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Stool-fermented *Plantago ovata* husk induces apoptosis in colorectal cancer cells independently of molecular phenotype

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Abstract

Several studies have suggested that the partially fermentable fibre *Plantago ovata* husk (PO) may have a protective effect on colorectal cancer (CRC). We studied the potentially pro-apoptotic effect of PO and the implicated mechanisms in CRC cells with different molecular phenotypes (Caco-2, HCT116, LoVo, HT-29, SW480) after PO anaerobic fermentation with colonic bacteria as it occurs in the human colon. The fermentation products of PO induced apoptosis in all primary tumour and metastatic cell lines, independent of p53, adenomatous polyposis coli, β -catenin or cyclo-oxygenase-2 status. Apoptosis was caspase-dependent and both intrinsic and extrinsic pathways were implicated. The intrinsic pathway was activated through a shift in the balance towards a pro-apoptotic environment with an up-regulation of B-cell lymphoma protein 2 homologous antagonist killer (*BAK*) and a down-regulation of B-cell lymphoma-extra large (*Bcl-xL*) seen in HCT116 and LoVo cells. This resulted in mitochondrial membrane depolarisation, increased expression of caspase activators second mitochondria-derived activator of caspases (*Smac*)/*Diablo*, death effector apoptosis-inducing factor, apoptosome member apoptotic protease activating factor 1 and down-regulation of inhibitors of apoptosis *Survivin* and X-linked inhibitor of apoptosis in most cells. The extrinsic pathway was activated presumably through the up-regulation of death receptor (*DR5*). Some important differences were seen between primary tumour and metastatic CRC cells. Thus, metastatic PO-treated LoVo cells had a remarkable up-regulation of *TNF- α* ligand along with death-inducing signalling complex components receptor interacting protein and TNF- α receptor 1-associated death domain protein. The extrinsic pathway modulator FLICE-inhibitory protein (FLIP), an inhibitor of both spontaneous death ligand-independent and death receptor-mediated apoptosis, was significantly down-regulated after PO treatment in all primary tumour cells, but not in metastatic LoVo. These findings suggest that PO could potentially be a useful chemotherapy adjuvant.

Key words: Colon cancer: Apoptosis: *Plantago ovata* husk

Dietary and other lifestyle factors substantially influence colorectal cancer (CRC) risk⁽¹⁾ and they seem to be responsible for the large differences in CRC incidence around the world⁽²⁾. Since Burkitt observed in 1969 an inverse correlation between fibre intake and CRC⁽³⁾, a significant number of studies have supported this protective role^(4–6). Several mechanisms have been suggested to explain the presumed preventive effect. Thus, reduction of toxin exposure to the colonic mucosa through dilution of luminal contents and/or faster transit time; bile acid adsorption; and the capacity to increase the generation of SCFA in the gut have all been

proposed as potential mechanisms⁽⁷⁾. SCFA are produced through the fermentation of fibre by colonic bacteria. The main SCFA generated are acetate, propionate and butyrate. The latter is the major energy source for healthy colonocytes and it is thought to be implicated in the presumable protective effect as it has been shown to promote differentiation, cell-cycle arrest and apoptosis in transformed colonocytes⁽⁸⁾. In an *in vivo* model of experimentally induced CRC, a higher faecal butyrate concentration was significantly associated with a lower number of tumours developed⁽⁹⁾.

Abbreviations: AIF, apoptosis-inducing factor; APAF 1, apoptotic protease activating factor 1; Bak, B-cell lymphoma protein 2 homologous antagonist killer; Bax, B-cell lymphoma protein 2-like protein 4; Bcl-2, B-cell lymphoma protein 2; Bcl-xL, B-cell lymphoma-extra large; Bid, Bcl-2 interacting domain; CRC, colorectal cancer; DAPI, 4',6-diamidino-2-phenylindole; DISC, death-inducing signalling complex; FLIP, FLICE-inhibitory protein; PO, *Plantago ovata* husk; RIP, receptor interacting protein; RQ, respiratory quotient; Smac, second mitochondria-derived activator of caspases; TNFR1, TNF receptor 1; TRADD, TNF receptor type 1-associated DEATH domain protein; XIAP, X-linked inhibitor of apoptosis.

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While there are plenty of convincing data, not all studies have been able to prove the chemo-preventive effect of fibre on CRC and in fact all randomised controlled trials have failed to do so^(10–12). Relatively short interventions, follow-up times and type of fibre consumed could underlie the lack of significant effect in these trials⁽¹³⁾. Regarding the type of fibre, several studies have supported the protective role of *Plantago ovata* husk (PO), a partially fermentable, 70% soluble fibre. Thus, in a paired case–control study, and after adjusting for diet, smoking, alcohol consumption, family history of cancer and socio-demographic factors, consumption of PO, physical exercise and aspirin were all significantly associated with a reduction in CRC risk⁽¹⁴⁾. A recent comparative ecological study showed an inverse correlation between PO consumption and mortality from CRC that reached statistical significance for the top quintile of PO consumption in the adjusted analysis⁽¹⁵⁾.

In the present study, we proposed to assess the capacity of the fermentation products of PO to induce apoptosis, and to study the mechanisms implicated in this effect. In order to maximally resemble a physiological environment, PO was incubated with human stool in anaerobic conditions and the totality of the resulting products was used to treat colonocytes and assess the effects. Immediately after fermentation, SCFA were measured in the fermentation samples. As reported before, PO fermentation with human faecal flora results in up to three times increase in SCFA production⁽¹⁶⁾, and we hypothesised that PO's chemo-preventive effects could be due to this significant increase in the generation of SCFA.

Materials and methods

Plantago ovata fermentation and SCFA quantification

PO fermentation and quantification were based on a modified protocol by Clausen *et al.*⁽¹⁷⁾. Thus, fresh stool samples were obtained from healthy human volunteers and homogenised with 150 mM-NaHCO₃ buffer in a 1:5 ratio. Stool was incubated with Plantaben® (Rottapharm, SL, Spain) and vortexed. Plantaben® contains PO (70% soluble fibre) and excipients (tartaric acid and sodium bicarbonate). Faecal homogenates had a final concentration of 20 mg/ml of PO as used in previous studies⁽¹⁸⁾. The concentration used is based on average concentrations found in stool after taking sachets of 5 g of Plantaben® containing a total of 7–10.5 g of PO (M Eastwood, unpublished results). Anaerobic fermentation was achieved by the exchange of air for nitrogen in the sealed tubes. Tubes were then incubated at 37°C in a shaking water bath for 72 h. Control tubes without PO powder addition were fermented in the same way. All tubes were stored at –80°C after fermentation.

For SCFA quantification, fermented samples were acidified with H₂SO₄ and centrifuged to take apart solid material. The supernatant was then recovered and SCFA were extracted by adding diethyl ether, vortexing for 1 min and allowing the phases to separate. The diethyl ether phase containing the fatty acids was recovered. Quantification was performed using a JEOL GCmate™ II GC/MS double-focusing mass

spectrometer (JEOL Limited Tokyo, Japan). As an internal standard, 2-methylvaleric acid was used. A standard curve was performed using six samples with known concentrations of acetic, propionic, butyric, isobutyric, isovaleric, valeric and caproic acids and extracted with diethyl ether. All samples were analysed twice each for acetate, butyrate and propionate concentrations. Table 1 shows the concentrations of SCFA in stool with no PO added and after 72 h of PO stool fermentation. The averages of butyrate measurements were used to determine cell treatment concentrations.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Hospital Universitari Germans Trias i Pujol ethics committee. Written informed consent was obtained from all subjects.

Cell cultures and supplements

To study the effects of PO, we used five human colon adenocarcinoma cell lines with different molecular phenotypes, such as wild-type *p53* (HCT116, LoVo) *v.* mutated *p53* (Caco-2, HT-29, SW480); microsatellite stable (Caco-2, HT-29, SW480) *v.* microsatellite unstable (HCT116, LoVo); wild-type β -catenin (LoVo, SW480, HT-29) *v.* mutated β -catenin (HCT116, Caco-2); primary tumour, non-metastatic (HCT 116, Caco-2, HT-29, SW480) *v.* metastatic (LoVo). Table 2 shows some of the main features of the cell lines studied. Doubling times in CRC cells ranged from 20 h for HCT116 to 62 h for Caco-2. Cell lines were grown as previously described⁽¹⁹⁾ and maintained at 37°C in a humidified atmosphere containing 5% CO₂. All cell lines were specifically bought for the present study from the American Type Culture Collection and none were used with more than fifty passes or 4 months. Cell cultures were tested monthly for *Mycoplasma* colonisation with LookOut™ Mycoplasma PCR Detection Kit (Sigma-Aldrich, St Louis, MO, USA).

For experiments, cells were plated and allowed to adhere overnight. For preparation of whole cell lysates, 4 000 000 cells were used per 10 mm cell culture dish. In all other experiments, 200 000 cells were used per well of sixteen-well plate or dual chamber slide. Fermented samples were prepared for supplementation by centrifugation to separate solid material and filtration of the supernatant using a 0.22 μ m filter. Cells were supplemented with faecal suspensions. These were set to contain a butyrate level of 4 mM for the PO treatment

Table 1. Generation of SCFA after stool fermentation with *Plantago ovata* husk (PO)*

SCFA	Concentration with no added PO (mmol)	Concentration 72 h after PO addition (mmol)
Acetic	56.91	358.59
Propionic	25.51	73.57
Isobutyric	0.34	1.87
Butyric	5.07	25.06
Isovaleric	0.30	1.70
Valeric	0.35	2.13
Caproic	0.21	1.26

* Data shows SCFA concentrations after fermentation for 72 h at 37°C in anaerobic conditions.

Table 2. Several characteristics of five different colorectal cancer cell lines and status of genes of interest

Cell lines...	HCT116	LoVo	SW480	HT-29	Caco2
Primary tumour or metastatic	Primary	Metastatic	Primary	Primary	Primary
MSI v. MSS	MSI	MSI	MSS	MSS	MSS
<i>p53</i>	Wild type	Wild type	Mutated	Mutated	Mutated
<i>APC</i>	Wild type	Mutated	Mutated	Mutated	Mutated
<i>CTNNB1</i> (β -catenin)	Mutated	Wild type	Wild type	Wild type	Mutated
<i>KRAS</i>	Mutated	Mutated	Mutated	Wild type	Wild type
<i>MSH 3</i>	Mutated	Wild type	Wild type	Wild type	Wild type
<i>MSH 6</i>	Mutated	Wild type	Wild type	Wild type	Wild type
<i>Cox-2</i> expression	Low expression	Normal	Not expressed	Over expression	Low expression

MSI, microsatellite instability; MSS, microsatellite stability; APC, adenomatous polyposis coli; CTNNB1, cadherin-associated protein β 1; KRAS, V-ki-ras2 kirsten rat sarcoma; MSH, Muts protein homolog; Cox-2, cyclo-oxygenase-2.

cells, which resulted in a level of 19 mM-propionate and 33 mM-acetate. In order to maintain the same concentration of the supernatant in the culture medium for the control cells, this resulted in SCFA levels of 0.8 mM-butyrate, 3.8 mM-propionate and 6.6 mM-acetate.

Experiments to assess apoptosis by 4',6-diamidino-2-phenylindole (DAPI) or cytometry were performed in all cells 24 h post-supplementation. Detection of mitochondrial membrane depolarisation was done 18 h after treatment in all cell lines. Caspase activity was assessed in all cell lines 8 and 18 h post-supplementation. Gene expression experiments were performed after 2, 4, 8 and 12 h of PO treatment in all cell lines. Protein expression was detected by Western blotting in HCT116 and LoVo cells after 8 and 18 h treatment.

Apoptosis assessment by 4',6-diamidino-2-phenylindole

Morphological assessment of apoptosis was performed 24 h post-supplementation with DAPI staining and mounted with Vectashield® mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) as previously described⁽²⁰⁾. Slides were visualised under a fluorescence microscope and apoptosis was assessed according to morphological criteria: cell shrinking, membrane blebbing, formation of apoptotic bodies and chromatin condensation.

Apoptosis quantification by flow cytometry

Cells were trypsinised 24 h post-supplementation, washed with 1 × PBS and stained with annexin-V/propidium iodine with Annexin-V-FLUOS (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. Apoptosis was quantified with a BD FACSCalibur flow cytometer (San Jose, CA, USA). Apoptosis was also assessed after the addition of several caspase inhibitors: z-VAD-FMK (broad-spectrum caspase inhibitor); Ac-DEVD-CHO (caspase-3 specific inhibitor); Ac-IETD-CHO (caspase-8 specific inhibitor); and Ac-LEHD-CHO (caspase-9 specific inhibitor), all from BIOMOL International (Plymouth Meeting, PA, USA) in order to determine the implication of the different apoptosis pathways.

Preparation of whole cell protein lysates

Lysates were prepared as previously described⁽¹⁹⁾. Briefly, 8 million cells were collected by scraping with 1 × PBS and

lysed with 200 μ l of lysis buffer. Incubation was carried out with agitation for 40 min at 4°C. Finally, cells were centrifuged at 16 000 g for 10 min at 4°C and the supernatant was collected. Protein concentration was measured by the Bradford assay (BioRad Laboratories, Hercules, CA, USA). Experiments were performed 8 and 18 h post-supplementation.

Caspase activity assay

Caspase activity was measured in whole cell lysates by fluorometric assay using DEVD-AMC, IETD-AMC and LEHD-AMC (Alexis Biochemicals, San Diego, CA, USA) as substrates for caspase-3, 8 and 9, respectively. A total of 75 μ g of protein lysate were incubated with 10 μ M of substrate in assay buffer (50 mM-HEPES pH 7.5, 20% sucrose, 0.2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, 10 mM-dithiothreitol) at 37°C. Caspase substrate cleavage releases 7-amino-4-methylcoumarin, which emits a fluorescent signal with 380 nm excitation and 460 nm emission. Fluorescence was registered from start of incubation for 3 h at 1 min intervals using a bonSAI fluorescence reader (Siemens, Deerfield, IL, USA). Fluorescence was calibrated using standard curves for 7-amino-4-methylcoumarin concentration. Experiments were performed 8 and 18 h post-supplementation.

Determination of mitochondrial membrane depolarisation

Cells were stained with the mitochondrial membrane potential marker JC1 (Sigma-Aldrich) for 1 h at 37°C and at a 4 μ M concentration as previously described⁽¹⁹⁾. Cells were then rinsed with medium and afterwards, fresh medium was added to the plates. Mitochondrial membrane potential was determined with fluorescence microscopy. Non-depolarised mitochondria display an orange colour and depolarised mitochondria display a green colour. Experiments were performed 18 h post-supplementation.

Determination of gene expression

Total RNA was extracted with RNAqueous®-4PCR (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions at 2, 4, 8 and 12 h post-supplementation. RNA (1 μ g) was reverse-transcribed to complementary DNA using a High-Capacity complementary DNA Reverse

Transcription kit (Applied Biosystems). TaqMan Low Density Microfluidic cards (Applied Biosystems) were loaded with 50 ng of complementary DNA plus 50 μ l of TaqMan gene expression master mix, per well. A total of four replicates were assayed for each gene: apoptosis-inducing factor (*AIF*), apoptotic protease activating factor 1 (*APAF1*), B-cell lymphoma protein 2 (*Bcl2*), Bcl-2-like protein 4 (*Bax*), B-cell lymphoma-extra large (*Bcl-xl*), Bcl-2 interacting domain (*Bid*), X-linked inhibitor of apoptosis (*XIAP*), *Survivin* FLICE-inhibitory protein (FLIP), second mitochondria-derived activator of caspases (*Smac*), Apo-1 (*PAS*), PAS-associated factor 1 (*FAF1*), Fas-associated death domain (*FADD*), PPAR α (*PPARG*), cyclo-oxygenase-2, receptor interacting protein (*RIP*), *TNF- α* , *death receptor (DR) 4*, *DR5*, TNF receptor 1 (*TNFR1*), TNF-related apoptosis-inducing ligand (*TRAIL*), TNF receptor type 1-associated DEATH domain protein (*TRADD*) and endogenous control (*TFRC*). Real-time PCR was performed with an ABI Prism 7900 (Applied Biosystems). Results were adjusted to endogenous control and normalised to experimental control. Relative expression (respiratory quotient, RQ) was calculated with the RQ manager program (Applied Biosystems). RQ values were calculated based on the comparative CT method⁽²¹⁾. Graphs reflect the plotted RQ and a relative expression of 1 means equal expression as control supplemented cells. ANOVA was used to assess statistically significant differences. *P* values of <0.05 were considered statistically significant.

Protein expression analysis

Protein expression was assessed as previously described⁽¹⁹⁾. Briefly, proteins from whole cell protein lysates were separated on a 10–12% SDS-PAGE and transferred to ImmobilonTM PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked and then incubated for 1 h with the primary antibody against B-actin (Cell Signaling Technology, Danvers, MA, USA). The overnight incubation was carried out with the following primary antibodies: Bcl-xL, Bid, XIAP (Cell Signaling Technology), Bcl-2 homologous antagonist killer (Bak) and Bax (Sigma-Aldrich). Incubation with secondary antibodies was carried out for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Cell Signaling Technology). Antibody binding was detected with SuperSignal West Pico (Pierce Biotechnology), and fluorescence was recorded on a blue sensitive X-ray film (Henry Schein, Melville, NY, USA). Blot density was analysed with ImageJ 1.44 (NIH, <http://rsbweb.nih.gov/ij/index.html>) using 8-bit gel images with background subtracted under standard settings of 50.0 pixels rolling bar radius.

Statistical analysis

All experiments were repeated at least three times and each experiment included at least three replicates per condition. Statistical analysis was performed using the Kruskal–Wallis one-way ANOVA by rank and the Mann–Whitney *U* test to assess significant differences in apoptosis. Statistical significance was assumed with a *P* \leq 0.05.

Results

Generation of SCFA after stool fermentation

The production of SCFA was approximately 1/5 in stool with no addition of PO as opposed to the stool where PO was added. Table 1 shows SCFA concentrations of acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids in stool with no added PO and 72 h after PO addition.

Apoptosis induction by *Plantago ovata* husk's fermentation products

Apoptosis induction was assessed by DAPI staining and quantified by flow cytometry. DAPI staining showed an important decrease in the number of cells treated with PO in relation to control cells. The remaining cells displayed clear apoptotic features, such as condensed chromatin, membrane blebbing and apoptotic bodies (Fig. 1(a)). Quantification by flow cytometry showed a significant increase in the percentage of apoptotic cells of between 1.8 \times in Lovo cells and 4.7 \times in SW480 cells (Fig. 1(b)). We then proceeded to examine different mechanisms leading to apoptosis in the CRC cells studied.

Plantago ovata-induced apoptosis is caspase-dependent. Implication of the intrinsic and extrinsic pathways

In order to establish the implication of the different caspase pathways, we quantified caspase activity using a fluorimetric assay. We assessed caspase-3, an executor caspase that is activated by both the intrinsic and the extrinsic pathways; caspase-8, the crucial initiator caspase of the extrinsic pathway, and caspase-9, essential in the activation of the intrinsic pathway. As shown in Fig. 1(c), the activity of all three caspases increased dramatically 18 h after supplementation with PO fermentation products in all the cells analysed, suggesting that both the intrinsic and extrinsic pathways are implicated in the pro-apoptotic effect of PO. The implication of caspases was further confirmed as the addition of a broad-spectrum caspase inhibitor z-VAD-FMK resulted in a significant decrease in the percentage of apoptotic cells in all but LoVo cells (Fig. 1(b)).

Implication of the B-cell lymphoma protein 2 family and activation of the mitochondrial pathway of apoptosis

The intrinsic pathway of apoptosis induction depends on a shift in the balance of pro- and anti-apoptotic members of the Bcl-2 family of proteins⁽²²⁾. Bax and 8 do have a pro-apoptotic effect and through their oligomerisation they permeabilise the mitochondrial outer membrane with the subsequent irreversible apoptotic cascade. On the other hand, anti-apoptotic elements such as Bcl-xL or Bcl-2 could prevent further oligomerisation and membrane permeabilisation functioning as defective constituents of Bax–Bak oligomers⁽²³⁾. As shown in Fig. 2, control cells showed a decrease in Bak protein expression that was significant at 8 and 18 h in HCT116 cells and at 8 h in LoVo cells in comparison to PO-treated

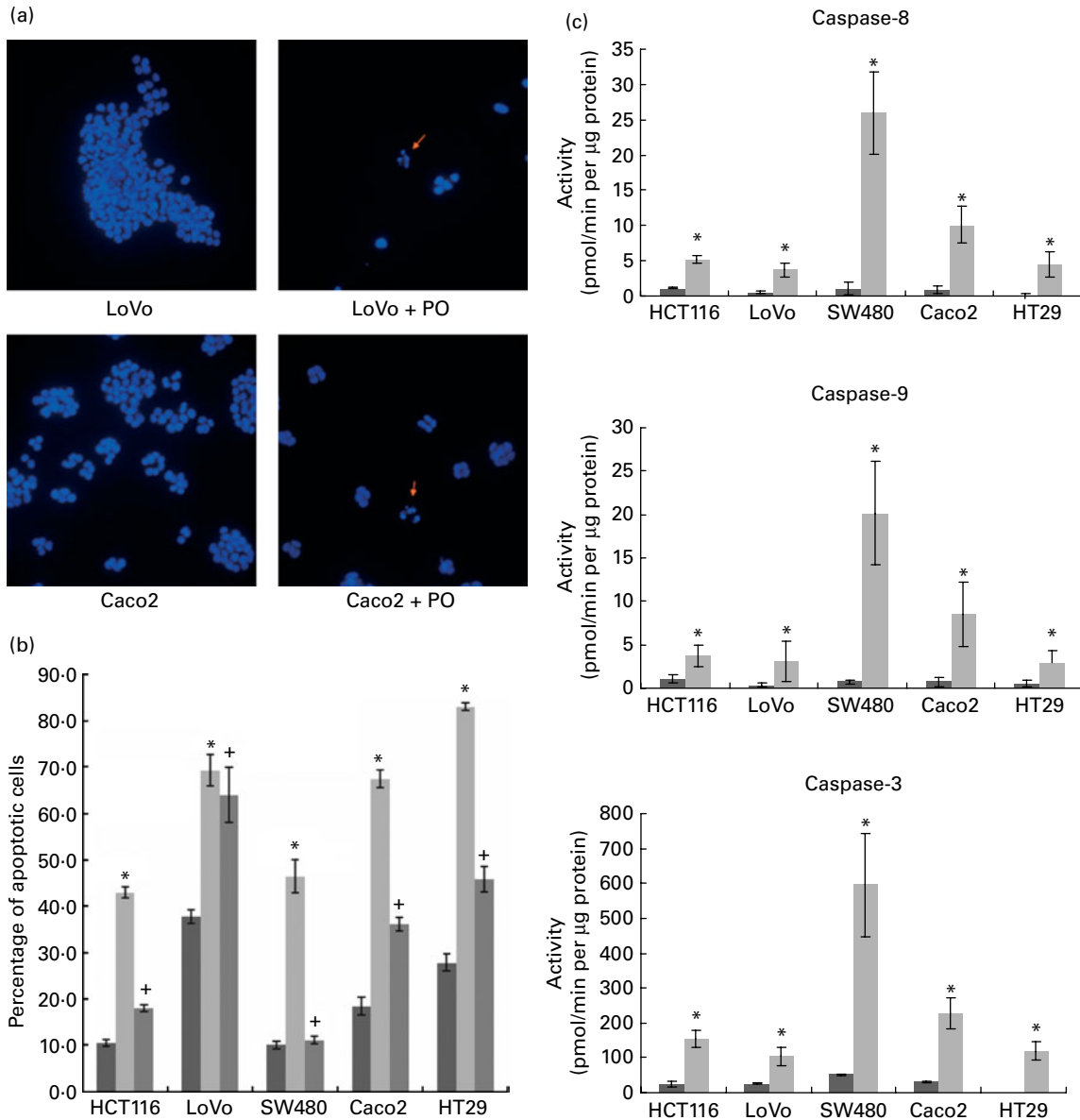


Fig. 1. (a) *Plantago ovata* husk (PO) fermentation products induced apoptosis. The panel shows a representative 4',6-diamidino-2-phenylindole staining of LoVo and Caco-2 cells after 24h treatment with PO. Orange arrows point to apoptotic bodies with condensed chromatin typical of cells undergoing apoptosis. (b) Flow cytometry quantification of apoptosis induction by PO fermentation products. The graph represents apoptosed cells as percentage of the total number of cells 24 h after supplementation of HCT116, LoVo, SW480, Caco-2 and HT-29 cells with Control (□), PO fermentation products (■) and PO + broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (zVAD) (▣). All PO-treated cells exhibited significantly greater levels of apoptosis *v.* control treatment, while PO + zVAD treatment resulted in a much lower apoptosis induction in all cells though it did not reach statistical significance in LoVo. Values are means with their standard errors represented by vertical bars. * Mean values were significantly different ($P < 0.05$). (c) Treatment with PO fermentation products induced caspase activity. Graphs represent specific caspase activity in pmol/min per µg of protein, 8 h (□) and 18 h (■) after PO supplementation in HCT116, LoVo, SW480, Caco-2 and HT-29 cells. Results are corrected for the baseline caspase activity values present in control cells. Values are means with their standard errors represented by vertical bars. * Mean values were significantly different from those of control ($P < 0.05$). Caspase 3, 8 and 9 activity was significantly greater at 18h compared to control in all cell lines.

cells. On the other hand, PO-treated cells had significantly lower levels of anti-apoptotic Bcl-xL protein at 8 and 18h in HCT116 and at 18h in LoVo cells, indicating a clear shift towards a pro-apoptotic balance. A decrease in *Bcl-xL* gene expression was significant in all five cell lines except in Caco-2 cells (Table 3). No significant changes were seen in Bax protein expression in HCT116 cells while LoVo cells' expression was undetectable. No significant changes in protein expression were seen in Bid. Finally, Bcl-2 protein

expression was not detected in any of the cell lines studied (data not shown).

The final result of a balance towards a pro-apoptotic environment results in mitochondrial membrane permeabilisation and subsequent release of key elements that trigger the apoptotic cascade. All PO-supplemented cells underwent mitochondrial membrane permeabilisation as assessed by JC1 stain, a mitochondrial potential marker. Control cells displayed mitochondria stained in red-orange, whereas

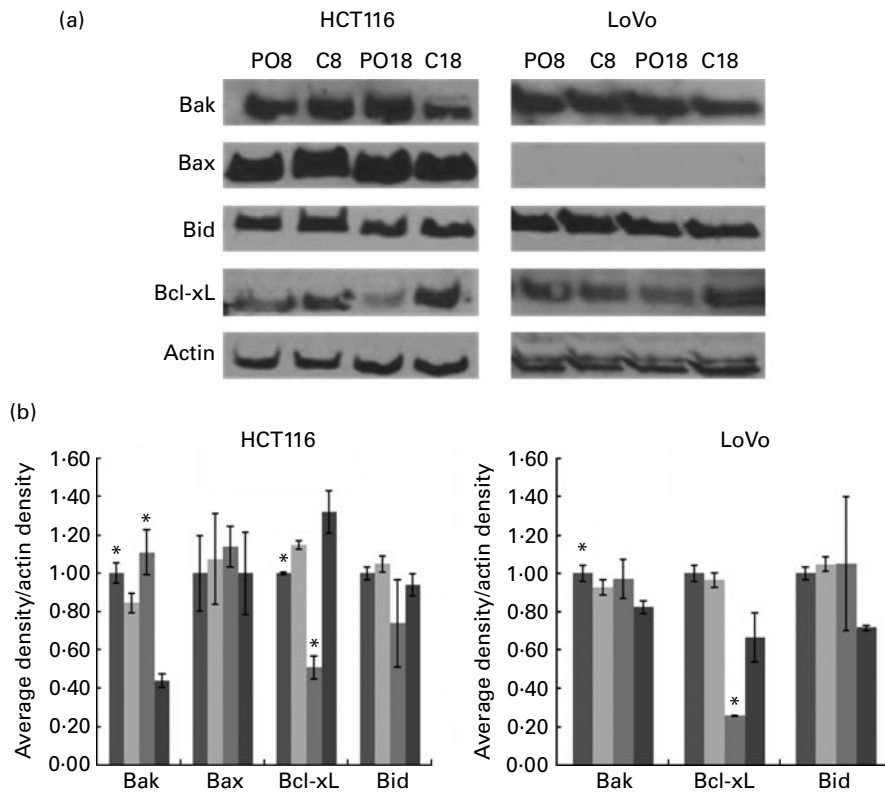


Fig. 2. *Plantago ovata* husk (PO) treatment resulted in a shift towards a pro-apoptotic state of the B-cell lymphoma protein 2 (Bcl-2) family of proteins. The figure shows immunoblots of Bcl-2 homologous antagonist killer (Bak), Bcl-2-like protein 4 (Bax), Bcl-2 interacting domain (Bid) and Bcl-extra large (Bcl-xL) after treatment of HCT116 and LoVo cells with PO and control (C) at 8 and 18 h. Actin was used as housekeeping control protein. Control cells showed a decrease in Bak protein expression that was significant at 8 and 18 h in HCT116 cells and at 8 h in LoVo cells in comparison to PO-treated cells. The expression of pro-apoptotic Bak was increased at 18 h in HCT116, and at 8 h in LoVo cells. The expression of anti-apoptotic Bcl-xL was decreased compared to control at 8 and 18 h after PO treatment in HCT116 and at 18 h in LoVo cells. Bid and Bax did not show any expression changes, but the latter was undetectable in LoVo cells. Blot density was analysed with ImageJ 1.44 (NIH, <http://rsbweb.nih.gov/ij/index.html>) using 8-bit gel images with background subtracted under standard settings of 50.0 pixels rolling bar radius. Values are means with their standard errors represented by vertical bars. ■, PO 8h; ▨, C 8h, ▩, PO 18h; ▪, C 18h.

PO-treated cells had a distinct green coloration clearly establishing induction of depolarisation by PO (Fig. 3(a)). Presumably as a consequence of the depolarisation, *APAF1* expression was increased in all cells after PO treatment though it did not reach significance in Caco-2. The expression of *AIF* was increased in metastatic Lovo, HT-29 and Caco-2 cells though it was not significant in the latter. To the contrary, *AIF* was down-regulated in HCT116. Smac/Diablo was up-regulated in the same cells as *AIF*, reaching significance also in Caco-2 but not in SW480. On the other hand, the IAP *Survivin* was down-regulated in all cells after PO treatment, reaching significance in LoVo and HCT116. HT29 had undetectable levels. Finally, the inhibitor of apoptosis protein *XIAP* gene expression was down-regulated in HCT116, SW480 and HT-29, while there was a late up-regulation in LoVo cells that was not detected at the protein level (Fig. 3(b) and (c); and Table 3).

Implication of cellular death receptors

In order to determine the potential implication of cellular death receptors in the induction of apoptosis by PO, we studied the gene expression of *FAS*, *DR5*, *DR4*, *TNF-R1*, as well as *TRADD*, *TNF α* and *RIP 2*, 4, 8 and 12 h post-supplementation.

All cell lines showed rapid up-regulation of *DR5* (2 h post PO supplementation) and a significant down-regulation of *DR4*, *Fas* and also *TNFR1*, although the latter was not significant in LoVo and Caco-2 cells (Fig. 4 and Table 3). On the other hand, while HCT 116 did not express *TNF- α* , only metastatic Lovo cells showed a significant increase of *TNF- α* expression, as early as 2 h post PO supplementation, accompanied by a significant up-regulation of two of the key elements of the death-inducing signalling complex (DISC): *TRADD* and *RIP*. Finally, *FLIP* was down-regulated after PO supplementation in all CRC cell lines, though it did not reach significance in metastatic Lovo cells (Fig. 4 and Table 3).

Discussion

The main objectives of the present study were: to assess if PO fibre supplementation can induce CRC cell apoptosis, and to investigate the mechanisms implicated in this anti-neoplastic effect. Apoptosis is one of the crucial anti-carcinogenic mechanisms by which damaged or mutated cells are eliminated. For this purpose, we used Plantaben®, which contains PO, a mucilaginous fibre that had been associated with a reduction in CRC risk⁽¹⁴⁾ and mortality⁽¹⁵⁾. Fermentation was carried out in anaerobic conditions using human

Table 3. Gene expression changes of different elements involved in apoptosis regulation after treatment with *Plantago ovata* husk (PO) fermentation products in five colorectal cancer cell lines

	Caco2				LoVo				SW480				HCT116				HT29			
	2h	4h	8h	12h	2h	4h	8h	12h	2h	4h	8h	12h	2h	4h	8h	12h	2h	4h	8h	12h
AIF	1.15	1.02	1.31	1.54	1.20*	1.00	1.22*	1.36*	1.14*	1.09	0.94	0.97	1.06	0.85	0.86	0.73*	1.15	1.07	1.58*	1.41*
APAF	1.41	1.47	1.71	1.58	1.38	1.32	1.87*	1.93*	1.63*	1.50	1.30	1.21	1.50*	1.38*	0.98	0.88	1.21	1.29	1.90*	1.84*
Bax	1.03	0.99	0.85	1.08	1.00	0.91	0.77*	0.85*	1.13	1.09	0.78*	0.85	1.09	0.90	0.66*	0.48*	Low expression†			
Bcl2	Low expression†				1.04	0.75	1.18	1.08	1.24	1.44	1.35	1.45	0.89	0.71	0.59	0.49*				
Bcl-xL	1.03	0.70	0.65	0.75	0.88	0.67*	0.80	0.90	0.87	0.63*	0.50*	0.46*	1.00	0.78	0.39*	0.17*	1.04	0.65*	0.67*	0.66*
BID	0.95	0.83	0.64*	0.67	1.02	0.82*	0.95	0.97	1.00	0.67	0.51*	0.35*	1.04	0.84	0.56*	0.36*	1.03	0.77	0.72	0.60*
XIAP	1.05	0.81	1.05	1.28	1.00	0.81	1.27	1.69*	0.82	0.59*	0.62	0.71	0.89	0.64*	0.44*	0.36*	0.88	0.63*	0.81	1.06
Survivin	0.97	0.88	0.89	1.07	0.92	0.67*	0.78*	0.70*	1.02	0.87	0.79	0.72	0.92	0.75*	0.73*	0.59*	Low expression†			
FLIP	1.11	0.88	0.64	0.47*	0.85	0.79	0.97	0.99	0.82	0.54*	0.32*	0.27*	0.82	0.54*	0.32*	0.19*	1.01	0.62*	0.54*	0.46*
Smac	1.05	1.22	1.31	1.56*	1.23	1.35*	1.86*	1.89*	1.02	1.13	1.04	1.15	1.08	1.01	1.12	0.97	1.03	1.22	1.46*	1.33*
FADD	0.82	0.82	0.68	1.04	0.77	0.82	1.19	1.32	0.85	1.01	0.69*	0.83*	1.22	0.73	0.71*	0.61*	0.76*	0.74*	0.86	0.92
FAF1	0.97	0.81	1.00	1.14	1.13	0.92	0.99	1.08	1.10	1.04	0.93	1.10	1.10	0.77*	0.88	0.87	Low expression†			
FAS	0.86	0.73*	0.46*	0.61*	0.88	0.67*	0.73*	0.74*	1.00	0.88	0.54	0.52	0.84	0.69	0.42*	0.20*	0.88	0.65*	0.54*	0.48*
PPARG	1.09	0.78	0.78	0.65	1.15*	1.05	1.20*	1.22*	1.14	1.12	1.17	0.81	1.02	0.79	0.62*	0.41*	0.99	0.82	0.83	0.58
Cox-2	0.91	0.63*	0.52*	0.87	1.53	1.17	1.36	1.30	Low expression†			1.55	1.28	2.08*	1.39	1.15	0.54*	0.58*	0.49	
RIP	1.32	1.39	1.37	1.18	1.98*	1.59	2.74*	3.14*	1.05	1.02	0.70*	0.76*	0.94	0.82	0.66*	0.62*	Low expression†			
TNF α	3.50	5.43	2.12	1.58	9.52*	10.54*	4.39*	3.09	1.64	1.55	1.47	1.74	Low expression†							
DR4	1.04	0.70*	0.59*	0.69*	0.91	0.70*	0.90	0.99	1.05	0.80	0.55*	0.63*	1.14	0.74*	0.49*	0.44*	1.05	0.65*	0.73	0.52*
DR5	1.91	1.70	1.62	1.79	1.65*	1.40	1.22	1.49	1.29*	1.21	1.08	1.17	1.48*	1.21*	1.21*	1.26*	1.50	1.28	2.18*	1.51
TNFR1	0.99	0.82	0.94	1.01	0.98	0.74	1.13	1.53	0.91	0.78	0.58*	0.60*	0.85	0.56*	0.53*	0.48*	1.03	0.70*	0.89	0.88
TRAIL	1.81	2.08*	1.94*	2.77*	0.97	0.73*	1.41*	1.51*	Low expression†				Low expression†				Low expression†			
TRADD	1.54	1.50	1.25	1.82	1.13	1.25	2.25*	2.69*	1.18	1.23	1.24	1.47	1.18	0.95	0.91	0.75				

AIF, apoptosis-inducing factor; APAF, apoptotic protease activating factor; Bax, B-cell lymphoma-2-like protein 4; Bcl2, B-cell lymphoma protein 2; Bcl-xL, Bcl-extra large; BID, Bcl-2 interacting domain; XIAP, X-linked inhibitor of apoptosis; FLIP, FLICE-inhibitory protein; Smac, second mitochondria-derived activator of caspases; FADD, Fas-associated death domain; FAF1, FAS-associated factor 1; FAS, Apo-1; PPARG, PPAR γ ; Cox-2, cyclo-oxygenase-2; RIP, receptor interacting protein; DR, death receptor; TNFR1, TNF receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; TRADD, TNF- α receptor 1-associated death domain protein.

* Fold changes demonstrating a significant difference between PO-treated cells and control-treated cells.

† Low expression indicates mRNA targets which required greater than or equal to forty PCR cycles for detection.

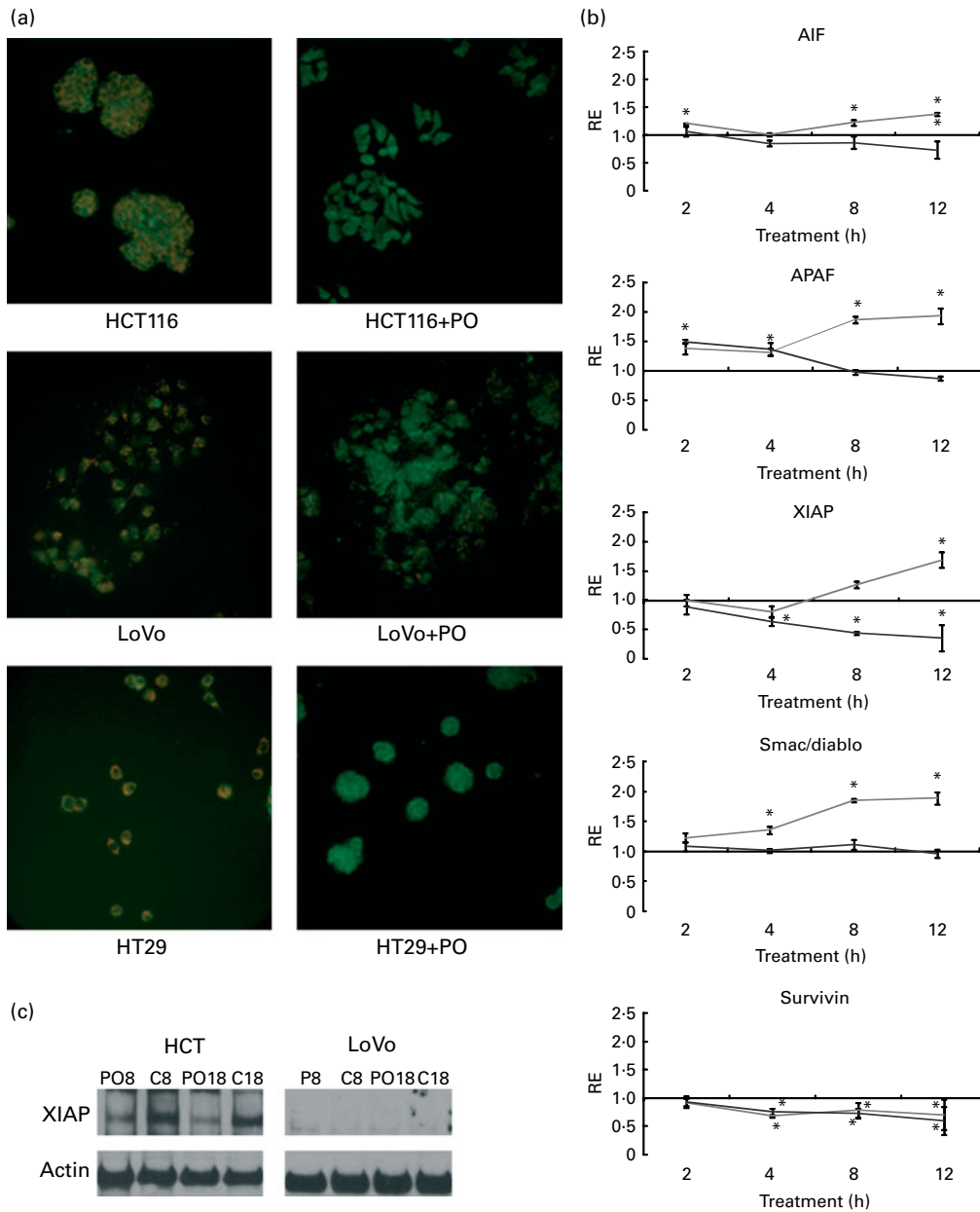


Fig. 3. (a) Mitochondrial membranes showed depolarisation 18 h after treatment with *Plantago ovata* husk (PO) fermentation products. Pictures show cells after JC-1 stain, a mitochondrial potential marker stain. Control cells show an orange colouration, indicating polarisation of the mitochondrial membrane, and PO-treated cells show a green colouration, as a result of mitochondrial membrane depolarisation. (b) Gene expression of intrinsic pathway regulators after treatment with PO fermentation products. Graphs represent variation of mRNA expression 2, 4, 8 and 12 h after PO supplementation, in respect to the control. Graphs reflect the plotted respiratory quotient (RQ) and a relative expression (RE) of 1 means equal expression as control supplemented cells. RQ value was calculated by the $\Delta\Delta CT$ method (endogenous control used was the *TFRC* or transferrin receptor gene). ANOVA was used to assess statistically significant differences. Values are means with their standard errors represented by vertical bars. Mean values were considered statistically significant different ($P < 0.05$). LoVo cells (—) and HCT116 cells (—). Intrinsic pathway activators apoptosis-inducing factor (*AIF*), apoptotic protease activating factor 1 (*APAF1*) and second mitochondria-derived activator of caspases (*Smac*/*Diablo*) were up-regulated in PO-treated LoVo cells. Only *APAF1* was significantly increased in HCT116 after PO. Anti-apoptotic *Survivin* was significantly down-regulated in both cell lines while X-linked inhibitor of apoptosis (*XIAP*) was down-regulated in HCT116 cells. (c) Regulation of anti-apoptotic *XIAP* protein by PO fermentation products. The figure shows immunoblots of *XIAP* 8 and 18 h after PO treatment and control (C). Actin was used as housekeeping protein. *XIAP* protein expression was down-regulated in HCT116 cells after PO treatment. LoVo cells had low *XIAP* protein expression.

faecal microbiota to mimic *in vivo* conditions and all products generated by this process were used to supplement cells and assess apoptosis induction. Therefore, cell supplements contained high concentrations of propionic and acetic, besides butyric acid. While butyric acid is well known to induce colonocyte apoptosis, to our knowledge

only another recent study by Borowicki *et al.*⁽²⁴⁾ has addressed this effect using all SCFA generated through anaerobic fibre fermentation.

Our present study results have shown that there is a significant induction of apoptosis in all CRC cells after supplementation with the fermentation products of PO. Apoptosis

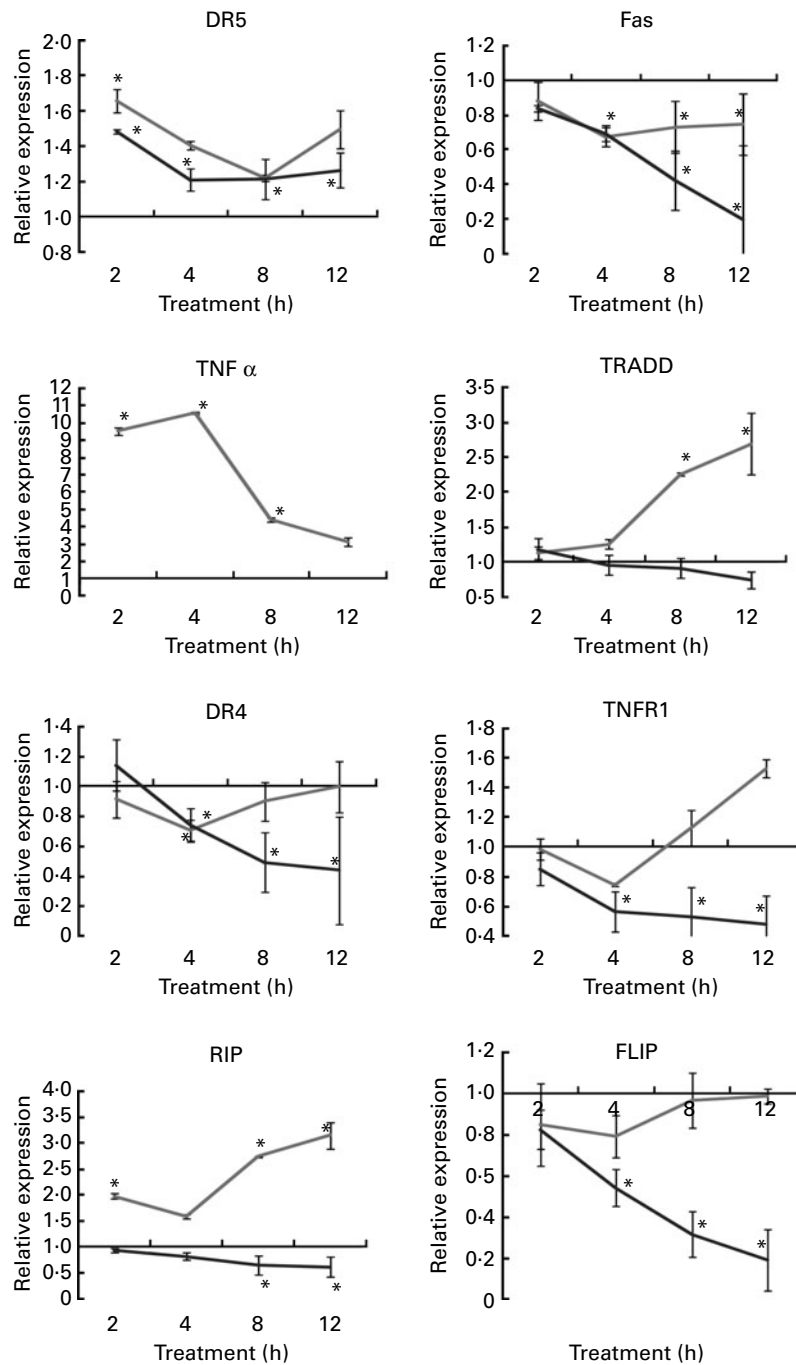


Fig. 4. Gene expression of extrinsic pathway regulators after treatment with *Plantago ovata* husk (PO) fermentation products. Graphs represent variation of mRNA expression 2, 4, 8 and 12 h after PO supplementation, respect to the control. Graphs reflect the plotted respiratory quotient (RQ) and a relative expression of 1 means equal expression as control supplemented cells. RQ value was calculated by the $\Delta\Delta CT$ method (endogenous control used was the *TFRC* or transferrin receptor gene). ANOVA was used to assess statistically significant differences. Values are means with their standard errors represented by vertical bars. Mean values were considered statistically significant different ($P < 0.05$) LoVo cells (—) and HCT116 cells (---). Death receptor (DR) 5 was significantly up-regulated as early as 2 h after PO treatment, while other receptors DR4, Fas and TNF receptor 1 (TNFR1) had decreased expression. TNF- α (ligand) was dramatically up-regulated in metastatic LoVo cells, but no significant expression was detected in HCT116 cells. Death-inducing signalling complex (DISC) members receptor interacting protein (RIP) and TNF receptor type 1-associated DEATH domain protein (TRADD) were up-regulated only in LoVo cells. Finally, the key extrinsic pathway inhibitor FLICE-inhibitory protein (FLIP) had decreased expression in HCT116 only.

induction is independent of *p53*, *APC*, cadherin-associated protein $\beta 1$ (β -catenin), mismatch repair or cyclooxygenase-2 expression status, as the studied cells have different levels of alteration in regards to these genes (Table 2), which suggests that this beneficial effect of PO is not limited to specific

colorectal carcinogenic pathways and it could be potentially useful as an anti-carcinogenic agent in a wide group of CRC. PO-induced apoptosis is mediated by caspases. Caspase 3, as well as caspase 8 and 9, activities are increased, demonstrating involvement by both the extrinsic and intrinsic pathways.

The involvement of the intrinsic pathway was further confirmed by the shift in the balance towards an increase in pro-apoptotic Bak expression and a decrease in anti-apoptotic Bcl-xL in both HCT116 and LoVo cells, in line with results previously reported in butyrate-treated Caco-2 cells^(25,26). The final result of a balance towards a pro-apoptotic environment results in mitochondrial membrane permeabilisation⁽²⁷⁾. Permeabilisation allows inter-membrane space proteins, including caspase activators (cytochrome-C and Smac/Diablo) as well as caspase-independent death effectors (AIF), to be released into the cytosol. Cytochrome-C binds to APAF1 and mediates its conformational change facilitating the activation of the initiator caspase-9 in a direct fashion via the assembly of the apoptosome causing the activation of effector caspases. Smac/Diablo favours the caspase cascade indirectly by antagonising the activity of endogenous inhibitors of caspases, such as IAP⁽²⁸⁾. In all PO-treated cell lines, we observed a depolarisation of the mitochondrial membrane along with a subsequent increase of caspase activator *Smac/Diablo* and death effector *AIF* expression in most cell lines, except in HCT116, presumably due to their release after mitochondrial depolarisation. We also observed a significant increase in the apoptosome member *APAF1* expression and a decrease in the inhibitors of apoptosis *Survivin* and *XIAP* expression, except in LoVo cells where there was a late increase in *XIAP* expression. Derangements of these intrinsic pathway components have well documented important clinical consequences. For example, decreased expression of *APAF1* in CRC correlates with tumour progression and aggressive phenotype^(29,30). *Survivin* over-expression in CRC has been associated with worse prognosis⁽³¹⁾, higher risk of relapse⁽³²⁾ and local recurrence⁽³³⁾, while *Survivin* down-regulation is key in the apoptosis induction of some chemotherapeutic agents^(34,35). *XIAP* expression has been correlated to poor clinical outcome and therapy resistance⁽³⁶⁾, but *XIAP* inhibition can increase sensitivity to some chemotherapeutic agents^(37,38) and was found to be useful in the treatment of chemo-refractory cancers⁽³⁹⁾. As down-regulation of these important apoptosis inhibitors had not been seen in a previous study on butyrate-treated cells⁽²⁶⁾, we cannot rule out that this could be related to the presence of the other fatty acids or even totally distinct products generated through PO fermentation. All together, PO's regulation of crucial aspects of the intrinsic pathway of apoptosis suggests important anticancer effects.

Several members of the TNF receptor family and their ligands are key elements in the induction of apoptosis via death receptors. Some of these receptors, such as TrailR2 (or DR5), TrailR1 (or DR4), Fas or TNFR1, contain death domains in their cytosolic tails. The activation of these receptors results in receptor clustering and recruitment of intracellular proteins, such as TRADD and RIP, to the cytosolic domains forming the DISC that triggers caspase activation and leads to apoptosis⁽⁴⁰⁾. On the other hand, there is evidence that some of these receptors, namely DR4 and TNFR1, engage in parallel pathways and besides apoptosis induction they can also send an anti-apoptotic signal through the activation of the NF- κ B pathway⁽⁴¹⁾. FLIP inhibits caspase-8 activation, blocking the apoptotic signal transduction through the extrinsic pathway. In the present study, the extrinsic pathway of apoptosis was also

induced in PO-treated cells. That is, most cells showed up-regulation of death receptor *DR5*, while *Fas* and *DR4* showed significant down-regulation. *TNFR1* was also significantly down-regulated in HCT116, HT-29 and SW480. As *DR4* and *TNFR1* may alternatively activate the pro-survival NF κ B pathway⁽⁴¹⁾, down-regulation by PO could further promote cell death by suppressing survival signals. Finally, *DR4* over-expression has been associated with worse disease-free and overall survival in some CRC patients⁽⁴²⁾.

While death receptors *DR4*, *DR5* and *Fas* were similarly regulated by PO in all cell lines, other important extrinsic pathway elements were differentially regulated in metastatic *v.* non-metastatic cells. As such, the remarkable up-regulation of *TNF- α* ligand along with DISC components *RIP* and *TRADD* in metastatic LoVo cells suggests a distinctive mechanism of apoptosis activation. This could be potentially important, as a higher endogenous expression of *TNF- α* has been associated with decreased metastatic potential and better prognosis in CRC^(43–45).

In primary tumour but not metastatic CRC cell lines, the extrinsic pathway modulator *FLIP* was significantly down-regulated after PO treatment (Table 3). *FLIP* may inhibit both spontaneous death ligand-independent and death receptor-mediated apoptosis in CRC cells⁽⁴⁶⁾. *FLIP* competes with pro-Caspase-8 and -10 for binding to FADD, thus squelching death receptor signalling. Several tumours, including CRC, contain inappropriately elevated levels of *FLIP*, rendering them resistant to apoptosis induction by death domain signalling. Interestingly, several important chemotherapeutic agents have been shown to induce apoptosis through down-regulation of *FLIP* expression^(47–49), which suggests an important anti-carcinogenic mechanism of PO in CRC.

In summary, using a setting that mimics physiological conditions in the gut, we have shown that supplementation with PO induces apoptosis in different types of CRC cells. This induction is mediated by both the intrinsic and extrinsic apoptosis pathways and is the result of modulating important regulators of these pathways. This regulation involves some distinct elements in primary tumour *v.* metastatic CRC cells. The presented results suggest that PO husk could potentially be useful as an adjuvant element in chemotherapy treatments.

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