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Abbreviations: hydroxyphenylacetic acid, HO-PAA; N-acetyl cystein, NAC; phenylacetic acid, PAA; reactive oxygen species, ROS

Abstract

Background: 4-hydroxyphenylacetic acid (HO-PAA) is produced by intestinal microbiota from L-tyrosine. High concentrations in human fecal water have been associated with cytotoxicity, urging us to test HO-PAA's effects on human colonocytes. We compared these effects with those of phenylacetic acid (PAA), phenol and acetaldehyde, also issued from amino acids fermentation.

Methods: HT-29 $Glc^{-/+}$ human colonocytes were exposed for 24h to metabolites at concentrations between 350-1000µM for HO-PAA and PAA, 250-1500µM for phenol and 25-500µM for acetaldehyde. We evaluated metabolites'cytotoxicity with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and DNA quantification assays, reactive oxygen species (ROS) production with H2DCF-DA, and DNA damage with the comet assay. We measured cell oxygen consumption and mitochondrial complexes activity by polarography.

Results: Although HO-PAA displayed no cytotoxic effect on colonocytes, it decreased mitochondrial complex I activity and oxygen consumption. This was paralleled by an increase in ROS production and DNA alteration. Cells pretreatment with N-acetylcysteine, a ROS scavenger, decreased genotoxic effects of HO-PAA, indicating implication of oxidative stress in HO-PAA's genotoxicity. PAA and phenol did not reproduce these effects, but were cytotoxic towards colonocytes. Last, acetaldehyde displayed no effect in terms of cytotoxicity and mitochondrial metabolic activity, but increased DNA damage.

Conclusions: Several bacterial metabolites produced from amino acids displayed deleterious effects on human colonocytes, in terms of genotoxicity (HO-PAA and acetaldehyde) or cytotoxicity (PAA and phenol).

General significance: This study helps understanding the consequences of intestinal microbiota's metabolic activity on the host since amino acids fermentation can lead to the formation of compounds toxic towards colonic epithelial cells.

Keywords
Colonocytes, bacterial metabolites, DNA damage, oxidative stress, cell respiration

1. Introduction

The colonic epithelium is facing the luminal content, which is characterized by a complex mixture of microorganisms and bacterial metabolites, resulting from the metabolic activities of these microorganisms towards undigested and partially digested dietary as well as endogenous compounds. Human dietary intervention studies and work with animal models have revealed that diet impacts the luminal environment of the colonic and rectal epithelial cells, notably regarding the concentrations of many bacterial metabolites (Blachier et al., 2017). More specifically, the amount and sources of undigestible polysaccharides and dietary proteins have been shown to impact, along with other parameters such as carbohydrate depletion in the colon, increased pH and prolonged transit time, the fecal concentrations of several amino acid-derived bacterial metabolites (Portune et al., 2016; Smith & Macfarlane, 1996). Such modifications can in turn impact the large intestine epithelium in terms of renewal, energy metabolism, DNA integrity or barrier function, with either positive or negative consequences (Russell, Hoyles, Flint, & Dumas, 2013). It is generally admitted that most of metabolites derived from undigestible polysaccharides, like short-chain fatty acids and particularly butyrate, display rather positive effects on the colonic epithelium in terms of energy metabolism, anti-inflammatory properties and gene expression regulation (Yin, Laevsky, & Giardina, 2001). On the contrary, metabolites originated from protein-derived amino acids, are for most (but not all) of them thought to have negative effects, when present in excess, on the colonic epithelial cell metabolism. For instance, *p*-cresol, ammonium or hydrogen sulfide have been shown to decrease oxygen consumption in colonocytes (Andriamihaja et al., 2015; Beaumont et al., 2016). However, few studies have been performed to document how changes of bacterial metabolite concentrations in vicinity of the colonic epithelial cells may impact the colonic epithelium in terms of beneficial over deleterious effect ratio (Nistal, Fernández-Fernández, Vivas, & Olcoz, 2015).

In a previous clinical study (Beaumont, Portune, et al., 2017), we evaluated the cytotoxicity of fecal samples recovered from volunteers on human colonocytes. We observed that the samples with the highest cytotoxicity were characterized, among other molecules, by higher contents of 4-hydroxyphenylacetic acid (HO-PAA) when compared with samples with lower cytotoxicity, suggesting that this compound may play a role in the overall cytotoxicity of the fecal water samples. This urged us to test this bacterial metabolite on colonic epithelial cells, since no study has investigated its individual effects as far as we know. HO-PAA is a bacterial metabolite produced from L-tyrosine (Windey, De Preter, & Verbeke, 2012) but it can also be derived from tea or berries polyphenols (Gao et al., 2006; Gill et al., 2010). Although evaluating the respective contributions of proteins and plants to HO-PAA's origin remains a difficult task (Chun, Chung, & Song, 2007; Institute of Medicine (U.S.) & Institute of Medicine (U.S.), 2005), a major part of HO-PAA is likely to be derived from proteins. Its colonic concentration has been evaluated by several studies, and the measured concentrations are usually in the micromolar range (Gill et al., 2010; Grün et al., 2008; Jenner, Rafter, & Halliwell, 2005; Russell et al., 2011). It is worth noticing that this concentration strongly differed between individuals, reaching up to 50 µM in some individuals without any particular dietary condition (Gill et al. 2010). In addition, the luminal concentration of HO-PAA is known to be affected by the diet (Gill et al., 2010). It is thus predictable that HO-PAA's concentration might, in some peculiar dietary situations, like the consumption of high-protein diet, reach concentrations well above 50 µM luminal concentration.

HO-PAA's chemical structure is very similar to the one of phenylacetic acid (PAA), another bacterial metabolite, except for a supplementary hydroxyl group. Then, we hypothesized that HO-PAA and PAA may exert common effects on colonocytes. PAA is a bacterial metabolic

end product of phenylalanine (Smith & Macfarlane, 1996). It is also known as an uremic toxin (Neirynck et al., 2013), like *p*-cresol that is produced by the microbiota from tyrosine, and which turned out to be deleterious for the colonic epithelium (Andriamihaja et al., 2015). The effects of PAA on the colonocytes are unknown. PAA colonic concentrations have been measured in several studies, with a wide range from 0.33 to 479 µM according to the different studies (Gao et al., 2006; Gill et al., 2010; Jenner et al., 2005; Karlsson et al., 2005; Russell et al., 2011). Interestingly, PAA fecal concentration increases in humans following high-protein diet consumption (Russell et al., 2011).

In addition to the study of HO-PAA and PAA, we decided to investigate the effects of phenol on colonocytes, as phenol is another (and the main) bacterial metabolite produced from tyrosine (Bone, Tamm, & Hill, 1976). Phenol has been classified as mutagen 2 by the European Chemicals Agency ('Phenol - Substance Information - ECHA', n.d.). However, toxicological studies on phenol have, so far, been mainly focused on cutaneous or pulmonary exposure, with the effect of luminal phenol on the colon epithelium being poorly investigated. Its colonic concentration in humans is highly variable, ranging from 58 up to 3000 µM (Verbeke et al., 2015). Besides, phenol concentration has been demonstrated to be dependent on the diet (Paturi et al., 2012; Taciak, Barszcz, Tuśnio, & Pastuszewska, 2015). *In vitro*, using a human epithelial cell line, it has been shown that phenol decreases epithelial barrier function (McCall et al., 2009).

Lastly, we evaluated the impact of acetaldehyde on our cellular model, as acetaldehyde represents the main bacterial metabolite produced from ethanol (Pöschl & Seitz, 2004) and since acetaldehyde can be also produced from pyruvate (Gray, Tompkins, & Taylor, 2014). This latter intermediary metabolite can be produced from several amino acids, including tyrosine (Kumagai, Utagawa, & Yamada, 1975). Accordingly, high-protein diet can increase the ethanol content in the colonic content of rats without any consumption of ethanol (Liu et al. 2014). Acetaldehyde has been classified by the international agency for research in cancer as probably carcinogen (2B carcinogen) when alone and known carcinogen (1 carcinogen) in combination with alcohol consumption (IARC, 1999). Acetaldehyde concentration in the colon ranges from 8 to 118 µM in the absence of ethanol consumption in the piglet model (Jokelainen, Matysiak-Budnik, Mäkisalo, Höckerstedt, & Salaspuro, 1996). However, in the rat model, acetaldehyde concentration can represent as much as 387 µM in the colon of rats after ethanol consumption (Homann, Tillonen, & Salaspuro, 2000). The effects of acetaldehyde on the colonic epithelium have been little studied but an *in vitro* study indicates that acetaldehyde disrupts epithelial barrier function in the Caco-2 cell line model (Elamin, Masclee, Dekker, & Jonkers, 2013).

In order to evaluate the effects of these 4 amino acid-derived bacterial metabolites on colonocytes, we used the human colonic adenocarcinoma cell line HT-29 $Glc^{-/+}$. These cells have retained major metabolic characteristics of normal colonocytes (Leschelle, Delpal, Goubern, Blottière, & Blachier, 2000). Cells were exposed to plausible concentrations of the bacterial metabolites for 24 hours. We investigated several cellular parameters which are known to be interconnected (Saha et al. 2017; Zorov et al. 2014), namely, mitochondrial activity, oxygen consumption, reactive oxygen species net production and DNA integrity alterations.

2. Materials and methods

2.1 Chemicals

Most chemicals including HO-PAA, PAA, phenol, acetaldehyde and N-acetyl cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). When not specified otherwise, chemicals were dissolved in ultrapure water.

2.2 Cell culture and exposure to bacterial metabolites

HT29-Glc$^{-/+}$ cells were cultured at 37°C under 5% CO_2 atmosphere in DMEM medium (ThermoFischer Scientific, Waltham, MA, USA) complemented with 10% (v/v) heat inactivated fetal bovine serum (GeLifeSciences, Chicago, IL, USA) and 4 mM L-glutamine (ThermoFischer Scientific). Cells were used between passages 43 and 60.
Cells were seeded into 96-well plates for cytotoxicity assays (6 wells per condition), into 24-well plates for comet assay (1 well per condition), into 12 well-plates for oxidative stress measurements (4 wells per condition), into 6-well plates for gene expression measurements (1 well per condition), and into 75 cm² flasks for oxygen consumption measurements, including respiratory complexes activity measurements (1 flask per condition).
Cells were exposed to the 4 metabolites dissolved in culture medium used at different concentrations for 24 hours, with cell confluency being around 90% at the end of the exposure period. These concentrations, prepared in culture medium, were respectively: 350-1000 µM for HO-PAA and PAA, 250-1500 µM for phenol and 25-500 µM for acetaldehyde. For the genotoxicity and the oxygen consumption experiments (including respiratory complexes activity), several cell samples were pretreated with 0.5 mM NAC (dissolved in culture medium) during 24 hours before exposure to HO-PAA. This concentration of NAC was not cytotoxic in our cell model and efficient in significantly reducing oxidative stress.

2.3 Cytotoxicity

Cytotoxicity was assessed using 2 complementary tests after metabolites exposure. First, we used the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, which measures mitochondrial activity. This test was also used to check the absence of NAC toxicity on colonocytes. To do so, after metabolites exposure, cell medium was replaced by 0.5 mg/mL MTT diluted in DMEM (from a 5 mg/mL PBS-dissolved solution). After a 1-hour incubation at 37°C, formazan crystals were dissolved in 100 µL dimethyl sulfoxide/well. Absorbance was measured at 570 nm (reference wavelength at 630 nm) and cell viability was determined as percentage of absorbance of the negative control (cells exposed to culture medium). The FluoReporter Blue Fluorimetric dsDNA quantitation kit (ThermoFischer Scientific) was used to measure the DNA content of adherent cells, according to manufacturer's protocol, with the results presented as percentage of untreated adherent control cells. For both protocols, positive control was obtained by replacing the medium of dedicated unexposed cells by 0.1% X100 triton DMEM-diluted solution for 30 minutes.

2.4 Oxidative stress measurement

Oxidative stress was evaluated through measurement of intracellular ROS formation, using the fluorescent 20,70-dichlorodihydrofluorescein diacetate acetyl ester (H2DCF-DA, ThermoFischer Scientific) probe.
After metabolites exposure, cells were washed twice with PBS and incubated for 30 min at 37°C with 80 µM H2DCF-DA (by diluting 8 mM DMSO-dissolved H2DCF-DA solution in culture medium). They were then harvested by scraping in 300 µL PBS. Two hundred microliters of each cell suspension were transferred for the immediate measurement of fluorescence intensity with excitation at 480 nm and emission at 530 nm, on an Infinite 200Pro spectrophotometer (Tecan, Männedorf, Switzerland). Relative fluorescence was normalized with respect to protein concentration, which was measured using the DC Protein Assay (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. Positive control was obtained by replacing the medium of dedicated unexposed cells by 500 µM H_2O_2 DMEM-diluted solution for 1 hour; and in order to demonstrate NAC ability to reduce oxidative stress, some cells were pretreated with 0.5 mM NAC for 24 hours before being exposed to H_2O_2 in the same conditions as positive controls.

2.5 Oxygen cell consumption

Oxygen consumption was measured by polarography, using a 2K Oroboros oxygraph (Oxygraph Hansatech Inst., Norfolk, UK) and a Clark-type electrode. Concerning the exposure conditions, in some experiments cells were pretreated with 0.5 mM NAC for 24 hours before treatment with HO-PAA for 24 hours. Cells were then isolated by trypsination, and 5×10^6 cells were resuspended in 2 mL air-saturated incubation mixture (20 mM Hepes buffer containing 200 mM mannitol, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.4, enriched with 0.1% bovine serum albumin) and placed in the oxygraph chamber at 37°C for oxygen consumption measurement. Oxygen consumption rates were obtained directly from the Datalab 4 software, and were calculated as the negative time derivative of oxygen concentration in the closed respirometry chamber; respiratory fluxes being corrected automatically for instrumental background by DatLab taking into account oxygen consumption of the oxygen sensor and oxygen diffusion out of or into the oxygraph (Gnaiger, 2001).

For intact cells, after stabilization of the basal oxygen consumption, which is considered as the 100% reference value, oligomycin (0.5 µg/mL), an inhibitor of mitochondrial ATP synthase (complex V), was added. This compound arrests mitochondrial respiration at a resting level. Oxygen flux measured in this leak state reflects the proton leak or futile respiration at maximum mitochondrial membrane potential. When the oxygen flux was stable, the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1.5 µg/mL) was added. This FCCP concentration was shown to induce optimal uncoupling conditions in our preliminary experiments on HT29 $Glc^{-/+}$ cells. Uncouplers dissipate the mitochondrial membrane potential and yields the maximum stimulated respiration referred to as the maximal respiratory capacity.

2.6 Activity of the mitochondrial respiratory complexes

We then investigated the activity of the mitochondrial respiratory complexes, using the cellular oxygen consumption method we described above before and after adding the inhibitors specific for each complex.
Cells were first placed in the glass oxygen chamber and permeabilized by adding digitonine (50 µg/5.10^6 cells). Basal mitochondrial oxygen consumption was measured in the presence

of 10 mM succinate. After the stabilization on this oxygen consumption, oxidative phosphorylation capacity was measured after addition of a saturating concentration of 1.5 mM ADP. Then 5 µM rotenone was added to inhibit complex I. When the respiratory rate was stabilized, 0.5 µg/ml oligomycin was added in the respiratory medium. When the resulting drop in oxygen consumption rate was stabilized, FCCP (1.5 µg/mL) was added. After the new stabilization of this high oxygen consumption rate, antimycin A (2 µg/mL) was then added to inhibit complex III. It led to a decrease of oxygen consumption. Complex IV activity was then estimated by adding concomitantly 4 mM ascorbate and 0.3 mM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride). When the majored plateau caused by stimulation in oxygen consumption rates was obtained, chemical background oxygen consumption induced by auto oxydation of ascorbate/TMPD reactions was assessed after inhibition of complex IV by adding 2 mM potassium cyanide and 1 mM sodium azide. Activity of this complex was assessed as cyanide-sensitive part of ascorbate/TMPD-induced stimulation in oxygen consumption rate.

2.7 Genotoxicity assay

Genotoxicity was measured using the comet assay, a well-known indicator of single and double DNA strand breaks, as well as alkali-labile sites (Singh, McCoy, Tice, & Schneider, 1988). The protocol used was the same as the one described previously (Beaumont, Andriamihaja, et al., 2017). Concerning the exposure conditions, in some experiments cells were pretreated with 0.5 mM NAC before treatment for 24 hours with HO-PAA. The software used for images analysis was Image J, with the Open Comet plugin.

2.8 Quantification of gene expression by real-time PCR

The expression of several relevant genes was evaluated in cells exposed to either culture medium (control cells), or 350 and 1000 µM HO-PAA for 24 hours with or without 24-hours pretreatment with 0.5 mM NAC. After exposure, total RNA extraction was performed from lysed cells using the RNeasy kit (Qiagen SAS, Les Ullis, France) with a DNA digestion step based on the manufacturer's protocol.
After cDNA synthesis from mRNA using High Capacity cDNA Reverse Transcription Kit (ThermoFischer Scientific), real-time PCR was performed subsequently on cDNA using the power SYBR Green PCR master mix and StepOne Real-Time PCR system (ThermoFischer Scientific and Life Technologies). Gene expression levels for each sample were normalized relative to 18S with $2^{-\Delta\Delta Ct}$ calculation. The studied genes were chosen in relationship with their involvement in oxidative stress response (catalase, glutathione reductase (GR)), DNA repair (poly(ADP-ribose) polymerase 1 (PARP-1), Xeroderma pigmentosum, complementation group C (XPC)) and endoplasmic reticulum stress response (inositol-requiring enzyme 1 (IRE-1)). The primers used are available on demand.

2.9 Statistics

For each assay, the number of biological replicates per experiment was at least 4. Data obtained from independent experiments were presented as mean ± SEM. Statistical significance was assessed by Kruskal–Wallis non-parametric one-way analysis of variance by ranks, using the Graphpad Prism 5.0 software (La Jolla, CA, USA). When significance was demonstrated ($p<0.05$), paired comparisons were run using Mann–Whitney tests.

3. Results

3.1 Effects of HO-PAA on the human colonocytes

We first used two viability assays in order to characterize the effects of HO-PAA on the human colonocytes HT-29 $Glc^{-/+}$ cells. Global mitochondrial metabolic activity was not affected after 24 hours-treatment with HO-PAA at 350, 700 and 1000 µM concentrations, as judged from the MTT assay (Figure 1A). Regarding the DNA quantification assay in adherent cells, HO-PAA did not exert any significant effect on the colonocytes (Figure 1B) reinforcing the view that HO-PAA is not cytotoxic for cells.

As oxidative stress is a frequent consequence of toxicant exposure and a demonstrated mechanism for many toxicological effects, we then tested the effect of 350, 700 and 1000 µM HO-PAA on the net production of reactive oxygen species (ROS). Intracellular ROS production, measured by the H2DCF-DA assay, was significantly higher (by about 60%) in HO-PAA-exposed cells compared to control (Figure 1C). However, the effect of HO-PAA on ROS production was presumably maximal at the 350 µM, as the highest concentrations of this bacterial metabolite failed to increase such production. We also noticed that pretreatment with 0.5 mM NAC for 24 hours was sufficient to significant decrease the oxidative stress caused by exposure to H_2O_2 for 1 hour, which demonstrated its efficiency in preventing oxidative stress.

We then investigated the effect of 350 and 1000 µM HO-PAA on cell oxygen consumption, using a polarographic technique. We observed that basal oxygen consumption was decreased by about 30% in cells exposed for 24 hours to 1000 µM HO-PAA when compared to control, whereas the 350 µM concentration failed to induce such results (Figure 1D). We then investigated the mitochondrial behavior of 1000 µM-exposed cells more in details by evaluating electron leaking and maximal respiratory capacity. In the presence of FCCP or oligomycin, oxygen consumption was not different between cells treated with HO-PAA and control cells (mean 1.28 ± 0.67 fold compared to control following oligomycin treatment with $p=0.61$, and 1.25±0.76 fold following FCCP treatment, with $p= 0.25$), indicating that HO-PAA displayed no uncoupling effect on cell mitochondria, and did not change the maximal respiratory capacity.

As impaired mitochondrial oxygen consumption can be associated with an increased net production of ROS, we then pretreated for 24 hours cells with 0.5 mM NAC, a known antioxidant, before HO-PAA exposure at 350 and 1000 µM. We observed that NAC pretreatment, although being effective in preventing ROS production as already observed, had no significant impact on the effect of HO-PAA on colonocytes oxygen consumption (Figure 1D), indicating that the inhibition of oxygen consumption by HO-PAA was not due to an increased ROS production.

DNA damage provoked by HO-PAA, at 350, 700 and 1000 µM concentrations was evaluated via the alkaline version of the comet assay, which detects primary lesions of DNA i.e. single strand breaks and double-strand breaks, as well as alkali-labile sites such as abasic sites. We observed that HO-PAA induced a significant increase in cell DNA damage (around 40% increase), for all concentrations tested (Figure 1E); indicating that the 350 µM concentration of HO-PAA already provoked the maximal effect.

As oxidative stress has been often linked to genotoxicity, we pretreated cells with NAC before HO-PAA treatment and measured DNA damage. It appeared that pre-exposure to NAC significantly decreased the DNA damage induced by 350 µM HO-PAA when compared to the non NAC-treated condition (Figure 1F). Exposure of HT-29 $Glc^{-/+}$ cells to NAC alone did not show any genotoxic effect on cells (1.14 ± 0.27 fold compared to control, arbitrary units,

p=0.57). It therefore appears that the genotoxicity we observed after HO-PAA exposure is partially linked to oxidative stress.

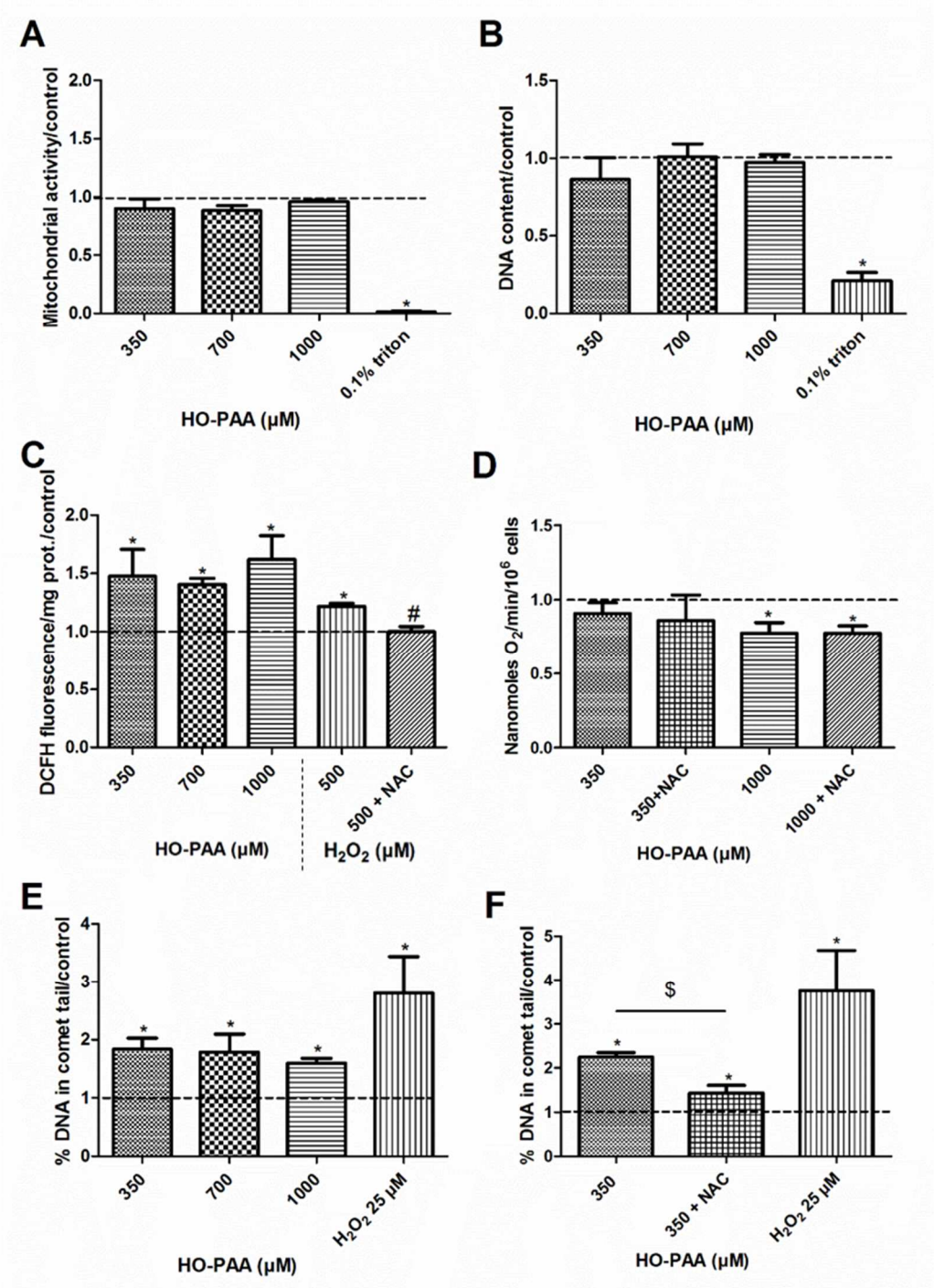

*Figure 1: Effects of 24 hours-exposure to HO-PAA in HT-29 Glc$^{-/+}$ cells. A: Cellular metabolic activity, assessed by the MTT assay. B: DNA content of adherent cells. C: Reactive oxygen species intracellular production, assessed by the DCFH-DA assay. D: Mitochondrial respiration, assessed by oxygen consumption. In experiments involving HO-PAA and NAC, some cells were pre-treated for 24 hours with NAC, and then treated for 24 hours with HO-PAA before oxygen consumption measurement. 1E: DNA damage, assessed by the comet assay. 1F: DNA damage in cells exposed to HO-PAA with or without 0.5 mM NAC pretreatment for 24 hours. Results are obtained from 4 (for Figures 1C and 1D) or 5 independent experiments and presented as mean values ± SEM. *: $p < 0.05$ versus control. $: $p<0.05$ vs equivalent non NAC-treated condition. #: $p<0.05$ vs H_2O_2 condition.*

We then decided to investigate deeper the mechanism responsible for the cellular respiration decrease in 1000 µM HO-PAA exposed-cells, by assessing the activity of several mitochondrial respiratory complexes namely complexes I, III and cytochrome oxidase (complex IV). Complex I activity was significantly (around 70%) decreased in HO-PAA-treated cells compared to control. Moreover, cellular pretreatment with NAC before HO-PAA treatment did not lead to significant difference in complex I activity, with p=0.85 (Figure 2) when compared with cells not pretreated with NAC. The activity of the other respiratory complexes measured was not significantly modified by HO-PAA exposure (p=0.51 for complex III, p=0.17 for complex IV), whatever the presence or absence of NAC (Figure 2). We concluded that HO-PAA impacted cell respiration by decreasing respiratory complex I activity, and this effect was apparently not caused by ROS production.

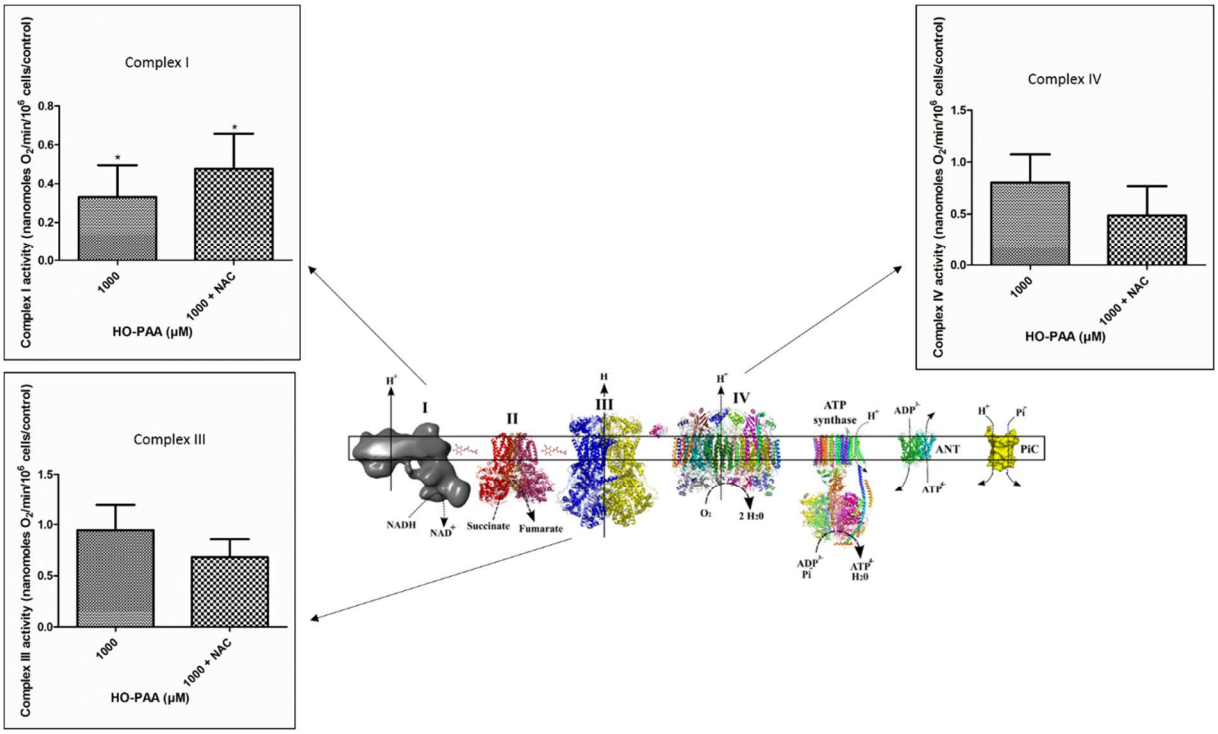

Figure 2: Mitochondrial respiratory complexes activity of cells exposed to 1000 µM HO-PAA for 24 h and cells pre-incubated with to 0.5 mM NAC for 24 hours and then incubated for 24 hours with HO-PAA. Differences in oxygen consumption without or with each complex specific inhibitor were measured (from (Mourier & Larsson, 2011)*). Results are obtained from 5 independent experiments and presented as mean values ± SEM. *: p < 0.05 versus control.*

Last, we evaluated the expression of several genes implicated in various mechanisms which, according to the previous results obtained, could have been affected by HO-PAA after exposure to 350 and 1000 µM concentrations, such as genes related to oxidative stress, reticulum stress or DNA damage responses. We did not observe, however, any expression modification of the studied genes after 24 hours-exposure to HO-PAA (Table 1).

Oxidative stress					
	Catalase			GSR	
HO-PAA (µM)	**without NAC**	**with NAC**	HO-PAA (µM)	**without NAC**	**with NAC**
0 (control)	2.49±0.54		**0 (control)**	1.02±0,21	
350	2.65±0.69	2.8±0.32	**350**	1.22±0.18	1.22±0.22
1000	2.55±0.85	2.3±0.69	**1000**	1.19±0.22	1.18±0.27

DNA damage					
	PARP1			XPC	
HO-PAA (µM)	**without NAC**	**with NAC**	HO-PAA (µM)	**without NAC**	**with NAC**
0 (control)	1.15±0.17		**0 (control)**	1.02±0.20	
350	1.30±0.29	1.34±0.34	**350**	1.15±0.29	1.19±0.46
1000	1.12±0.21	1.14±0.23	**1000**	1.09±0.30	1.04±0.23

Reticulum stress		
	IRE-1	
HO-PAA (µM)	**without NAC**	**with NAC**
0 (control)	0.84±0.20	
350	0.85±0.16	0.76±0.05
1000	0.89±0.28	0.81±0.23

Table 1: Effects of 24 hours-exposure to HO-PAA on genes expression in HT-29 Glc$^{-/+}$ cells: catalase, glutathione reductase (GR), poly(ADP-ribose) polymerase 1 (PARP-1), Xeroderma pigmentosum, complementation group C (XPC) and inositol-requiring enzyme 1 (IRE-1). Some cells were pretreated for 24 hours with NAC, and then treated for 24 hours with HO-PAA before oxygen consumption measurements. Results are obtained from 5 independent experiments and presented as mean values ± SEM.

3.2 Effects of PAA on the human colonocytes

The MTT assay showed that PAA displayed a significant inhibitory effect on the HT-29 $Glc^{-/+}$ mitochondrial activity; that appears not vastly different whatever the concentrations tested (350, 700 and 1000 µM, Figure 3A). However, the DNA quantification assay showed no effect of PAA on the number of adhering cells (Figure 3B), supporting the idea that PAA was mildly cytotoxic.

According to the DCFH assay, neither 350 nor 1000 µM PAA induced any significant increase in oxidative stress (Figure 3C), therefore we did not perform the NAC experiments on PAA as the one we performed with HO-PAA. PAA did not significantly altered oxygen consumption, since 1000 µM PAA exposure did not significantly modify this parameter (0.83 ±0.1for the PAA-treated samples compared to 1 for control (arbitrary unit)). Last, PAA did not induce DNA breaks whatever the concentration tested (Figure 3D).

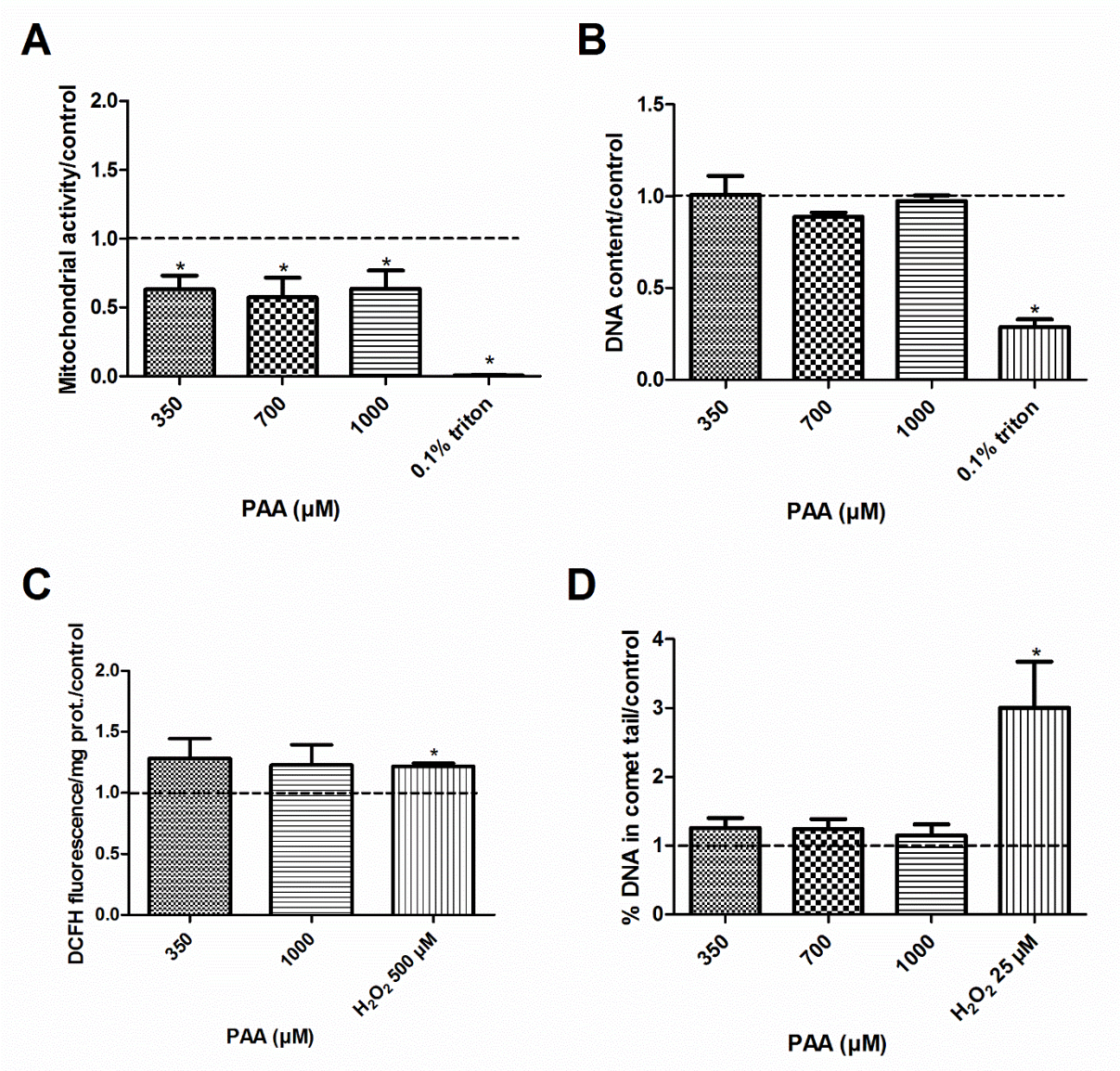

*Figure 3: Effects of 24 hours-exposure to PAA in HT-29 $Glc^{-/+}$ cells. A: Cellular metabolic activity, assessed by the MTT assay. B: DNA content of adherent cells. C: Reactive oxygen species intracellular production, assessed by the DCFH-DA assay. D: DNA damage, assessed by the comet assay. Results are obtained from 4-5 independent experiments (as indicated in Figure 1) and presented as mean values ± SEM. *: $p < 0.05$ versus control.*

3.3 Effects of phenol on the human colonocytes

As observed for PAA, phenol induced a slight but significant decrease in the mitochondrial activity of HT-29 $Glc^{-/+}$ cells at 250, 500, 1000 or 1500 µM concentrations (Figure 4A), but did not modify the amount of DNA in adherent cells (Figure 4B). We can therefore conclude that phenol was mildly cytotoxic. It did not modify intracellular ROS production at 500 or 1500 µM (Figure 4C). Phenol exposure at 1500 µM did not affect oxygen consumption rate (1.01±0.2) for treated cells compared to the control (1, arbitrary unit, data not shown). Last, phenol exposure did not result in any measurable increase in DNA breaks at either 500 or 1000 µM (Figure 4D).

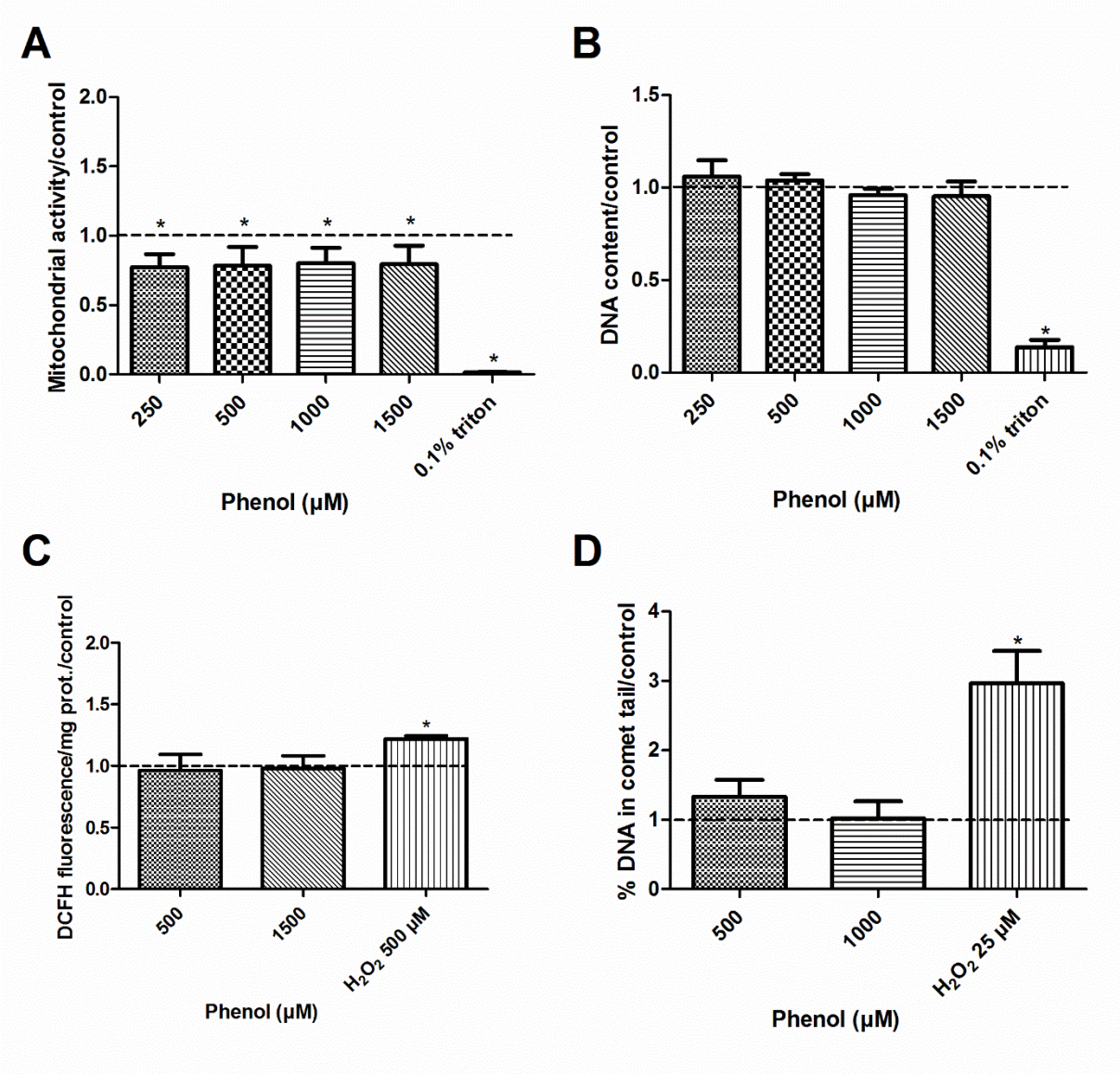

*Figure 4: Effects of 24 hours-exposure to phenol in HT-29 $Glc^{-/+}$ cells. A: Cellular metabolic activity, assessed by the MTT assay. B: DNA content of adherent cells. C: Reactive oxygen species intracellular production, assessed by the DCFH-DA assay. D: DNA damage, assessed by the comet assay. Results are obtained from 4-5 independent experiments (as indicated in Figure 1) and presented as mean values ± SEM. *: $p < 0.05$ versus control.*

3.4 Effects of acetaldehyde on the human colonocytes

Acetaldehyde exerted no cytotoxic effects on HT-29 $Glc^{-/+}$ cells as judged from the MTT or the DNA quantification assays, at 25, 100, 200 and 500 µM (Figures 5A and 5B). It did not increase intracellular ROS production at 200 or 500 µM (Figure 5C). Cell exposure to acetaldehyde had no effect on oxygen consumption rate (1.05±0.05 for treated cells compared to 1 for the control, data not shown). However, acetaldehyde increased in the human colonocytes the DNA strand-breaks as assessed by the comet assay, without significant difference between the effects obtained for the 200 and 500 µM concentrations (Figure 5D).

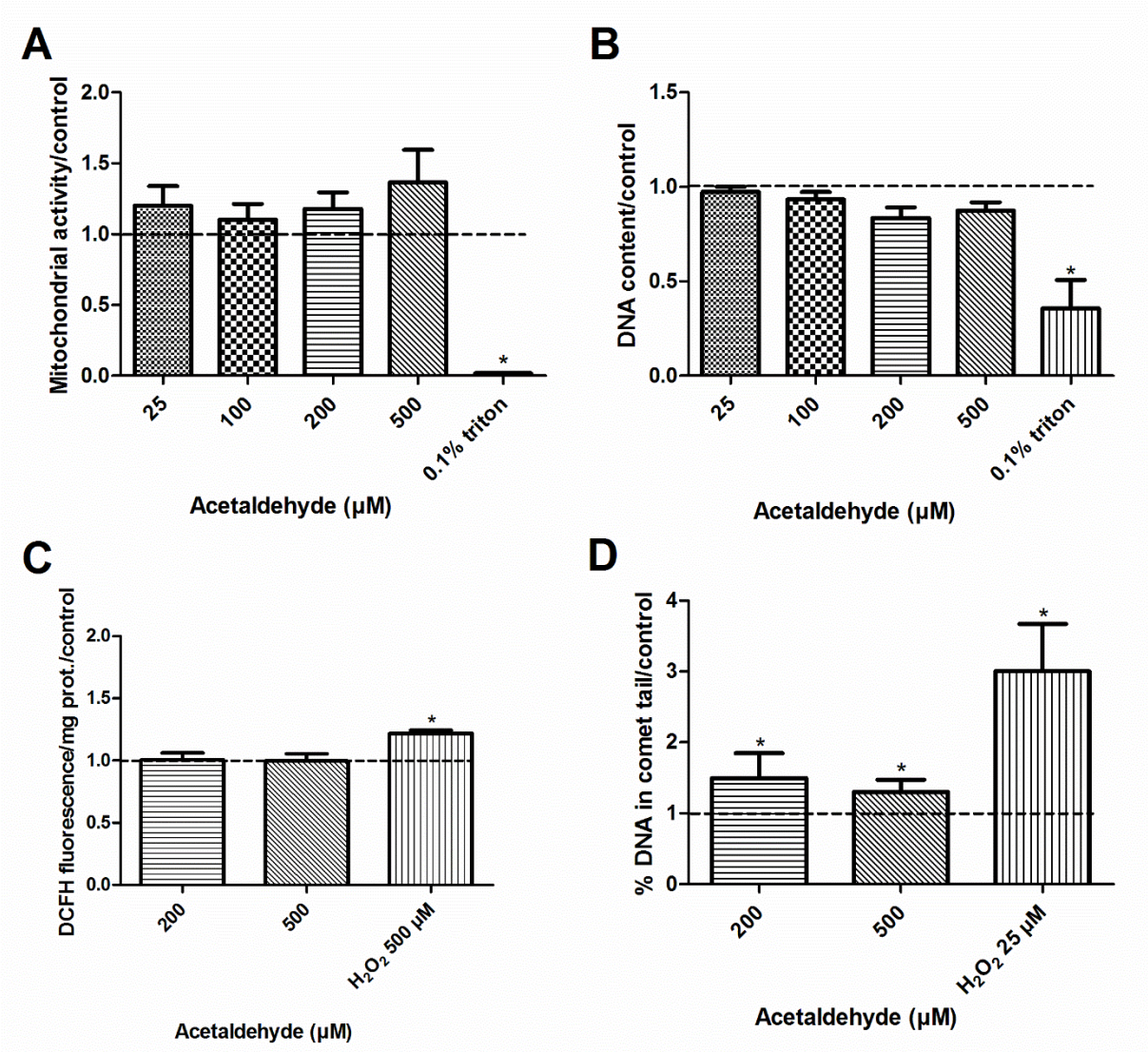

*Figure 5: Effects of 24 hours-exposure to acetaldehyde in HT-29 $Glc^{-/+}$ cells. A: Cellular metabolic activity, assessed by the MTT assay. B: DNA content of adherent cells. C: Reactive oxygen species intracellular production, assessed by the DCFH-DA assay. D: DNA damage, assessed by the comet assay. Results are obtained from 4-5 independent experiments (as indicated in Figure 1) and presented as mean values ± SEM. *: $p < 0.05$ versus control.*

4. Discussion

Our work, aiming at documenting the effects of several amino acid-derived bacterial metabolites on the human colonocytes HT-29 $Glc^{-/+}$ cells, clearly showed that HO-PAA was not cytotoxic in our model, but it impaired cellular respiration and increased both oxidative stress and DNA damage. A recapitulation of HO-PAA effects is presented in Figure 6.

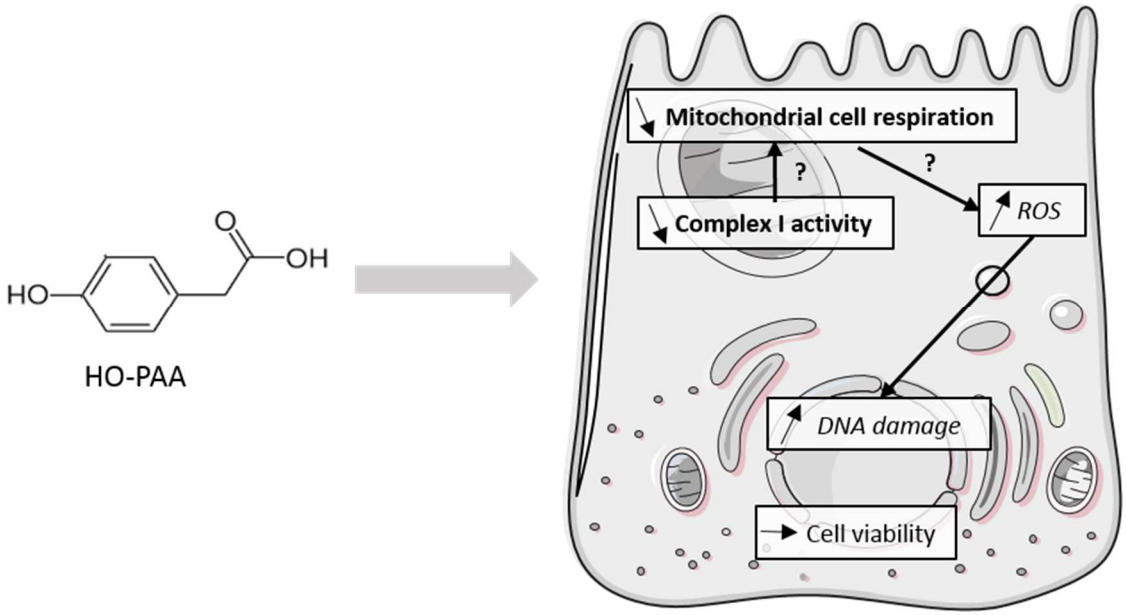

Figure 6: Recapitulative scheme of HO-PAA effects. HO-PAA, possibly by inhibiting the mitochondrial complex I activity decreases oxygen consumption and increases ROS net production. Such increase is partially implicated in the alteration of DNA integrity in colonocytes. Italics: increase caused by HO-PAA exposure; Bold: decrease due to HO-PAA exposure; Normal font: no modification caused by HO-PAA exposure.

Phenol and PAA were mildly cytotoxic but did not induce any of the other effects we investigated. Last, acetaldehyde did not decrease cell viability in our model, did not modify oxygen consumption or increase oxidative stress, but induced significant DNA damages.

Regarding the methodology used in our study, the MTT assay has been used as a cytotoxicity assay for several years as representative of mitochondrial activity, assuming that formazan reduction was exclusively due to respiratory complex II activity. However, it has been demonstrated lately that other mitochondrial or even cytoplasm enzymatic activities can also reduce formazan (Berridge, Herst, & Tan, 2005; van Tonder, Joubert, & Cromarty, 2015). Therefore, it is largely conceivable that HO-PAA decreases global cellular respiration, as observed in this study, without impacting MTT reduction, or that phenol or PAA are cytotoxic using the MTT assay without modifying basal oxygen consumption. Incidentally, HO-PAA was found to decrease the mitochondrial oxygen consumption in cells only at the highest concentration tested.

Another aspect of this work that is worth discussing is the absence of dose-dependent effects of several bacterial metabolites on the human colonocytes, notably regarding HO-PAA. This can be interpreted by considering that the lowest concentration tested was already close to the one that give the maximal effect on the cells under our experimental conditions. In addition, this absence of dose response may be related to the semi-quantitative characteristic of the assays used, notably regarding the comet assay used for the estimation of the bacterial metabolite genotoxicity which, even if it detects several kinds of DNA damage, is known to

be a semi-quantitative technique (Collins et al., 2014). It has to be noted that we did, however, found that the 1000 µM concentration induced a cellular respiration decrease while the 350 µM did not.

Considering the potentially cytotoxic effect of PAA on cells, different results have been obtained, depending on cell types. Although PAA has been defined as an uremic toxin, as patients with end-stage renal failure presented a higher urinary PAA concentration compared to controls, PAA was not cytotoxic on RAW264.7 (macrophages) cells using the Trypan blue assay (Jankowski et al., 2003). In our study, PAA was found to be moderately cytotoxic towards the human colonocytes HT-29 $Glc^{-/+}$ cells.

Very few studies have focused on specific phenol impact on colonic cells. McGall and coworkers found that phenol did not induce cytotoxicity on the SK-CO15, a human intestinal epithelial cell line (McCall et al., 2009). However, in this latter study, cells were exposed to phenol for only 60 minutes, in contrast with the 24 hours-exposure of the present study. Pedersen and coworkers observed that a 24 hours exposure to phenol significantly impaired the viability of primary human colonic epithelial cell cultures, at concentrations between 1 and 10 mM (Pedersen, Brynskov, & Saermark, 2002), which is in line with our results.

Acetaldehyde effects on the colon epithelium have been studied previously. Acetaldehyde was not cytotoxic after 3 hours exposure in a 3D model involving Caco-2 cells (Elamin et al., 2013). Acetaldehyde is also known to be genotoxic on cultured mammalian cells (Speit, Fröhler-Keller, Schütz, & Neuss, 2008). Globally these results are in line with the ones we obtained in the present study, even though the model and exposure conditions are different. It therefore seems that acetaldehyde displays the same genotoxic effects on the colonic epithelium as it does on other mammalian cells (Yu et al., 2010).

Despite their similarity in terms of chemical structure, PAA and HO-PAA displayed largely different effects on human colonocytes in our study, indicating that minor difference in the structure of a given bacterial metabolite may lead to different effects on the colonic epithelial cells. Incidentally, the role played by the hydroxyl group in HO-PAA for its effect on colonocytes may be linked to its high polarity, that is likely to modify global molecule hydrophilicity and therefore toxicity (Screnci et al., 2000). Indeed, PAA and HO-PAA lipophilicities are different, with logP(PAA) = 1,41 and logP(HO-PAA) = 0,74 (which means that HO-PAA is more hydrophilic that PAA). More studies, outside of the scope of the present work, are needed to link the chemical structure of metabolites produced by the intestinal microbiota and their effects on colonocytes.

HO-PAA, although not cytotoxic, displayed several deleterious effects in our conditions. Some of these effects seemed to be partially due to its capacity to increase the production of ROS, as previously found in other cell types (Saha et al., 2017). We observed, however, that the decrease in oxygen consumption caused by HO-PAA was not due to oxidative stress. As a decrease in complex I activity can lead to production of ROS (Ward, Poff, Koutnik, & D'Agostino, 2017), we propose that, by inhibiting the mitochondrial complex I activity, HO-PAA may increase the net mitochondrial production of ROS, that finally impact DNA integrity in colonocytes (Figure 6). However, since NAC did limit partially the genotoxic effect of HO-PAA, other mechanisms, out of the scope of this study, might explain HO-PAA's effects in our model.

Concerning the experiments dedicated to the effect of HO-PAA on the expression of some genes related to oxidative stress, DNA damage and reticulum stress in colonocytes after 24h-treatment, our negative results indicate that the effects of this metabolite on the mitochondrial respiratory chain, ROS production and DNA integrity are not associated to any detectable modification of the expression of these genes.

Last but not least, in opposition with our initial working hypothesis postulating that the presence of HO-PAA in human fecal water is linked to its cytotoxic potential towards colonocytes (Beaumont, Portune, et al., 2017), the results of the present *in vitro* study strongly suggest that HO-PAA does not play any major role in the observed fecal water cytotoxicy. However, it is worth underlining that fecal water represents a very complex mixture of compounds including bacterial metabolites and undigested dietary compounds (Pearson, Gill, & Rowland, 2009), and then it is likely that a given bacterial metabolite like HO-PAA would behave differently when tested individually or in a mixture. Therefore the effect of mixtures of several bacterial metabolites on colonocytes remain an important but still largely ignored challenge for future research.

Our study has some limitations. First, the results were obtained using an *in vitro* model, and thus they need confirmation using an *in vivo* experimental model, like for instance the model of intra-colonic instillation of bacterial metabolites in anesthetized rodents (Beaumont et al., 2016). Particularly, it is necessary to take into consideration that the colonic and rectal epithelia are protected from the deleterious compounds present in the luminal content by the mucus layers which may limit the accessibility of such compounds to the intracellular content of epithelial cells (Desai et al., 2016). Secondly, if we have used in the present study plausible concentrations of bacterial metabolites taking into account the available data in the literature, it has to be recognized that the luminal concentrations of the bacterial metabolites in their free (unbound) form in the different regions of the human large intestine are still poorly known and estimation of luminal concentrations are often extrapolated from animal models and from concentrations measured in fecal water from humans with large inter-individual values, as discussed above.
With these reservations in mind, our study clearly demonstrates that the metabolites in our study exert either cytotoxic or genotoxic effects on human colonocytes. This is notably the case for acetaldehyde, which has been shown to be genotoxic in several studies, and HO-PAA, which displayed genotoxic properties in association with disturbances of oxygen consumption and ROS production in colonocytes, without however cytotoxic effect.

If we replace the observed effects of the different bacterial metabolites on the human colonocytes in a physiological and physiopathological perspective, we can predict that, depending on the cell phenotype that are affected by the luminal compounds in the colonic epithelium, the consequences of such alterations, notably those affecting the DNA integrity, are likely to be different. Indeed, if such compounds affect the progenitor cells situated at the bottom of the colonic crypts (Barker, van de Wetering, & Clevers, 2008), more deleterious effects may be envisaged as these cells can accumulate DNA alterations as observed in some forms of colorectal cancer. However, mostly for technical reasons, the concentrations of individual bacterial metabolites in close vicinity with the progenitor cells are not known, thus rendering difficult to document at what threshold concentration the suspected bacterial metabolites may become hazardous.

In conclusion, our study demonstrated that several metabolites which are synthesized by the intestinal microbiota from amino acids displayed deleterious properties towards colonic

epithelial cells, either in terms of genotoxicity (HO-PAA and acetaldehyde) or of cytotoxicity (PAA and phenol). Since HO-PAA can be derived from multiple food sources (Gill et al., 2010; Windey et al., 2012), we hope that our present results will stimulate future studies on the long-term *in vivo* effects of HO-PAA, in order to characterize the future cellular consequences of HO-PAA impact on DNA integrity we observed in colonocytes, notably regarding the possible implication in the process of neoplasia.

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5. References

Andriamihaja, M., Lan, A., Beaumont, M., Audebert, M., Wong, X., Yamada, K., … Blachier, F. (2015). The deleterious metabolic and genotoxic effects of the bacterial metabolite p-cresol on colonic epithelial cells. *Free Radical Biology & Medicine*, *85*, 219–227. https://doi.org/10.1016/j.freeradbiomed.2015.04.004

Barker, N., van de Wetering, M., & Clevers, H. (2008). The intestinal stem cell. *Genes & Development*, *22*(14), 1856–1864. https://doi.org/10.1101/gad.1674008

Beaumont, M., Andriamihaja, M., Armand, L., Grauso, M., Jaffrézic, F., Laloë, D., … Lan, A. (2017). Epithelial response to a high-protein diet in rat colon. *BMC Genomics*, *18*. https://doi.org/10.1186/s12864-017-3514-z

Beaumont, M., Andriamihaja, M., Lan, A., Khodorova, N., Audebert, M., Blouin, J.-M., … Blachier, F. (2016). Detrimental effects for colonocytes of an increased exposure to luminal hydrogen sulfide: The adaptive response. *Free Radical Biology and Medicine*, *93*, 155–164. https://doi.org/10.1016/j.freeradbiomed.2016.01.028

Beaumont, M., Portune, K. J., Steuer, N., Lan, A., Cerrudo, V., Audebert, M., … Blachier, F. (2017). Quantity and source of dietary protein influence metabolite production by gut microbiota and rectal mucosa gene expression: a randomized, parallel, double-blind trial in overweight humans. *The American Journal of Clinical Nutrition*, *106*(4), 1005–1019. https://doi.org/10.3945/ajcn.117.158816

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. In *Biotechnology Annual Review* (Vol. 11, pp. 127–152). Elsevier. https://doi.org/10.1016/S1387-2656(05)11004-7

Blachier, F., Beaumont, M., Andriamihaja, M., Davila, A.-M., Lan, A., Grauso, M., … Tomé, D. (2017). Changes in the Luminal Environment of the Colonic Epithelial Cells and Physiopathological Consequences. *The American Journal of Pathology*, *187*(3), 476–486. https://doi.org/10.1016/j.ajpath.2016.11.015

Bone, E., Tamm, A., & Hill, M. (1976). The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer. *The American Journal of Clinical Nutrition*, *29*(12), 1448–1454.

Chun, O. K., Chung, S. J., & Song, W. O. (2007). Estimated Dietary Flavonoid Intake and Major Food Sources of U.S. Adults. *The Journal of Nutrition*, *137*(5), 1244–1252. https://doi.org/10.1093/jn/137.5.1244

Collins, A. R., El Yamani, N., Lorenzo, Y., Shaposhnikov, S., Brunborg, G., & Azqueta, A. (2014). Controlling variation in the comet assay. *Frontiers in Genetics*, *5*. https://doi.org/10.3389/fgene.2014.00359

Desai, M. S., Seekatz, A. M., Koropatkin, N. M., Kamada, N., Hickey, C. A., Wolter, M., … Martens, E. C. (2016). A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell*, *167*(5), 1339-1353.e21. https://doi.org/10.1016/j.cell.2016.10.043

Elamin, E. E., Masclee, A. A., Dekker, J., & Jonkers, D. M. (2013). Ethanol metabolism and its effects on the intestinal epithelial barrier. *Nutrition Reviews*, *71*(7), 483–499. https://doi.org/10.1111/nure.12027

Gao, Xu, A., Krul, C., Venema, K., Liu, Y., Niu, Y., … Henning, S. M. (2006). Of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements, only 3,4-dihydroxyphenylacetic acid has antiproliferative activity. *The Journal of Nutrition*, *136*(1), 52–57.

Gill, C. I. R., McDougall, G. J., Glidewell, S., Stewart, D., Shen, Q., Tuohy, K., … Rowland, I. R. (2010). Profiling of Phenols in Human Fecal Water after Raspberry Supplementation.

Journal of Agricultural and Food Chemistry, *58*(19), 10389–10395. https://doi.org/10.1021/jf1017143
Gnaiger, E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respiration Physiology*, *128*(3), 277–297. https://doi.org/10.1016/S0034-5687(01)00307-3
Gray, L. R., Tompkins, S. C., & Taylor, E. B. (2014). Regulation of pyruvate metabolism and human disease. *Cellular and Molecular Life Sciences*, *71*(14), 2577–2604. https://doi.org/10.1007/s00018-013-1539-2
Grün, C. H., van Dorsten, F. A., Jacobs, D. M., Le Belleguic, M., van Velzen, E. J. J., Bingham, M. O., … van Duynhoven, J. P. M. (2008). GC–MS methods for metabolic profiling of microbial fermentation products of dietary polyphenols in human and in vitro intervention studies. *Journal of Chromatography B*, *871*(2), 212–219. https://doi.org/10.1016/j.jchromb.2008.04.039
Homann, N., Tillonen, J., & Salaspuro, M. (2000). Microbially produced acetaldehyde from ethanol may increase the risk of colon cancer via folate deficiency. *International Journal of Cancer*, *86*(2), 169–173. https://doi.org/10.1002/(SICI)1097-0215(20000415)86:2<169::AID-IJC4>3.0.CO;2-3
IARC. (1999). *Consumption of alcoholic beverages* (IARC Monogr Eval Carcinog Risks Hum No. 36, Sup 7, 71) (pp. 1–128).
Institute of Medicine (U.S.), & Institute of Medicine (U.S.) (Eds.). (2005). *Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids*. Washington, D.C: National Academies Press.
Jankowski, J., van der Giet, M., Jankowski, V., Schmidt, S., Hemeier, M., Mahn, B., … Tepel, M. (2003). Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression. *Journal of Clinical Investigation*, *112*(2), 256–264. https://doi.org/10.1172/JCI200315524
Jenner, A. M., Rafter, J., & Halliwell, B. (2005). Human fecal water content of phenolics: The extent of colonic exposure to aromatic compounds. *Free Radical Biology and Medicine*, *38*(6), 763–772. https://doi.org/10.1016/j.freeradbiomed.2004.11.020
Jokelainen, K., Matysiak-Budnik, T., Mäkisalo, H., Höckerstedt, K., & Salaspuro, M. (1996). High intracolonic acetaldehyde values produced by a bacteriocolonic pathway for ethanol oxidation in piglets. *Gut*, *39*(1), 100–104.
Karlsson, P. C., Huss, U., Jenner, A., Halliwell, B., Bohlin, L., & Rafter, J. J. (2005). Human fecal water inhibits COX-2 in colonic HT-29 cells: role of phenolic compounds. *The Journal of Nutrition*, *135*(10), 2343–2349.
Kumagai, H., Utagawa, T., & Yamada, H. (1975). Studies on tyrosine phenol lyase. Modification of essential histidyl residues by diethylpyrocarbonate. *The Journal of Biological Chemistry*, *250*(5), 1661–1667.
Leschelle, X., Delpal, S., Goubern, M., Blottière, H. M., & Blachier, F. (2000). Butyrate metabolism upstream and downstream acetyl-CoA synthesis and growth control of human colon carcinoma cells: Butyrate metabolism and colon cell growth. *European Journal of Biochemistry*, *267*(21), 6435–6442. https://doi.org/10.1046/j.1432-1327.2000.01731.x
McCall, I. C., Betanzos, A., Weber, D. A., Nava, P., Miller, G. W., & Parkos, C. A. (2009). Effects of phenol on barrier function of a human intestinal epithelial cell line correlate with altered tight junction protein localization. *Toxicology and Applied Pharmacology*, *241*(1), 61–70. https://doi.org/10.1016/j.taap.2009.08.002
Mourier, A., & Larsson, N.-G. (2011). Tracing the Trail of Protons through Complex I of the Mitochondrial Respiratory Chain. *PLoS Biology*, *9*(8), e1001129. https://doi.org/10.1371/journal.pbio.1001129

Neirynck, N., Vanholder, R., Schepers, E., Eloot, S., Pletinck, A., & Glorieux, G. (2013). An update on uremic toxins. *International Urology and Nephrology*, *45*(1), 139–150. https://doi.org/10.1007/s11255-012-0258-1
Nistal, E., Fernández-Fernández, N., Vivas, S., & Olcoz, J. L. (2015). Factors Determining Colorectal Cancer: The Role of the Intestinal Microbiota. *Frontiers in Oncology*, *5*. https://doi.org/10.3389/fonc.2015.00220
Paturi, G., Nyanhanda, T., Butts, C. A., Herath, T. D., Monro, J. A., & Ansell, J. (2012). Effects of Potato Fiber and Potato-Resistant Starch on Biomarkers of Colonic Health in Rats Fed Diets Containing Red Meat. *Journal of Food Science*, *77*(10), H216–H223. https://doi.org/10.1111/j.1750-3841.2012.02911.x
Pearson, J. R., Gill, C. I., & Rowland, I. R. (2009). Diet, fecal water, and colon cancer – development of a biomarker. *Nutrition Reviews*, *67*(9), 509–526. https://doi.org/10.1111/j.1753-4887.2009.00224.x
Pedersen, G., Brynskov, J., & Saermark, T. (2002). Phenol Toxicity and Conjugation in Human Colonic Epithelial Cells. *Scandinavian Journal of Gastroenterology*, *37*(1), 74–79. https://doi.org/10.1080/003655202753387392
Phenol - Substance Information - ECHA. (n.d.). Retrieved 10 July 2018, from https://echa.europa.eu/substance-information/-/substanceinfo/100.003.303
Portune, K. J., Beaumont, M., Davila, A.-M., Tomé, D., Blachier, F., & Sanz, Y. (2016). Gut microbiota role in dietary protein metabolism and health-related outcomes: The two sides of the coin. *Trends in Food Science & Technology*, *57*, 213–232. https://doi.org/10.1016/j.tifs.2016.08.011
Pöschl, G., & Seitz, H. K. (2004). ALCOHOL AND CANCER. *Alcohol and Alcoholism*, *39*(3), 155–165. https://doi.org/10.1093/alcalc/agh057
Russell, W. R., Gratz, S. W., Duncan, S. H., Holtrop, G., Ince, J., Scobbie, L., … Flint, H. J. (2011). High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *American Journal of Clinical Nutrition*, *93*(5), 1062–1072. https://doi.org/10.3945/ajcn.110.002188
Russell, W. R., Hoyles, L., Flint, H. J., & Dumas, M.-E. (2013). Colonic bacterial metabolites and human health. *Current Opinion in Microbiology*, *16*(3), 246–254. https://doi.org/10.1016/j.mib.2013.07.002
Saha, S. K., Lee, S. B., Won, J., Choi, H. Y., Kim, K., Yang, G.-M., … Cho, S. (2017). Correlation between Oxidative Stress, Nutrition, and Cancer Initiation. *International Journal of Molecular Sciences*, *18*(7). https://doi.org/10.3390/ijms18071544
Screnci, D., McKeage, M. J., Galettis, P., Hambley, T. W., Palmer, B. D., & Baguley, B. C. (2000). Relationships between hydrophobicity, reactivity, accumulation and peripheral nerve toxicity of a series of platinum drugs. *British Journal of Cancer*, *82*(4), 966–972. https://doi.org/10.1054/bjoc.1999.1026
Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, *175*(1), 184–191. https://doi.org/10.1016/0014-4827(88)90265-0
Smith, E. A., & Macfarlane, G. T. (1996). Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *The Journal of Applied Bacteriology*, *81*(3), 288–302.
Speit, G., Fröhler-Keller, M., Schütz, P., & Neuss, S. (2008). Low sensitivity of the comet assay to detect acetaldehyde-induced genotoxicity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *657*(2), 93–97. https://doi.org/10.1016/j.mrgentox.2008.07.010

Taciak, M., Barszcz, M., Tuśnio, A., & Pastuszewska, B. (2015). Interactive Effects of Indigestible Carbohydrates, Protein Type, and Protein Level on Biomarkers of Large Intestine Health in Rats. *PLOS ONE*, *10*(11), e0142176. https://doi.org/10.1371/journal.pone.0142176
van Tonder, A., Joubert, A. M., & Cromarty, A. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Research Notes*, *8*(1), 47. https://doi.org/10.1186/s13104-015-1000-8
Verbeke, K. A., Boobis, A. R., Chiodini, A., Edwards, C. A., Franck, A., Kleerebezem, M., … Tuohy, K. M. (2015). Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutrition Research Reviews*, *28*(1), 42–66. https://doi.org/10.1017/S0954422415000037
Ward, N. P., Poff, A. M., Koutnik, A. P., & D'Agostino, D. P. (2017). Complex I inhibition augments dichloroacetate cytotoxicity through enhancing oxidative stress in VM-M3 glioblastoma cells. *PLOS ONE*, *12*(6), e0180061. https://doi.org/10.1371/journal.pone.0180061
Windey, K., De Preter, V., & Verbeke, K. (2012). Relevance of protein fermentation to gut health. *Molecular Nutrition & Food Research*, *56*(1), 184–196. https://doi.org/10.1002/mnfr.201100542
Yin, L., Laevsky, G., & Giardina, C. (2001). Butyrate Suppression of Colonocyte NF-κB Activation and Cellular Proteasome Activity. *Journal of Biological Chemistry*, *276*(48), 44641–44646. https://doi.org/10.1074/jbc.M105170200
Yu, H.-S., Oyama, T., Isse, T., Kitagawa, K., Pham, T.-T.-P., Tanaka, M., & Kawamoto, T. (2010). Formation of acetaldehyde-derived DNA adducts due to alcohol exposure. *Chemico-Biological Interactions*, *188*(3), 367–375. https://doi.org/10.1016/j.cbi.2010.08.005