are structurally and
4 functionally different from the differentiated enterocytes in the villi (Beaulieu, 1999;
5 Gordon and Hermiston, 1994; Montgomery et al., 1999; Tan and Barker, 2014).
6 Although the adverse effects of mycotoxins, especially those of DON, on the
7 intestinal functions are well described (Payros et al., 2016; Pinton et al., 2015, 2012)
8 the comparative effect of DON, and more generally of any toxic compound, on villus
9 and crypt cells is largely unknown. The aim of this study was thus to describe the
10 effects of DON on proliferative and differentiated intestinal cells by measuring
11 cytotoxicity, trans-epithelial electrical resistance (TEER), and gene expression
12 profiles. This comparison was performed on the human Caco-2 enterocyte-like model,
13 as these cells spontaneously differentiate to polarized monolayer with the
14 morphological and biochemical properties of small intestinal enterocytes (Luo et al.,
15 2019; Pinton et al., 2009; Sambruy et al., 2001).

16 In the present study, we observed that DON caused higher toxicity in proliferating
17 cells than in differentiated ones. We also observed that DON at concentration of 1 μM
18 and above delayed the differentiation process. In line with our results, Wang and al.,
19 showed that DON at 5 μM reduced protein expression of claudin-3, 4 and occludin in
20 proliferative cells, whereas it only reduced protein expression in claudin-4 in
21 differentiated cells (Wang et al., 2019). Two other studies reported similar toxicity of
22 zinc in Caco-2 cells whatever their differentiation status (Scarino et al., 1992; Zödl et
23 al., 2003).

24 We compared the cytotoxicity of DON for proliferative and differentiated Caco-2
25 cells using three different methods, LDH release, ATP content and neutral red uptake.
26 Whatever the test, proliferative cells were found to be more sensitive to DON than
27 differentiated cells. The high sensitivity of proliferative cells to DON has already
28 been reported in Caco-2 cells as well as in the IPEC-J2 porcine intestinal epithelial
29 cell line (Awad et al., 2012; Pierron et al., 2016; Ying et al., 2019). The greater

1 sensitivity of proliferative cells could be due to the primary feature of DON toxicity.
2 Indeed, the ribotoxic stress response-mediated inhibition of protein synthesis (Pan et
3 al., 2014; Zhou et al., 2014), is more important for dividing cells than for
4 differentiated cells as proliferating cells need a large amount of RNA, DNA and
5 protein synthesis (Fortin-Magana et al., 1970, Pestka et al 2010).
6 Perturbation of proliferation affects cell migration along the villus. Indeed, using cell-
7 labelling, Parker et al. (2017) demonstrated a relation between cell proliferation rates
8 and epithelial cell migration velocities and concluded that cell proliferation within the
9 crypt is the primary force driving cell migration along the villus. These toxic effects
10 may cause morphological alterations of the intestinal tissue. Alterations in villus
11 architecture (increased villus fusion and shorter villi) associated with necrosis and
12 edema have already been observed both in intestinal explants (Pinton et al., 2012) and
13 *in vivo* (Luo et al., 2019).
14 Measurement of TEER was used to assess the effect of DON on the differentiation
15 process as well as on the establishment of mature and functional monolayer. In
16 differentiated Caco-2 cells, TEER values were significantly reduced after 20 days
17 exposure to 3 and 10 μ M DON, as previously described in several intestinal cell lines
18 (Luo et al., 2019; Maresca et al., 2002; Pinton et al., 2009). This reduced intestinal
19 barrier function was associated with the disruption of tight junctions, mediated by
20 activation of DON-induced MAPKs (Pinton et al., 2010; Springler et al., 2016).
21 Increased intestinal permeability exposes the sub-epithelial tissues to luminal
22 pathogens including bacterial translocation and can trigger inappropriate immune and
23 inflammatory responses (Alassane-Kpembi et al., 2017b; Cano et al., 2013). In the
24 proliferative cells, low doses of DON (1–3 μ M) delayed the establishment of the
25 TEER. We hypothesize that this will reduce the renewal and repair of the epithelium
26 and reduce intestinal absorption and the barrier function (Piche, 2014; Wu et al.,
27 2014). It is noteworthy that these effects can be linked to the growth deficiency and
28 anorexia observed in presence of DON (Terciolo et al., 2018; Yunus et al., 2012) but
29 also to the susceptibility of the host to develop bacterial infections and intestinal
30 chronic disorders (Maresca, 2013; Payros et al., 2020).

1 A transcriptome analysis was performed to further delineate the toxicity of DON in
2 differentiated and proliferative cells. We observed that DON modulates the
3 expression of a larger number of genes (25% increase) in proliferative cells than in
4 differentiated intestinal cells. The amplitude of the effect was also higher in
5 proliferative than in differentiated cells.

6 In response to DON exposure, genes involved in ribosome biogenesis, mRNA
7 surveillance, and in the MAPK signaling pathway were up-regulated in both
8 proliferative and differentiated Caco-2 cells. Indeed, DON-induced ribotoxic stress
9 impacts the regulation of the transcription as well as epigenetic factors, which may
10 affect all the processes of RNA maturation and translation (mRNA surveillance,
11 ribosome biogenesis, and mRNA transport). Among the genes involved in these
12 signaling pathways, ribosome biogenesis is a fine-tuning of translation that likely
13 occurs early before the onset of translation inhibition (Pan et al., 2013). We observed
14 that DON increased gene expression of RNA biosynthesis (*EMG1*), RNA processing,
15 and RNA maturation (*NOB1*, *WDR33*, *RIOK2*) as well as of Eukaryotic Initiation
16 Factors (eIFs) kinases that control the translation initiation process (Roux and
17 Topisirovic, 2018). DON controls gene expression by activating PKR that inhibits
18 global translation but induces specific translation of pro-inflammatory genes by
19 phosphorylating eIF2 α (Piazzi et al., 2019). Modulation of transcriptional activity is a
20 hallmark of cell response to stress. Integrated stress response leads to the restoration
21 of cellular homeostasis to compensate for excessive apoptosis and for the reduced
22 proliferation rate observed in intestinal epithelia cells, which, in turn, leads to a delay
23 in TEER establishment. If this stress cannot be resolved and general translation
24 remains inhibited, the cell will likely die due to the apoptosis mechanism (Piazzi et al.,
25 2019). In addition, increased expression of some genes involved in mRNA transport
26 (*PRMT5*) was observed after DON exposure. PRMT5-regulated processes caused
27 MAPk activation. An increase in the number of genes involved in these pathways was
28 associated with increased cytotoxicity in proliferative cells. What is more, ribotoxic
29 stress induced by DON binding to ribosomes can rapidly activate the MAPK signaling

1 pathway leading to inflammation or ribosome RNA cleavage and hence, to cell
2 apoptosis (He et al., 2012; Pestka, 2008).

3 Genes involved in cholesterol metabolism, protein digestion and absorption, and in
4 metabolic pathways were down-regulated upon DON exposure. Cholesterol is a key
5 component of cell membrane and is therefore necessary for cell growth, proliferation
6 and differentiation, and excessive synthesis of cholesterol induced differentiation
7 defects has been reported (Stange et al., 1988; Suzuki et al., 2019). In the present
8 study, the DON-suppression of genes involved in cholesterol efflux such as *LIPA* and
9 *CYP27A1* might lead to accumulation of cholesterol in cells, thereby inhibiting cell
10 differentiation.

11

12 **5 Conclusion**

13 Our study highlights the fact that the state of differentiation of the cell layer is an
14 important determinant of the response to exogenous substances and food
15 contaminants. DON affects not only the ability of Caco-2 cells to proliferate but also
16 their ability to form an impermeable barrier to protect the underlying tissue. Defective
17 epithelial differentiation and altered function of the mucosal barrier may lead to the
18 translocation of luminal bacteria within the body and potentially to chronic disorders
19 such as inflammatory bowel disease. Disturbance of these processes can result in
20 chronic inflammation and in anorexigenic effects of DON (Payros et al., 2020; Pinton
21 and Oswald, 2014).

22

23 **Acknowledgements:** We are grateful to Ms Goodfellow for the English correction.

24 This work was supported, in part, by the Genofood ANR project (19-CE34). Su Luo
25 was supported by the China Scholarship Council.

26

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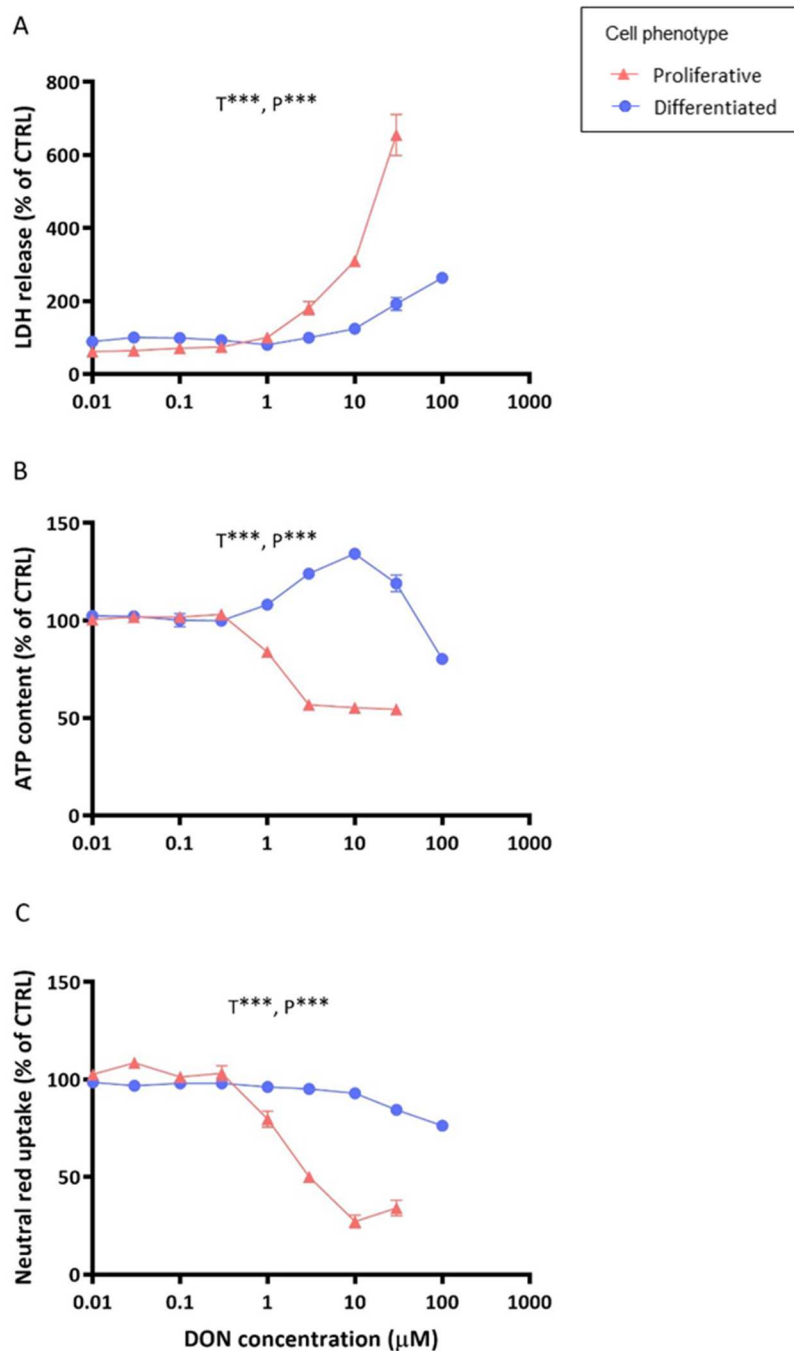
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2 Figure 1. Cytotoxicity of DON on proliferative and differentiated Caco-2 cells

3 Cells were seeded in 96-well plates and cultured for 2 d (proliferative cells) or 21 d

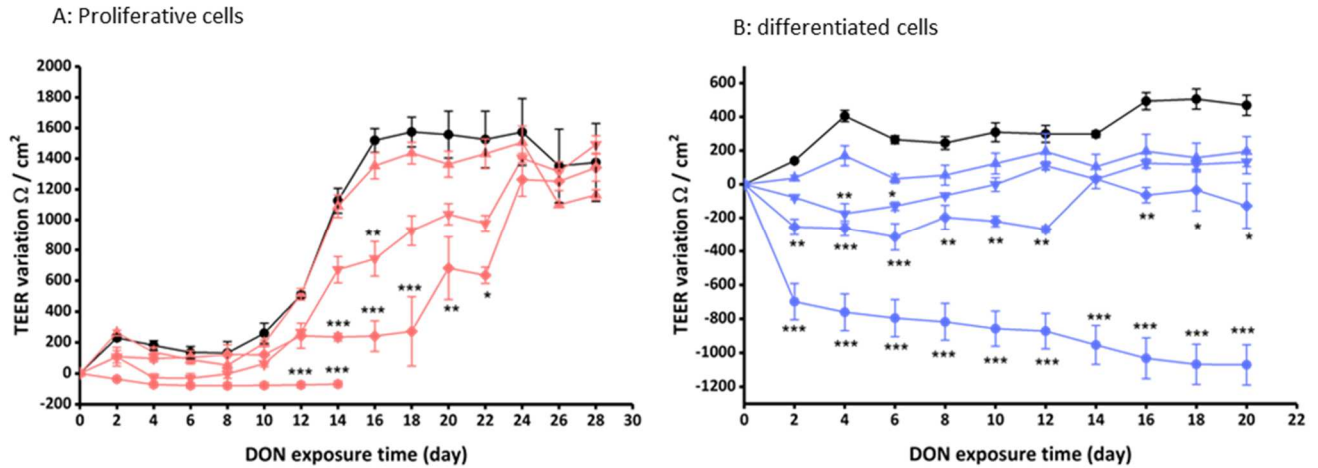
4 (differentiated cells) before DON treatment. After 48h, LDH release (A), ATP content

5 (B) and neutral red uptake (C) were measured. Values are expressed as percent of

6 untreated cells. Results are expressed as the mean of 3 independent experiments \pm

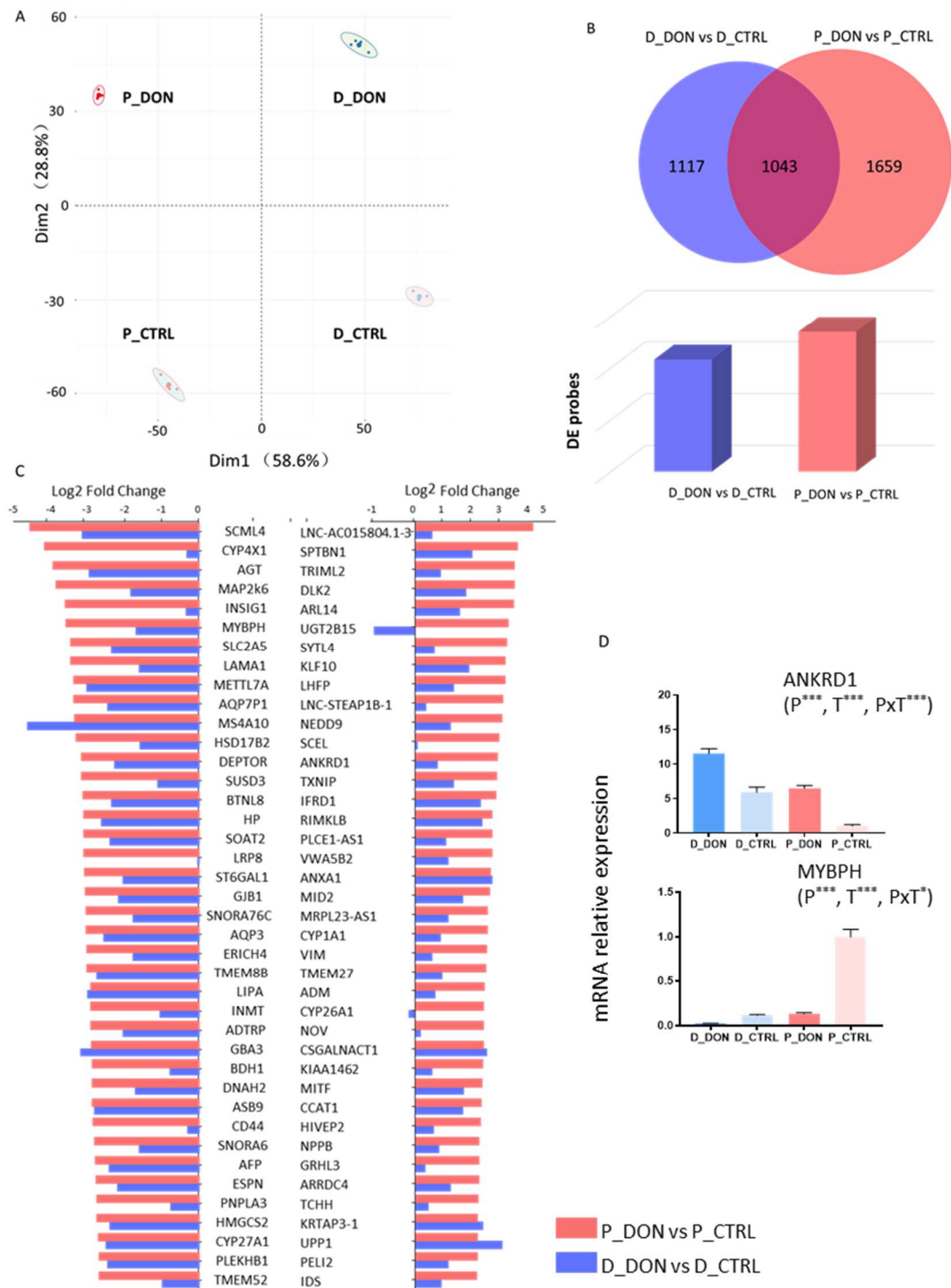
7 SEM. Data were analyzed by two-way ANOVA with Bonferroni post-test, T:

8 treatment effect; P: phenotype effect, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



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Figure 2. Effects of DON on the establishment and maintenance of TEER in Caco-2 cells. Proliferative (A) or differentiated (B) Caco-2 cells cultivated on insert were treated apically with 0 (●), 0.3 (▲), 1 (▼), 3 (◆) and 10 (●) μM DON, and the TEER measured. Results are expressed as TEER variation after DON addition, mean of 3 independent experiments ± SEM. Data were analyzed by non-parametric one-way ANOVA; *p < 0.05; **p < 0.01; ***p < 0.001.

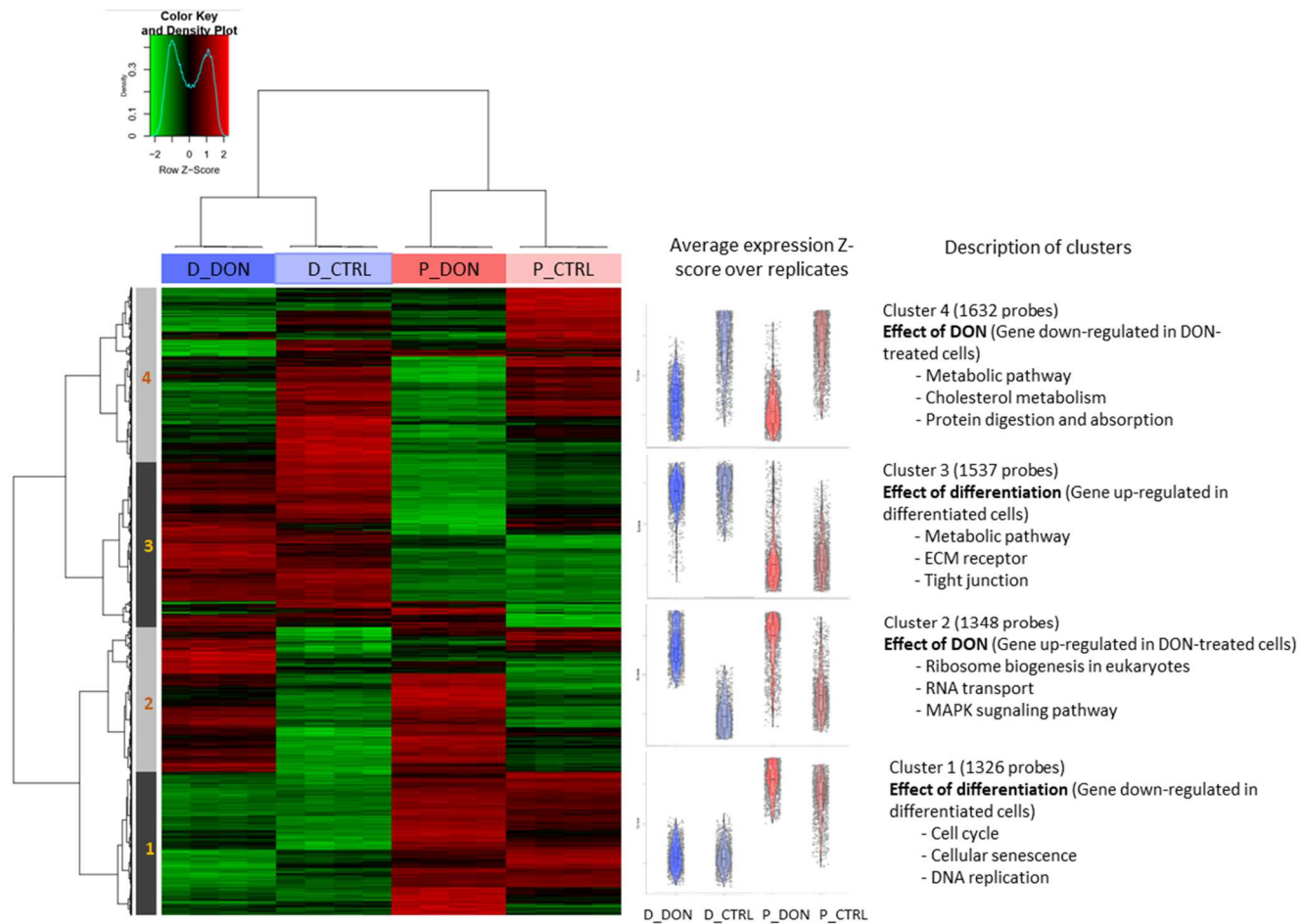


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3 Figure 3. Comparative effect of DON on the genes expression of proliferative and
 4 differentiated intestinal epithelial cells.
 5 Proliferative and differentiated cells were exposed to 3 μ M DON for 24h. (A)
 6 Principal component analysis of samples relationship. (B) Venn diagram comparing
 7 differentially expressed genes (FDR p-value \leq 0.05, FC \geq 2) in response to DON

1 exposure in proliferative and differentiated Caco-2 cells. Number of DE genes in the
2 differentiated and proliferative cells upon DON exposure (bottom panel). (C) Top 40
3 significantly down-regulated and up-regulated genes in proliferative (red bars) and
4 differentiated (blue bars) cells. The values expressed as fold change (in log₂)
5 relatively to control cells. (D) Relative mRNA expression by qPCR of two
6 representative genes: ANKRD1 (up-regulated gene) and MYBPH (down-regulated
7 gene). Data were analyzed by non-parametric t-test; T: treatment effect; P: phenotype
8 effect, TxP: interaction *p < 0.05; **p < 0.01; ***p < 0.001.

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3 Figure 4. Heat-map depicting the differentially expressed genes between proliferative
 4 and differentiated Caco-2 cells exposed to DON.

5 Proliferative and differentiated cells were exposed to 3 μ M DON for 24h. (A) Gene
 6 expression was analyzed with a 60K microarray. Red and green indicate values above
 7 and below the mean averaged centered and scaled expression values (Z score),
 8 respectively. Black indicates values close to the mean. The probe clustering (left
 9 panel), delineated 4 gene clusters that are described on the right of the heatmap.

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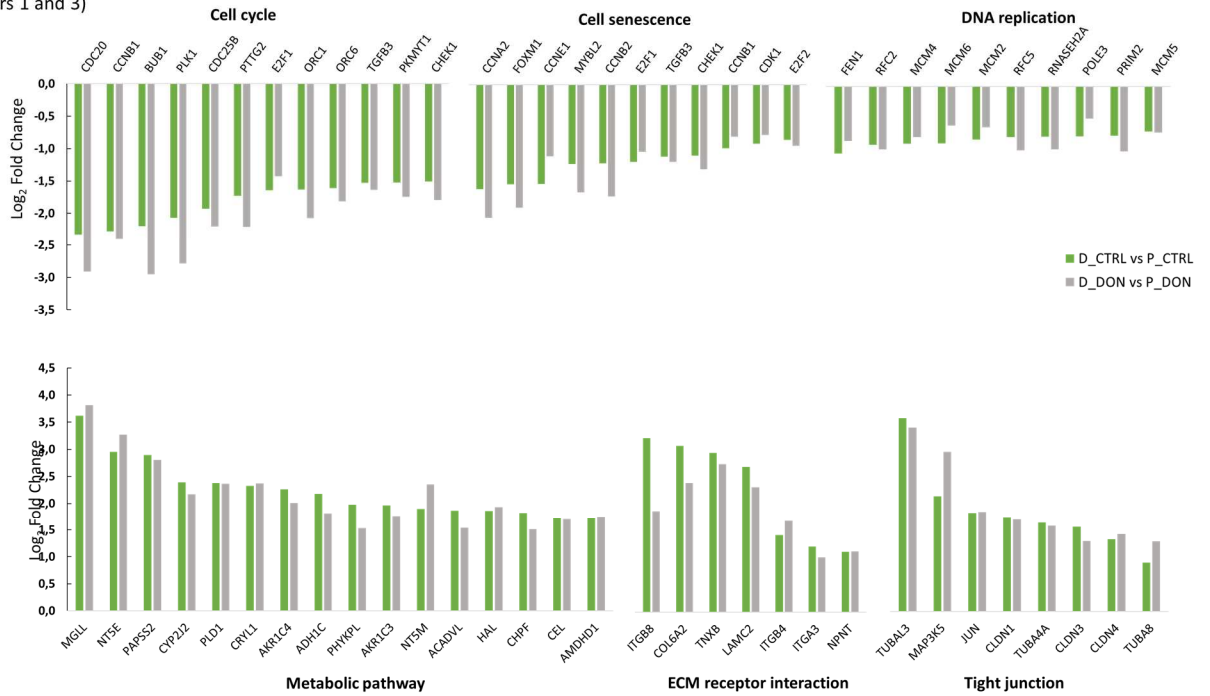
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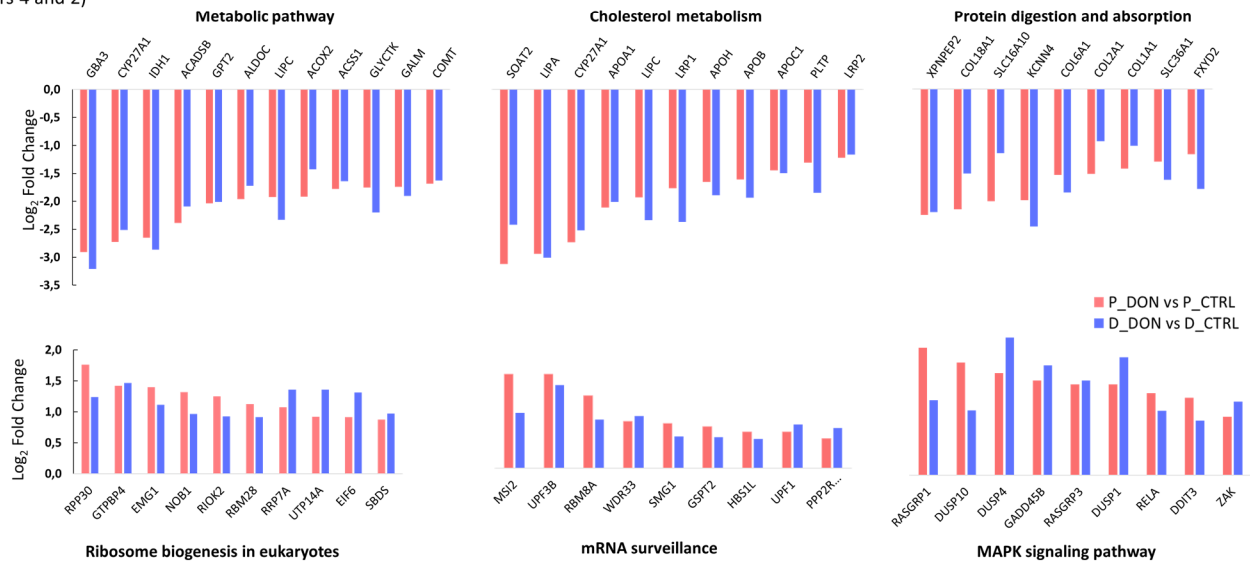
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Effect of differentiation
(Clusters 1 and 3)



Effect of DON
(Clusters 4 and 2)

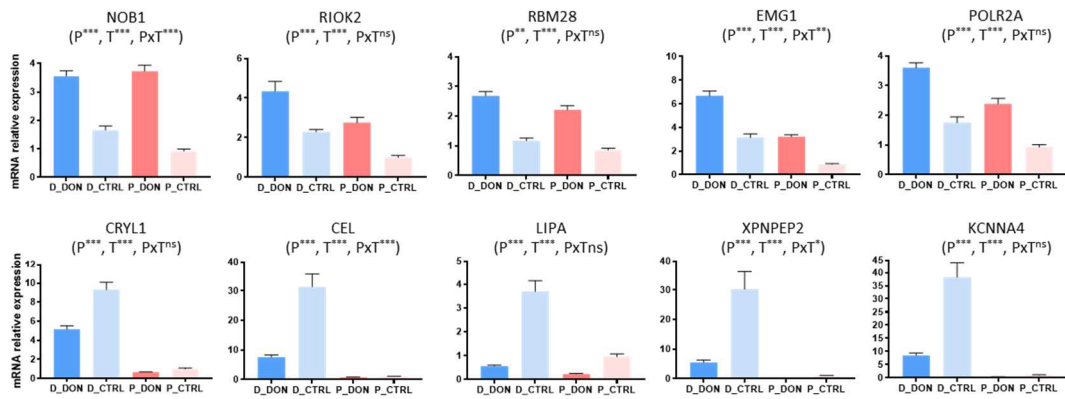


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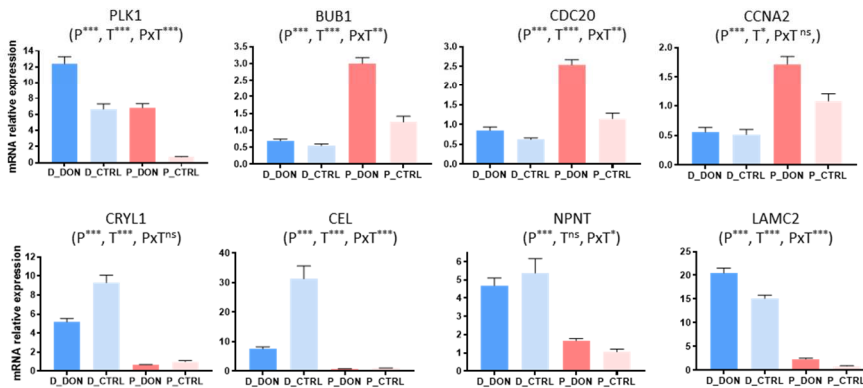
2 Figure 5. Gene expression analysis of the different clusters.

3 Proliferative and differentiated cells were exposed to 3 μ M DON for 24h and gene
 4 expression was analyzed by microarray. 4 clusters depicting the effect of the cell
 5 status (cluster 1 and 3) and the effect of the DON treatment (cluster 2 and 4) were
 6 identified by a supervised hierarchical clustering analysis. Enriched KEEG pathways
 7 ($p \leq 0.05$) was used to analyze in each cluster the relative mRNA expression of
 8 differentiated genes each cluster are presented.

Effect of DON (Clusters 2 and 4)



Effect of differentiation (Clusters 1 and 3)



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3 Figure 6. qPCR analysis of representative genes from each cluster.

4 Proliferative and differentiated cells were exposed to 3 μ M DON for 24h and gene

5 expression was analyzed by qPCR. Results are expressed as the mean of 4

6 independent experiments \pm SEM. Data were analyzed by two-way ANOVA after log

7 transformation, T: treatment effect; P: phenotype effect, TxP: interaction *p < 0.05;

8 **p < 0.01; ***p < 0.001.

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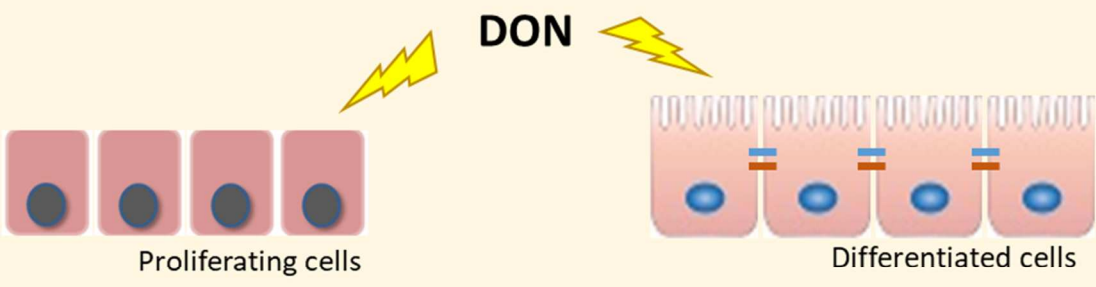
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Gene symbol	Gene name	Primer sequence	Reference
<i>TREML2</i>	Triggering Receptor Expressed on Myeloid cells Like 2	F: CATCTGGACAACATCCTCAA R: CCATCACACCAGTGGTAAA	ENST00000483722.2
<i>ANKRD1</i>	Ankyrin D1	F: CAATCCAGATGTTTGTGATGAG R: GCTCCAGCTTCCATTAAC	ENST00000371697.3
<i>PLK1</i>	polo-like kinase 1	F: CAATCCAGATGTTTGTGATGAG R: GCTCCAGCTTCCATTAAC	ENST00000300093.9
<i>BUB1</i>	Mitotic Checkpoint Serine/Threonine Kinase	F: CCACAATGACCCAAGATTCA R: GGATGACAGGGTTCCAATC	ENST00000302759.11
<i>MYBPH</i>	Myosin Binding Protein H	F: CATCGGCAACTCGTACTC R: CTGAGAAGTCTCGCTCAATAA	ENST00000255416.9
<i>CDC20</i>	Cell Division Cycle 20	F: AAGACCTGCCGTTACATTC R: ACATTCCCAGAACTCCAATC	ENST00000372462.1
<i>CCNA2</i>	Cyclin A2	F: ACTGCTGCTATGCTGTTAG R: ACTTGTTTCTTGGTGTAGGT	ENST00000618014.1
<i>LAMC2</i>	laminin gamma2	F: GGCTCACCAAGACTTACAC R: CGCAGTAACCTTCGATACTC	ENST00000264144.5
<i>NPNT</i>	Nephronectin	F: CGATGCAAACATGGTGAATG R: GCATAACCAGGATGACACTT	ENST00000379987.6
<i>CRYL</i>	Crystallin Lambda 1	F: ACATTGAGCAACAGCAGATA R: AGGGAGCCTTTCAGAGAA	ENST00000298248.12
<i>CEL</i>	Carboxyl Ester Lipase	F: AGACTGCCTGTACCTCAA R: CCTCCATAGATCCAGATCATAAC	ENST00000673714.1
<i>EMG1</i>	Ribosomal RNA Small Subunit Methyltransferase NEP1	F: ACACAAGCTCAGTGTTTCG R: TGCCAACCTTTCATACATCCA	ENST00000599672.6
<i>NOB1</i>	NIN1 (RPN12) Binding Protein 1 Homolog	F: GGTTCAAGGAGCCCTTAC R: CAACAACTCTGCTTCCAAC	ENST00000268802.10
<i>RIOK2</i>	RIO Kinase 2	F: TCACAGACTAGGAAGAACCT R: TGGCAGAGAGACGAGATAA	ENST00000283109.8
<i>RBM28</i>	RNA Binding Motif Protein 28	F: CAGAAGAGAGCAGTCAAGAG R: AGTTCCTCTCCATCATCAATAC	ENST00000223073.6
<i>POLR2A</i>	RNA Polymerase II Subunit A	F: GAGGACTCTCAGGAGAAGAA R: CCATGCCCAGCACAAA	ENST00000572844.1
<i>LIPA</i>	lipase A	F: CTGTGTGGATTTAATGAGAGAAA R: CTGGCTCCAGTGTAAACAT	ENST00000456827.5
<i>XPNPEP2</i>	X-Prolyl Aminopeptidase 2	F: AGGGCAGGATGTGAGAAA R: GGATGATGTAGGCTGAGAGA	ENST00000371106.4
<i>KCNN4</i>	Potassium Calcium-Activated Channel subfamily N member 4	F: GAGAGGCAGGCTGTTAATG R: GATGGTCAGGAATGTGATGG	ENST00000648319.1
<i>GAPDH</i>	Glyceraldehyde-3-Phosphate dehydrogenase	F: TCAAGGCTGAGAACGGGAAG R: CCACTTGATTTTGGAGGGATCTC	ENST00000229239.1
<i>PSMB6</i>	Proteasome Subunit Beta-Type 6	F: CAGAACAACCACTGGGTCCTACA R: AGCAGAAAATGCGGTCGTG	ENST00000270586.8
<i>ACTIN</i>	Actin Beta	F: AGGCCCCCTGAACCC R: ATCACGATGCCAGTGGTACG	ENST00000432588.17

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2 Table 1. Nucleotide sequences of primers for real-time qPCR



+++	Cytotoxicity	+
Delayed establishment	TEER	↓
+++	Gene expression	+
	Number of gene modified	
+++	Amplitude of the effect	+

- Pathways modified**

 - Metabolic pathway
 - Cholesterol metabolism
 - Protein digestion and absorption

 - Ribosome biogenesis in eukaryotes
 - mRNA surveillance
 - MAPK signaling pathway