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Human Gut Flora-Fermented Nondigestible Fraction from Cooked Bean (*Phaseolus vulgaris* L.) Modifies Protein Expression Associated with Apoptosis, Cell Cycle Arrest, and Proliferation in Human Adenocarcinoma Colon Cancer Cells

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ABSTRACT: Metabolism of the nondigested fraction (NDF) from common bean (*Phaseolus vulgaris* L.) by the human gut flora (hgf) produces short-chain fatty acids (SCFAs) that may benefit cancer by reducing colorectal tumor risks. This paper reports the effect of fermentation products (FP) by hgf (FP-hgf) from NDF of cooked beans on survival and protein expression associated with apoptosis, cell cycle arrest, and proliferation in human adenocarcinoma colon cancer cells. FP-hgf was the only inoculum eliciting butyrate production after 24 h of NDF fermentation using different bacterial sources. FP-hgf inhibited HT-29 cell growth and modulated protein expression associated with apoptosis, cell cycle arrest, and proliferation, as well as morphological changes linked to apoptosis evaluated by TUNEL and hematoxylin and eosin stains, confirming previous results on gene expression. The current results suggest that fermentation of NDF from common beans can elicit beneficial chemoprotective effects in colon cancer by modulating protein expression in HT-29 cells.

KEYWORDS: *colon cancer, common bean, in vitro fermentation, nondigestible fraction, protein modulation*

■ INTRODUCTION

Beans are a staple food in Mexico and other Latin American countries,[1] containing numerous bioactive substances including enzyme inhibitors, lectins, phytates, oligosaccharides, and phenolic compounds as well as nondigestible compounds that play an important metabolic role in humans and animals that frequently consume these foods.[2]

Dietary fiber fermentation in the human large intestine modifies the species and composition of the colonic microbiota and manifests several health-promoting properties related to potential anticancer activities, lipid metabolism, and anti-inflammatory and other immune effects, including atopic disease. Many of these phenomena can be linked to their digestion and short-chain fatty acids (SCFAs) production by bacteria in the large intestine.[3] Metabolism of the nondigestible fraction (NDF) from common bean (*Phaseolus vulgaris* L.) by the human gut flora (hgf) produces SCFAs such as acetate, propionate, and butyrate.[4] SCFAs, mostly propionate and butyrate, induce differentiation, growth arrest, and apoptosis in colon cancer cells. Butyrate's anticarcinogenic effects are in contrast with the effects of this compound in normal enterocytes.[5] In fact, butyrate stimulates the physiological pattern of proliferation in the basal crypt in the colon, whereas it reduces the number and size of aberrant crypt foci, which are the earliest detectable neoplastic lesions in the colon. These contradictory patterns of butyrate represent the so-called "butyrate paradox".[5] Our previous study[6] showed that fermentation products (FP) by hgf (FP-hgf) from the NDF up-regulated gene expression (evaluated by PCR array) in human colon cancer HT-29 cells involved in cell cycle arrest and the induction of apoptosis and proliferation inhibition. Protein modulation linked to progression and malignancy, cell cycle, and actin-associated proteins also results from butyrate effects on HT-29 cells.[7] In vitro assays of SCFAs have shown growth arrest induction in malignant cell lines, reverse neoplasic changes, and similar effects exerted in the colonic epithelium in vivo.[8,9]

The objective of this research was to evaluate the chemopreventive effect of FP from NDF of cooked bean cv. 'Bayo Madero' formed by in vitro colon model (simulating human colon fermentation) on human colon adenocarcinoma HT-29 cell survival and to evaluate the effects of different bacterial isolates from humans [hgf and the commercial cell cultures Lactipan (L) and Danisco (D)] on intestinal epithelial growth, potentially through SCFAs, mainly butyrate, generation, from NDF. FP-hgf was evaluated on HT-29 cells to investigate protein modulation linked to apoptosis, cell cycle arrest, and proliferation, as well as apoptotic induction by

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TUNEL and hematoxylin and eosin assays for their chemopreventive effect against the development of colon cancer.

■ MATERIALS AND METHODS

Chemicals. Butyrate was purchased from Fluka (Sigma-Aldrich, Canada Ltd.). Acetate, propionate, formic acid, and other chemicals were purchased from J. T. Baker (Mexico). Danisco culture (bulk set Y 216 LYO 500) was purchased from Danisco (Copenhagen, Denmark), and Lactipan culture was obtained from a commercial market.

Dry Bean Seeds. Seeds of cultivar 'Bayo Madero' were harvested in 2005 at the Bajio Experimental Station of the National Research Institute for Forestry, Agriculture and Livestock (INIFAP), Celaya, Guanajuato, Mexico, and cooked using a traditional cooking process.[10]

Extraction of Nondigestible Fraction. AOAC Method 991.43[11] was used to obtain total dietary fiber (TDF) considered here as the NDF.

In Vitro Lower Gastrointestinal Fermentation. This study used a human gut flora fermentation method to estimate the effects of NDF digestion in the colon representing a simulated large bowel model. Although this is a model of what actually occurs in the large bowel,[12] it provides useful data to form hypotheses for in vitro studies.[13] In vitro fermentation was performed according to the procedure described earlier.[14] Two commercial mixtures, Lactipan and Danisco, were used as bacterial source to compare the SCFAs production with human gut flora (Table 1). Briefly, fermentations were performed in duplicate in a water bath at 37 °C. Raffinose was used as a control for fermentable sugar under the same conditions. Fecal inoculum was prepared from stool supplied by a healthy subject, who had not consumed antibiotics for at least 3 months and had no history of gastrointestinal diseases. Sterile tubes (15 mL capacity) were filled with 9 mL of sterile basal culture medium. Sealed tubes were maintained under a headspace containing H_2–CO_2–N_2 (10:10:80, by volume), O_2-free, for 24 h. Fecal slurries were prepared by homogenizing 2 g of fresh stool with 18 mL of 0.1 M sodium phosphate buffer, pH 7.0. Danisco culture was prepared by rehydrating 1 mg of dry culture in 50 mL of 0.1 M sodium phosphate buffer, pH 7.0. Lactipan culture (2 pills) was homogenized in 5 mL of 0.1 M sodium phosphate buffer, pH 7.0. Lactipan/Danisco mixtures were prepared by homogenizing 0.5 mL of each solution. The tubes containing the mixture of basal culture medium were inoculated with 1 mL of fecal slurries, Lactipan, Danisco, or Lactipan/Danisco, and the NDF (100 mg) was added after inoculation, except for blanks. The samples were vortexed for 30 s and placed in a water bath at 37 °C. At the end of each fermentation time, the pH of the sample and SCFAs production were assessed (6, 12, and 24 h). Fermentation was stopped by placing the tubes in a freezer at −70 °C.

Table 1. Commercial Bacterial Mixture Composition

Lactipan	mo[a]/g	Danisco[b]
Lactobacillus acidophilus	2.0×10^8	*Streptococcus thermophilus*
Lactobacillus bulgaricus	2.0×10^8	*Lactobacillus delbrueckii* subsp. *bulgaricus*
Lactobacillus caseii	2.0×10^{10}	*Lactobacillus acidophilus*
Lactobacillus lactis	5.0×10^7	*Bifidobacterium lactis*
Lactobacillus plantarum	1.6×10^8	
Streptococcus lactis	2.0×10^3	

[a]mo = microorganism. [b]No specified amount.

SCFAs Analysis. The short-chain fatty acids were determined according to the procedure of Campos-Vega et al.[14] The frozen fermentation tubes were rapidly thawed in warm water, and a sample of 1.5 mL was centrifuged (2264*g*, 15 min, 4 °C; Hermle Z 323 K, Hermle Labortechnik GmbH, Wehingen, Germany); 800 μL of the supernatant was transferred to an 8 mL tube and adjusted to pH 2.9–3.1 with 1 M HCl; 750 μL of the sample was transferred to a flask containing 120 μL of formic acid. An aliquot (1.5 μL) of the supernatant was loaded in the injection port of an Agilent 6890 gas chromatography Plus coupled to a flame ionization detector (FID) (Agilent Technologies Wilmington, DE, USA) and separated on a Carbowax 20 M column (25 m × 320 μm × 0.30 μm nominal). Helium was supplied as the carrier gas at a 1.0 mL/min flow rate. The initial oven temperature of 95 °C was maintained for 2 min and ramped at 20 °C/min to 180 °C. The temperatures of the FID and the injection port were 240 and 200 °C, respectively. The flow rates of hydrogen, air, and nitrogen as makeup gas were 30, 300, and 20 mL/min, respectively. Acetate, propionate, and butyrate were used as external standards to quantify SCFAs in the fermentation mixtures.

Cell Growth Inhibitory Assay. Human colorectal cancer cells, HT-29, that had been established by Fogh and Trempe from a colon adenocarcinoma of a Caucasian female[15] containing a mutated p53 gene were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. The cells were grown and maintained in McCoy's 5A medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% antibiotic–antimicotic (Gibco) at 37 °C in 95% air and 5% CO_2. Subculture of the HT-29 cell line was performed by enzymatic digestion (trypsin/EDTA solution: 0.05/0.02%) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada).

HT-29 cells (passage 6) were cultured in 24-well plates at a density of 3×10^4cells/well under the growth conditions indicated above for 24 h. The medium was changed by adding McCoy's 5A medium containing 0.5% BSA (Sigma-Aldrich Canada Ltd.) plus different concentrations of 100% fermented extract (FP-hgf, 24 h; the frozen fermentation tubes were rapidly thawed in warm water, a sample of 1.5 mL was centrifuged (Hermle Z 323 K, Hermle Labortechnik GmbH) at 2264*g* for 15 min at 4 °C and sterilized by filtration) or fermented extract without NDF addition as control. After incubation (24 h), cells were harvested, hemocytometer counts were performed, and the growth inhibition rate was plotted to determine cell survival LC_{50} (lethal concentration 50) value. McCoy's 5A medium containing 0.5% BSA was also added to control cell culture. Synthetic mixtures mimicking the LC_{50}/FP-hgf in SCFAs (5.1, 0.68, and 1.19 mmol/mL of acetic, propionic, and butyric acids, respectively) were also evaluated. All data points were performed in duplicate, and each experiment was repeated independently in triplicate for statistical evaluation.

Measurement of Cellular Injury. Cellular injury was measured by lactate dehydrogenase (LDH) release.[16] LDH content was determined by an LDH-Cytotoxicity Assay Kit (BioVision, Mountain View, CA, USA). Cytotoxicity in experimental samples measured as percent LDH release was compared with cells treated with 1% Triton X-100.

Hematoxylin and Eosin Staining. HT-29 cells were cultured in 60 mm Petri dishes at 5×10^4 cells/well density under the growth conditions indicated above until confluence and exposed to treatments (LC_{50}/FP-hgf, SCFAs solution, and control) for 24 h; adherent cells were used for morphological studies. For this purpose, coverslips were posited on the bottoms of the dishes. The cultured cells growing on the coverslips were fixed with methanol and stained with hematoxylin and eosin (H&E). Samples were taken for cell count and for counting apoptosis 24 h after treatment. Apoptotic cells were identified by characteristic morphology, according to the criteria of Ben-Sasson et al.,[17] and the apoptotic index (AI) was calculated.

$$\text{AI} = (\text{no. of apoptotic cells})/\text{no. of total cells})$$

TUNEL Assay. DNA fragmentation was detected by the TUNEL method. Once LC_{50} was determined, cells were treated with the LC_{50}/FP-hgf and with a synthetic mixture of SCFAs mimicking LC_{50} for 24 h. Afterward, the adherent treated and untreated cells were harvested and centrifuged to remove the medium. The pellet was resuspended in 1× phosphate-buffered saline and then laid on a coverslip until dry. Cells were fixed with methanol to be stained by TUNEL, which identifies apoptotic cells by transferring biotin-dUTP to the strand breaks of cleaved DNA during apoptosis using terminal deoxynucleotidyl transferase. The biotin-labeled cleavage sites were then detected by reaction with horseradish peroxidase conjugated streptavidin and visualized by 3,3-diaminobenzidine. The brown spots representing apoptotic cells were counted at 10 arbitrarily selected microscopic

Table 2. Effect of NDF on pH and SCFAs Production during in Vitro Fermentation with Different Bacterial Inoculums[a]

bacterial source	pH			
	0 h	6 h	12 h	24 h
human gut flora	6.69 ± 0.08 x	6.04 ± 0.01 x	5.79 ± 0.15 y	5.82 ± 0.01 x
Lactipan	6.70 ± 0.05 x	6.90 ± 0.02 y	6.90 ± 0.03 x	6.04 ± 0.29 y
Danisco	6.74 ± 0.03 x	7.00 ± 0.005 y	6.35 ± 0.05 x	6.08 ± 0.36 y
Lactipan/Danisco mixture	6.73 ± 0.05 x	6.99 ± 0.05 y	6.66 ± 0.3 x	5.72 ± 0.005 x

[a]Means in the same column with different letters are different ($p < 0.05$, Tukey's test). Results are the average of three independent experiments ± standard error.

Table 3. Short-Chain Fatty Acids (SCFAs, Millimoles per Milliliter) in Fermentation Products of Cooked Bean Polysaccharides Using Different Bacterial Inoculums[a]

bacterial source	6 h			12 h			24 h		
	acetate	propionate	butyrate	acetate	propionate	butyrate	acetate	propionate	butyrate
human gut flora	29 ± 0.0 w	1 ± 0	1 ± 0.1	30 ± 0.5 w	2 ± 0	7 ± 0.5	30 ± 0.5 w	4 ± 0.0 a	7 ± 0.5
Lactipan	17 ± 0.0 x	UDL[b]	UDL	18 ± 0.0 x	UDL	UDL	20 ± 1 x	UDL	UDL
Danisco	19 ± 0.5 x	UDL	UDL	22 ± 1.0 y	UDL	UDL	23 ± 0.5 y	UDL	UDL
Lactipan/ Danisco	18 ± 0.1 x	UDL	UDL	20 ± 0.5 x	UDL	UDL	26 ± 1.5 y	2 ± 0.5 b	UDL

[a]Means in the same column for varieties and SCFAs with different letters are different ($p < 0.05$, Tukey's test). Results are the average of three independent experiments ± SEM. [b]UDL, under detection limit.

fields (~95 cells per field) at ×400 magnification, together with total number of cells, and the apoptotic index was calculated as the number of apoptotic cells × 100/total number of cells.

Western Blot. HT-29 cells (2×10^5) were plated in 60 mm Petri dishes in 10% FBS McCoy's 5A medium. Cells were incubated and medium was refreshed every other day until preconfluence, and they were treated as described above. After treatment (24 h), adherent cells were washed with PBS and 2×10^6 cells were resuspended in 1 mL of lysis buffer (70 mM sucrose, 1 mM EDTA, 10 mM HEPES, 1% Nonidet P40, 1 mM sodium ortovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptine). Cells were lysed, and the total cell extract was analyzed for protein content according to the Bradford method.[18] Samples (150 μg) were electrophoresed in a 10% (for pRb, ppRb, caspase 3, HDAC1, cyclin E, NFκB, p21, cyclin D, PCNA and β-actin, proteins) or 12% (for p53, Bax, Bcl-2, SMH2 and β-actin, proteins) SDS-PAGE gel (120 V, 180 min) and transferred onto a polyvinyldifluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA) (15 V, 10 min). The membrane was placed in Millipore SNAP id Protein Detection System, and the membranes were blocked in 0.5% milk in TBS-T buffer. Primary incubation of the membranes was carried out using mouse monoclonal [β-actin (3:1500) (sc-8432), Bax (3:1500) (sc-7480), p21(15:1500) (sc-6246), cyclin D (15:1500) (sc-56302), HDAC1 (7.4:1500) (sc-8410), Bcl-2 (15:1500) (sc-7282), PCNA (10:1500) (sc-56)] or rabbit polyclonal [cleaved caspase 3 (10:1500) (sc-7148), SIAH 1/2 (15:1500) (sc-22765), cyclin E (15:1500) (sc-20684), pRb/ppRb (3:1500) (10:1500) (sc-16670)] antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Rabbit anti-NFκB (20:1500) (51−3500, Zymed, Invitrogen, Carlsbad, CA, USA), mouse anti-MSH2 (15:1500) (33−7900, Invitrogen, Camanillo, CA, USA), and goat polyclonal p53 (15:1500) (sc-1314, Santa Cruz Biotechnology) antibodies were also evaluated. Secondary incubation of the membrane was then carried out using a 2:1500 dilution of goat anti-mouse, goat anti-rabbit, and goat anti-rat antibodies (sc-2005, sc-2004, and sc-2006, respectively, Santa Cruz Biotechnology, CA). Visualization of immunoreactive bands was accomplished using a chemiluminiscence kit (MB Chemiluminiscence Blotting Substrate (POD), Roche Diagnosis Co., Indianapolis, IN, USA) and Kodak photographic film (BioMax Light Film, Eastman Kodak Co., Rochester, NY, USA). The blots were scanned using the Epson system (1650; Epson America, Long Beach, CA, USA). The band intensity was evaluated using the Image-J program (http://rsb.info.nih.gov/ij/). The protein expression was normalized to β-actin (it was run on both 10 and 12% gels).

Statistical Analysis. The results are expressed as the mean ± standard error. Tukey's (multiple comparisons) or Dunnet's (treated versus control cells) test was used for comparisons at a 95% probability level. The statistical package JMP V. 5.0 (SAS Institute Inc., Cary, NC, USA) was used for all analyses.[19]

RESULTS AND DISCUSSION

Effect of Bacterial Inoculums on pH and SCFAs Production during NDF in Vitro Fermentation. Bacterial inoculums affected the pH during in vitro fermentation of bean polysaccharides (Table 2). Differences in pH were insignificant at initial (0 h) incubation time, whereas the highest statistical differences were observed between 6 and 12 h for fermented products by human gut flora (FP-hgf), Lactipan (FP-L), and Lactipan/Danisco mixture (FP-L/D). However, after 24 h of fermentation, FP-hgf and FP-L/D displayed the lowest pH values (5.82 and 5.72, respectively). Lowered pH may reflect the increased total concentration of the three SCFAs, acetate, propionate, and butyrate. The pH of the proximal colon varies considerably (4.0−7.0),[20] falling dramatically when unabsorbed carbohydrate enters from the small intestine. Thus, changes in pH may have a major influence upon the health of colonic enterocytes and the efficiency of the colon to salvage unabsorbed carbohydrate by conversion into rapidly absorbed and utilized volatile fatty acids.

Walker et al.[21] suggested that pH reduction resulting from substrate fermentation in the colon by the microflora induced butyrate production and populations of butyrate-producing bacteria decreased the growth of bacteroides. The production of SCFAs was inoculum dependent (Table 3). FP-hgf was the only butyrate producer (7 mmol/mL) after 24 h of fermentation, with the highest acetate and propionate production (30 and 4 mmol/mL, respectively). This molar ratio (73, 10, and 17) of the three SCFAs (acetic, propionic, and butyric acids, respectively) is in the range commonly found (from ~75:15:10 to 40:40:20) in humans.[22] The Lactipan/Danisco mixture (FP-L/D) displayed comparatively lower acetate and propionate production (26 and 2 mmol/mL, respectively) and no butyrate production, suggesting greater carbohydrate fermentation capacity by fresh human fecal

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bacteria. The different metabolic cross-feeding between strains could be another reason for the missing fermentation efficiency of Lactipan and Danisco because lactic acid bacteria, including lactobacilli, bifidobacteria, enterococci, and streptococci, present into this mixtures are L-lactate and/or D-lactate producers, whereas other lactate-utilizing bacteria produce butyrate as a major fermentation product.[23] Also, gut microflora from human inoculum produce more total SCFAs than those from some other animal species due to the presence of different microbial populations and/or the fermentative capacity of the microflora.[24] SCFAs data in this study suggest that differences exist in the ability of microflora from different bacterial sources to ferment fiber in vitro (Table 3).

The concentration of SCFAs may also stimulate water absorption and mineral uptake in the colon, resulting in faster recovery from diarrhea and prevention of mineral deficiency.[25] SCFAs produced in the gut lumen as a consequence of microbial fiber fermentation are important in relation to fiber intake and colon cancer prevention.[26]

Effect of Fermented Products from NDF by Human Gut Microbiota and Mixture of SCFAs on HT-29 Cell Proliferation and Cytoxicity. Biological assays were conducted with fermented products from NDF by human gut flora (FP-hgf) because it was the only bacterial source that produced butyrate that has been associated with the prevention and inhibition of colon carcinogenesis.[27] FP-hgf inhibited the growth of HT-29 cells in a concentration-dependent manner (Figure 1). The log LC_{50} value after 24 h treatment was 17%,

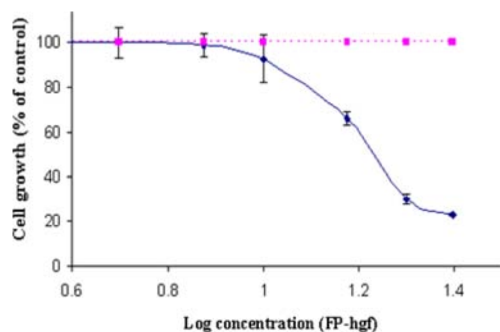

Figure 1. Concentration−response curve of FP-hgf on HT-29 colon carcinoma cells after incubation (24 h). The effect of FP-hgf (■) was normalized to the blank control (FP-hgf without cells) and to the nontreated control (0%/mL, 100%) cells (◆). IC_{50} was calculated from the antilog of the *x*-axis value at the inflection point of the sigmoid curve fit generated for the treatment (JMP V. 5.0). Values are the mean of three independent experiments with standard errors depicted by vertical bars.

whereas the control (without NDF) showed no significant ($p \leq 0.05$) inhibition of cell growth (data not shown). Growth inhibition of HT-29 cells at LC_{50}/FP-hgf was not cytotoxic compared to the positive control (Triton), which induced 100% cytotoxicity based on LDH activity (date not shown). The synthetic SCFAs mixtures of LC_{50}/FP-hgf (5.1, 0.68, and 1.19 mmol/mL of acetate, propionate, and butyrate, respectively) were less effective in growth inhibition of cells; nevertheless, the results suggest that SCFAs in the FP-hgf are key components responsible for inhibiting HT-29 cell survival (35%) (Figure2). Similar results were reported by Beyer-Sehlmeyer et al.,[28] who found that the complete fermentation products inhibited the growth of HT-29 cells more effectively than synthetic SCFAs mixtures. The so-called "added value" of

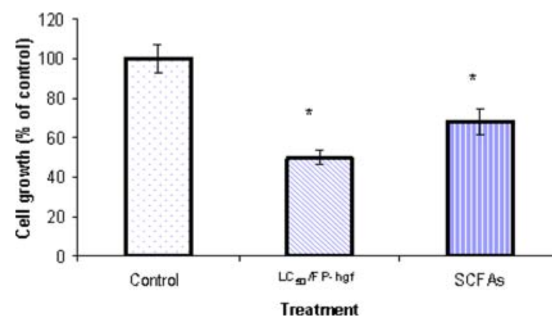

Figure 2. Growth inhibition of HT-29 colon carcinoma cells after 24 h of incubation with LC_{50}/FP-hgf and SCFAs standards found in the LC_{50}/FP-hgf. The effect of LC_{50}/FP-hgf was normalized to the blank control (FP-hgf without cells) and to the nontreated control (0%/mL, 100%) cells. The values are the mean of three independent experiments with standard errors shown with vertical bars (*, $p < 0.05$, versus control group).

the complete fermentation samples probably reflects the growth-inhibitory properties of numerous other plant ingredients and nutrients.[29] This could be attributed to differences in the magnitude of biological activities from different compounds, even though the SCFAs are consumed by the cells at similar rates.[30] Butyrate and, to a lesser extent, propionate induced alkaline phosphatase (AP) activity, whereas acetate had no effect. This is in line with previous results on the modulation of cell growth, where butyrate and propionate were most effective and acetate least active.[30]

The overall results therefore provide preliminary, but novel, information that complete fermentation products from common bean NDF could have different phytoprotectants that enhance the SCFAs properties. This indicates that NDF from common bean has an "added value" for inhibiting the growth of transformed colon tumor cells.

Morphological Changes (H&E) and DNA Breaks (TUNEL) Induced by LC_{50}/FP-hgf and Synthetic SCFAs Mixture on HT-29 Cells. Both LC_{50}/FP-hgf and synthetic SCFAs mixture induced considerable morphological characteristics (hematoxylin and eosin staining) of apoptosis relative to control cells (Figure 3). These included cellular shrinkage, nuclear condensation, and cytoplasmic blebbing. Synthetic SCFAs mixture induced morphologic signs of apoptosis in more cells than LC_{50}/FP-hgf.

The TUNEL assay was used to confirm the pro-apoptotic effect of LC_{50}/FP-hgf and synthetic SCFAs mixture on HT-29 cells. As shown in Figure 4, untreated HT-29 cells were minimally stained by the TUNEL method. In contrast, 28 and 51% of HT-29 cells were TUNEL-positive after treatment with LC_{50}/FP-hgf and synthetic SCFAs mixture, respectively, suggesting the occurrence of apoptosis. Interestingly, the fermentation sample was more effective in mediating growth inhibition than their corresponding SCFAs mixture (Figure 3). This could mean that the activity of fiber derived from beans is mainly based on the SCFAs produced during fermentation. However, itis important to emphasize that only adherent cells were evaluated, so that excluding floating cells could have influenced the results. Recently, fermented wheat aleurone (with higher levels of potentially chemopreventive SCFAs) has been shown to inhibit growth and induce apoptosis in HT-29 cells.[31] Ruemmele et al.[32] reported that butyrate induced CaCo-2 apoptosis, confirmed by morphological changes and TUNEL assay. These results suggest that apoptosis induced by LC_{50}/

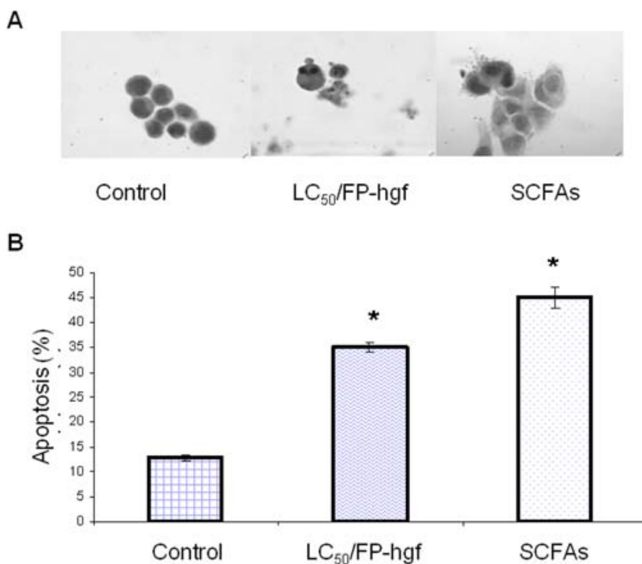

Figure 3. Detection of apoptosis by hematoxylin and eosin (H&E) staining: (A) black arrows indicate the apoptotic cell (original magnification ×400); (B) quantitative analysis of apoptosis on HT-29 colon carcinoma cells after 24 h of incubation with LC_{50}/FP-hgf and SCFAs mixture found in the LC_{50}/FP-hgf. The H&E-positive rate is presented with the mean ± standard error based on 10 randomly selected microscopic fields (~95 cells per field) for each group in three independent experiments. (*) Statistical differences versus control $p < 0.05$.

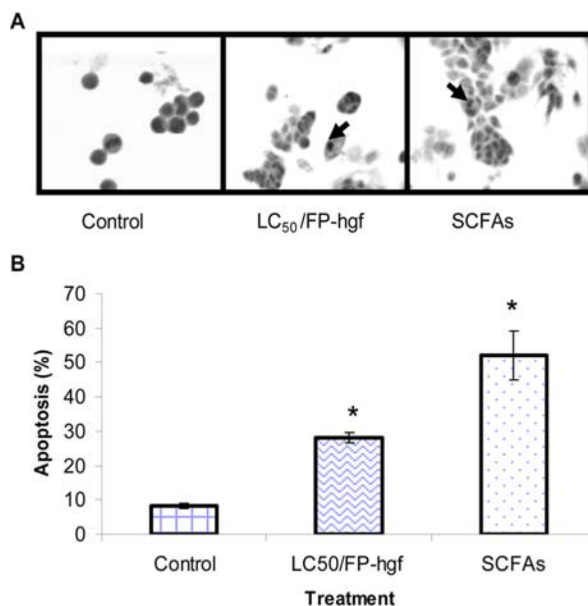

Figure 4. Result of TUNEL staining: (A) brown signals show the TUNEL-positive cells [black arrows indicate the apoptotic cell (original magnification ×400)]; (B) quantitative analysis to apoptosis on HT-29 colon carcinoma cells after 24 h of incubation with LC_{50}/FP-hgf and SCFAs mixture found in the LC_{50}/FP-hgf. The TUNEL-positive rate is presented with the mean ± standard error based on 10 randomly selected microscopic fields (~95 cells per field) for each group in three independent experiments. (*) Statistical differences versus control $p < 0.05$.

FP-hgf is primarily mediated by SCFAs, mainly butyrate, as reported earlier,[28,30] where the growth-inhibitory properties of SCFAs were directly related to the butyrate content.

Protein Modulation Linked to Apoptosis, Cell Cycle, DNA Repair, Inflammation, Proliferation, and Transcription. In our earlier study[6] of LC_{50}/FP-hgf effects on HT-29 cells, an extensive screening by RT-PCR array was performed. Significant differences in gene expression were detected in 72 of 84 human p53-mediated signal transduction response genes involved in apoptosis, cell cycle, and cell proliferation. On the basis of these results, the main modulated and some related genes were selected to evaluate LC_{50}/FP-hgf and SCFAs mixtures on HT-29 protein expression. The levels of SIAH1 and caspase 3 (cleaved) (apoptotic proteins) were significantly higher in LC_{50}/FP-hgf and SCFAs mixtures treated cells compared with untreated cells (Figure 5). Bax, another

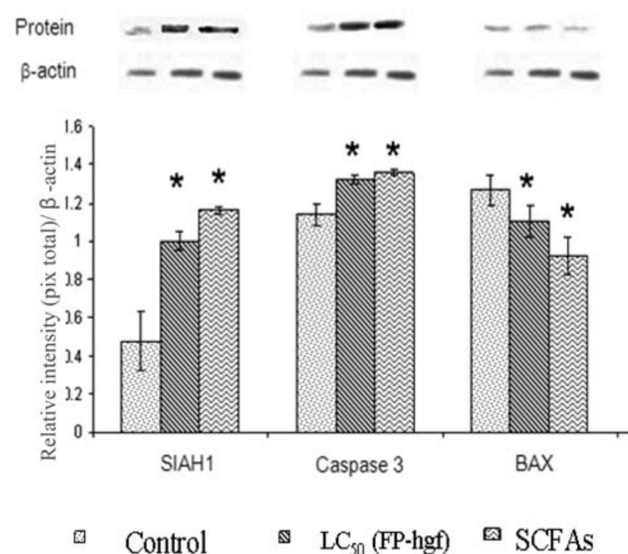

Figure 5. Expression of apoptosis-related proteins in HT-29 cells after 24 h of treatment with LC_{50}/FP-hgf and SCFAs mixture found in the LC_{50}/FP-hgf. Expression was analyzed by Western blot using specific antibodies. Control: protein expression in cells without any treatment. The blot was tested with anti-actin antibody to confirm equal protein loading. The protein expression was normalized to β-actin. Data are the mean ± standard errors of three independent experiments ($p < 0.05$ versus control).

apoptotic protein, was suppressed in both treatments compared to the control. Bcl-2 level was undetectable in HT-29 cells, which do not express the protein spontaneously, as reported previously[33,34] (data not shown).

The resultant low expression of Bax observed in our studies and HT-29-Bcl-2-may suggest that LC_{50}/FP-hgf and SCFAs do not stimulate adenocarcinoma colon cancer cell apoptosis via the intrinsic or mitochondrial Bcl-2/Bax pathway. Furthermore, other pro- and anti-apoptotic proteins should be evaluated. The result probably reflects LC_{50}/FP-hgf and SCFAs mixture involvement in an alternative apoptosis-mediated pathway by SIAH1 and caspase 3 activation, because those proteins have been associated with tumor suppression and apoptosis.[31]

Mutations and deletions of the tumor suppressor gene p53 have been identified in about 50% of colorectal carcinomas and are associated with poor prognosis due to its weaker ability to inhibit cell proliferation.[35] Rodrigues et al.[36] showed that overexpression of p53 is synonymous with mutation and that HT-29 cells have mutations in codon 273 of the p53 gene, so HT-29 cells overproduce mutant p53 antigen. In our study, the abnormal expression of p53 protein decreased compared to

control ($P < 0.05$) in LC_{50}/FP-hgf and SCFAs mixture-treated cells (Figure 6), indicating that these treatments reduce the expression of mutant p53 protein. Although MDM-4 protein (p53 essential regulator and inhibitor)[37] was not evaluated in this study, the transcriptional up-regulation observed in our previous research[6] may support the observed LC_{50}/FP-hgf -p53 inhibition.

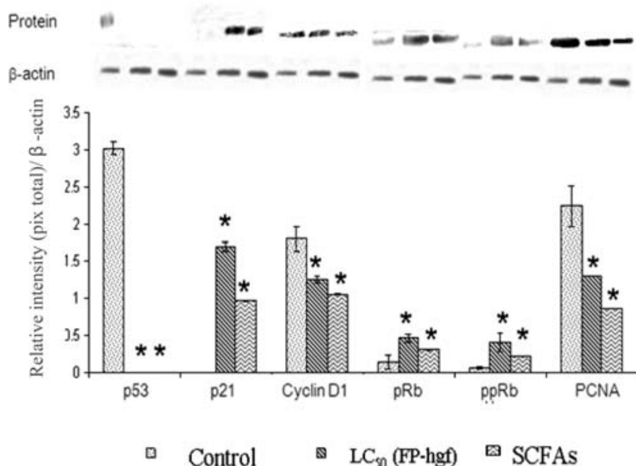

Figure 6. Expression of cell cycle-related proteins in HT-29 cells after 24 h of treatment with LC_{50}/FP-hgf and SCFAs mixture found in the LC_{50}/FPhgf. Expression was analyzed by Western blot using specific antibodies. Control: protein expression in cells without any treatment. The blot was tested with anti-actin antibody to confirm equal protein loading. The protein expression was normalized to β-actin. Data are the mean ± standard errors of three independent experiments ($p < 0.05$ versus control).

In the present study, p53 polyclonal antibody was not specified for mutant p53 but responded to many antigenic determinants, including wild p53, indicating that treatment down-regulates the expression of mutant p53 to control cell cycle check-point and prevents progression of cells to the DNA synthesis phase (S phase) of the cell cycle, as reported by Tian and Song.[38] Cyclins are key cell cycle control molecules with specific, periodic expression associated with cell cycle progression. Other cell cycle control molecules include cyclin-dependent kinase (cdk) inhibitors, such as p21CIP1 and p27KIP1, which tightly regulate the activities of cyclin/CDK enzyme complexe genes.[39] The expression of p21 was significantly higher, whereas cyclin D1 was lower than control (Figure 6). Interestingly, p21 showed no expression in the control group, similar to those reported for colon cancer cells (HT-29, Caco-2, and HCT-15), where the p21 expression increased in a time-dependent manner after Celecoxib treatment.[40] Because cyclin D1 is the rate-limiting component in promoting G1 phase progression,[35] the inhibition of cyclin D1 expression and p21 induction by LC_{50}/FP-hgf and SCFAs mixtures suggests that they may induce G1 phase cell cycle arrest. Previous studies have shown that butyrate could induce cell cycle arrest and inhibit cell proliferation via p21 and cyclin D1 modulation.[41] Therefore, inhibition of cyclin D1 and p21 overexpression could be a potential mechanism of growth inhibition by LC_{50}/FP-hgf mediated by SCFAs.

Because p21 WAF1/CIP1 inhibits CDKs, it results in inhibition of both G1-to-S and G2-to-mitosis transitions by hyper (or hypo) phosphorylated Rb (retinoblastoma) and prevention of the release of E2F, resulting in cell cycle arrest.[42] In this way, unclear results were obtained based on ppRb (hyperphosphorylated) and pRb (hypophosphorylated) on HT-29 treated cells compared to the control, because both proteins increased their expression (Figure 6). Overexpression of p21 WAF1/CIP1 is growth inhibitory, possibly by binding to the C-terminal domain of PCNA and resulting in a p21−PCNA complex blocking the ability of PCNA to process DNA polymerase in DNA replication. Thus, p21 WAF1/CIP1 may act as a tumor suppressor because of its role in growth control.[43] In the present study, p21 expression increased after 24 h treatments ($P < 0.05$) (Figure 6). High expression of p21 WAF1/CIP1 leads to decreased nuclear expression of PCNA, which is in agreement with our results (Figure 6).

Heterozygous germline mutations in genes such as MSH2 that encode components of the DNA mismatch repair (MMR) pathway predispose individuals to cancer, particularly hereditary nonpolyposis colorectal cancer (HNPCC).[44] Compared to the control, MSH2 expression decreased after treatments (Figure 7), results that differ from those observed at the transcriptional level (early studies), where the LC_{50}/FP-hgf significantly increased MSH2 gene expression. Because HT-29 cells are MMR-proficient, our result suggests that treatments could have a mechanism like apoptosis and cell cycle arrest induction instead of DNA repair. Coxhead et al.[45] reported that butyrate inhibits cell proliferation, but the effects were more pronounced on MMR-deficient colorectal cancer cells.

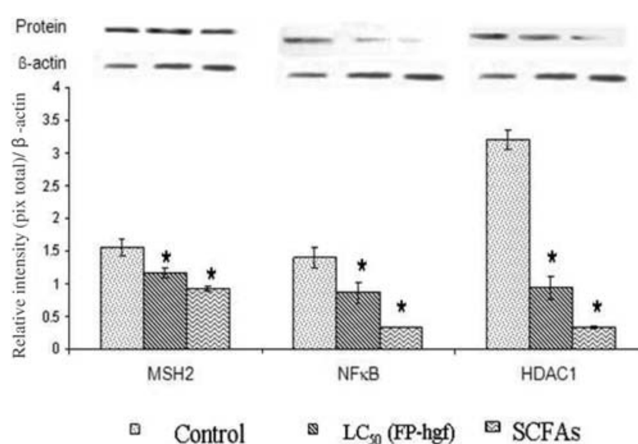

Figure 7. Expression of MSH2, NFκB, and HDAC1 proteins in HT-29 cells after 24 h of treatment with LC_{50}/FP-hgf and SCFAs mixture found in the LC_{50}/FP-hgf. Expression was analyzed by Western blot using specific antibodies. Control: protein expression in cells without any treatment. The blot was tested with anti-actin antibody to confirm equal protein loading. The protein expression was normalized to β-actin. Data are the mean ± standard errors of three independent experiments ($p < 0.05$ versus control).

The effects of LC_{50} (FP-hgf) and SCFAs mixtures on HT-29 cells may be related to survival inhibition and anti-inflammatory process. This is supported by the down-regulation of NFκB (Figure 7), a central pro-inflammatory transcription factor regulator.[46] The survival of some cancer cells, such as hematological malignancies and prostate cancer, clearly depends on NFκB.[47] Therefore, inhibition of NFκB activity is an attractive approach to cancer therapy and, indeed, has been attempted for many types of cancer.[48] Partial suppression of NFκB activity by butyrate with immune-suppressing and anti-inflammatory properties was produced during fiber colonic fermentation,[49] and the reduced NFκB activity by butyrate on

inflammatory bowel disease[50] supports our hypothesis. On the other hand, NFκB activation has been associated with multiple aspects of oncogenesis, including the control of apoptosis, cell cycle, differentiation, and cell migration, that should be considered.[51]

The result of this research may be linked to histone deacetylase (HDAC) down-regulation by both treatments (Figure 7), which may be SCFAs mediated. Weichert et al.[52] suggested that short interfering RNA-based inhibition of HDAC1 suppressed in vitro colon cancer cell growth. Class I HDACs, such as HDAC1, are highly expressed in a subset of colorectal carcinomas; the expression is significantly enhanced strongly proliferating and dedifferentiated tumors. High HDAC expression levels have been implicated in significantly reduced patient survival.[52]

Butyrate is well established as a HDAC inhibitor, resulting in hyperacetylation of core histone proteins,[53] and as such has the potential to modify gene expression.[54] However, recent study suggests that butyrate could be acting as a product inhibitor of deacetylation.[55] Further research is needed to understand the involvement of LC_{50}/FP-hgf in regulating HDAC expression on HT-29 cells.

Our findings suggest that HT-29 colon carcinoma cell proliferation is significantly inhibited by compounds generated during in vitro fermentation of NDF from common beans (*P. vulgaris* L.) cultivar 'Bayo Madero', mainly SCFAs. FP-hgf modulated protein expression associated with apoptosis, cell cycle arrest, and proliferation, as well as morphological changes linked to apoptosis indicated by TUNEL and hematoxylin and eosin results. Collectively, these results imply that the antiproliferative effect by FP-hgf may contribute to its overall chemoprotective function against colon carcinogenesis. Additional research is needed to explore the mechanisms of FP-hgf-NDF-common bean on cell proliferation, apoptosis, and potential chemopreventive value. Translated to the in vivo situation, the results suggest that fermentation products can suppress the growth of tumor cells by a number of components including butyrate, propionate, and unidentified phytochemicals as well as by different molecular mechanisms.

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ABBREVIATIONS USED

NSP, nonstarch polysaccharides; HGF, human gut flora; SCFAs, short-chain fatty acids; FP, fermentation products; cv., cultivar; L, Lactipan; D, Danisco; INIFAP, Institute for Forestry, Agriculture and Livestock; AOAC, Association of Official Analytical Chemists; TDF, total dietary fiber; ATCC, American Type Culture Collection; FBS, fetal bovine serum; LDH, lactate dehydrogenase; LC_{50}, lethal fifty concentration; H&E, hematoxylin & eosin; AI, apoptotic index; TUNEL, terminal deoxynucleotide transferase nick end labeling; MMR, mismatch repair; HNPCC, hereditary nonpolyposis colorectal cancer; HDAC, histone deacetylase

REFERENCES

(1) Agurs-Collins, T.; Smoot, D.; Afful, J.; Makambi, K.; Adams-Campbell, L. Legume intake and reduced colorectal adenoma risk in African-Americans. *J. Natl. Black Nurses Assoc.* **2006**, *17*, 6−12.

(2) Beninger, C. W.; Hosfield, G. L. Antioxidant activity of extracts, condensed tannin fractions, and pure flavonoids from *Phaseolus vulgaris* L. seed coat color genotypes. *J. Agric. Food Chem.* **2003**, *51*, 7879−7883.

(3) Macfarlane, G. T.; Macfarlane, S. Fermentation in the human large intestine: Its physiologic consequences and the potential contribution of prebiotics. *J. Clin. Gastroenterol.* **2011**, *45*, S120−S127.

(4) Hoover, R.; Zhou, Y. *In vitro* and *in vivo* hydrolysis of legume starches by α-amylase and resistant starch formation in legumes − a review. *Carbohydr. Polym.* **2003**, *54*, 401−417.

(5) Canani, R. B.; Costanzo, M. D.; Leone, L.; Pedata, M.; Meli, R.; Calignano, A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* **2011**, *17* (12), 1519−1528.

(6) Campos-Vega, R.; Guevara-Gonzalez, R. G.; Guevara-Olvera, B. L.; Oomah, B. D.; Loarca-Piña, G. Bean (*Phaseolus vulgaris* L.) polysaccharides modulate gene expression in human colon cancer cells (HT-29). *Food Res. Int.* **2010**, *43*, 1057−1064.

(7) Fung, K. Y. C.; Lewanowitsch, T.; Henderson, S. T.; Priebe, I.; Hoffmann, P.; McColl, S. R.; Lockett, T.; Head, R.; Cosgrove, L. J. Proteomic analysis of butyrate effects and loss of butyrate sensitivity in HT29 colorectal cancer cells. *J. Proteome Res.* **2009**, *8*, 220−1227.

(8) Henningsson, A. M.; Nyman, E. M.; Bjorck, I. M. Content of short-chain fatty acids in the hindgut of rats fed processed bean (*Phaseolus vulgaris*) flours varying in distribution and content of indigestible carbohydrates. *Br. J. Nutr.* **2001**, *86* (3), 379−389.

(9) Wong, J. Colonic health: fermentation and short chain fatty acids. *J. Clin. Gastroenterol.* **2006**, *40*, 235−243.

(10) Aparicio-Fernández, X.; Manzo-Bonilla, L.; Loarca-Piña, G. F. Comparison of antimutagenic activity of phenolic compounds in newly harvested and stored common beans *Phaseolus vulgaris* against aflatoxin B_1. *J. Food Sci.* **2005**, *70*, S73−S78.

(11) Association of Official Analytical Chemists. *Official Methods of Analysis*, 17th ed.; AOAC: Arlington, VA, 2002.

(12) Macfarlane, G. T.; Macfarlane, S. Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut. *Curr. Opin. Biotechnol.* **2007**, *18*, 156−162.

(13) McBurney, M. I.; Thompson, L. U. Effect of human inoculum on *in vitro* fermentation variables. *Br. J. Nutr.* **1987**, *58*, 233−243.

(14) Campos-Vega, R.; Reynoso-Camacho, R.; Pedraza-Aboytes, G.; Acosta-Gallegos, J. A.; Guzman-Maldonado, S. H.; Paredes-Lopez, O.; Oomah, B. D.; Loarca-Piña, G. Chemical composition and *in vitro* polysaccharide fermentation of different beans (*Phaseolus vulgaris* L.). *J. Food Sci.* **2009**, *74* (7), T59−T65.

(15) Fogh, J.; Trempe, X. *Human Tumor Cells in Vitro*: Plenum Press: New York, 1975; pp 115−15.

(16) Decker, T.; Lohmann-Matthes, M. L. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods* **1998**, *115*, 61−69.

(17) Ben-Sasson, S. A.; Sherman, Y.; Gavrieli, Y. Identification of dying cells − *in situ* staining. *Methods Cell Biol.* **1995**, *46*, 29−39.

(18) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(19) Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters*; Wiley: New York, 1978.

(20) Bown, R. L.; Gibson, J. A.; Sladen, G. E.; Hicks, B.; Dawson, A. M. Effects of lactulose and other laxatives on ileal and colonic pH measured by a radiotelemetry device. *Gut* **1974**, *15*, 999−1004.

(21) Walker, W.; Duncan, H. S.; Leitch, McW. C. E.; Child, W. M.; Flint, J. H. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Appl. Environ. Microbiol.* **2005**, *71* (7), 3692−3700.

(22) Cummings, J. H.; Macfarlane, G. T. Role of intestinal bacteria in nutrient metabolism. *(JPEN) J. Parenter. Enteral. Nutr.* **1997**, *21*, 357−365.

(23) Duncan, S. H.; Louis, P.; Flint, H. J. Lactate-utilizing bacteria, isolated from human feces that produce butyrate as a major fermentation product. *Appl. Environ. Microbiol.* **2004**, *70* (10), 5810−5817.

(24) Sunvold, G. D.; Hussein, H. S.; Fahey, G. C.; Merchen, N. R.; Reinhart, G. A. *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *J. Anim. Sci.* **1995**, *73*, 3639−3648.

(25) Younes, H.; Coudray, C.; Bellanger, J.; Demigné, C.; Rayssiguier, Y.; Rémésy, C. Effects two fermentable carbohydrates (inulin and resistant starch) and their combination on calcium and magnesium balance in rats. *Br. J. Nutr.* **2001**, *86*, 479.

(26) Aune, D.; Chan, D. S.; Lau, R.; Vieira, R.; Greenwood, D. C.; Kampman, E.; Norat, T. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *BMJ* **2011**, *10*, 343.

(27) Hamer, H. M.; Jonkers, D.; Venema, K.; Vanhoutvin, S.; Troost, F. J.; Brummer, R. J. The role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* **2008**, *27*, 104−119.

(28) Beyer-Sehlmeyer, G.; Glei, M.; Hartmann, E.; Hughes, R.; Persin, C.; Böhm, V.; Rowland, I.; Schubert, R.; Jahreis, G.; Pool-Zobel, B. L. Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fiber sources. *Br. J. Nutr.* **2003**, *90*, 1057−1070.

(29) World Cancer Research Fund and American Institute for Cancer Research. *Food, Nutrition and the Prevention of Cancer: A Global Perspective*; American Institute for Cancer Research: Washington, DC, 1997.

(30) Mariadason, J. M.; Velchich, A.; Wilson, A. J.; Augenlicht, L. H.; Gibson, P. R. Resistance to butyrate-induced cell differentiation and apoptosis during spontaneous Caco-2 cell differentiation. *Gastroenterology* **2001**, *120*, 889−899.

(31) Borowicki, A.; Stein, K.; Scharlau, D.; Scheu, K.; Brenner-Weiss, G.; Obst, U.; Hollmann, J.; Lindhauer, M.; Wachter, N.; Glei, M. Fermented wheat aleurone inhibits growth and induces apoptosis in human HT29 colon adenocarcinoma cells. *Br. J. Nutr.* **2010**, *103* (3), 360−369.

(32) Ruemmele, F. M.; Schwartz, S.; Seidman, G. E.; Dionea, S.; Lentze, E. L.; Lentze, M. J. Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. *Gut* **2003**, *52*, 94−100.

(33) Lacour, S.; Micheau, O.; Hammann, A.; Drouineaud, V.; Tschopp, J.; Solary, E.; Dimanche-Boitrel, M. T. Chemotherapy enhances TNF-related apoptosis-inducing ligand DISC assembly in HT29 human colon cancer cells. *Oncogene* **2003**, *22* (12), 1807−1816.

(34) Pattingre, S.; Tassa, A.; Qu, X.; Garuti, R.; Liang, X. H.; Mizushima, N.; Packer, M.; Schneider, M. D.; Levine, B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **2005**, *122* (6), 927−939.

(35) Culotta, E.; Koshland, D. E. p53 sweeps through cancer research. *Science* **1993**, *262*, 1958−1961.

(36) Rodriguez, N. R.; Rowan, A.; Smith, M. E.; Kerr, I. B.; Bodmer, W. F.; Gannon, J. V.; Lane, D. P. p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7555−7559.

(37) Toledo, F.; Geoffrey, M.; Wahl, G. M. MDM2 and MDM4: p53 regulators as targets in anticancer therapy. *Int. J. Biochem. Cell Biol.* **2007**, *39* (7−8), 1476−1482.

(38) Tian, Y.; Song, Y. Effects of inositol hexaphosphate on proliferation of HT-29 human colon carcinoma cell line. *World J. Gastroenterol.* **2006**, *12* (26), 4137−4142.

(39) Tenderenda, M. A study on the prognostic value of cyclins D1 and E expression levels in resectable gastric cancer and on some correlations between cyclins expression, histoclinical parameters and selected protein products of cell-cycle regulatory genes. *J. Exp. Clin. Cancer. Res.* **2005**, *24*, 405−414.

(40) Grösch, S.; Tegeder, I.; Niederberger, E.; Bräutigam, L.; Geisslinger, G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. *FASEB J.* **2001**, *15*, 2742−2744.

(41) Kles, K.; Chang, E. Short-chain fatty acids impact on intestinal adaptation, inflammation, carcinoma, and failure. *Gastroenterology* **2006**, *130* (2), S100−S105.

(42) Mundle, S. D.; Saberwal, G. Evolving entricacies and implications of E2F1 regulation. *FASEB J* **2003**, *17* (6), 569−574.

(43) Zheng, Q. H.; Ma, L. W.; Zhu, W. G.; Zhang, Z. Y.; Tong, T. J. p21(Waf1/Cip1) plays a critical role in modulating senescence through changes of DNA methylation. *J. Cell Biochem.* **2006**, *98* (5), 1230−1248.

(44) Poulogiannis, G.; Frayling, I. M.; Arends, M. J. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology* **2010**, *56*, 167−179.

(45) Coxhead, J. M.; Williams, E. A.; Mathers, J. C. DNA mismatch repair status may influence anti-neoplastic effects of butyrate. *Biochem. Soc. Trans.* **2005**, *33*, 728−729.

(46) Inan, M. S.; Rasoulpour, R. J.; Yin, L.; Hubbard, R. K.; Rosenberg, D. W.; Giardina, C. The luminal short-chain fatty acid butyrate modulates NFκB activity in a human colonic epithelial cell line. *Gastroenterology* **2000**, *118*, 724−734.

(47) Kikuchi, E.; Horiguchi, Y.; Nakashima, J.; Kuroda, K.; Oya, M.; Ohigashi, T.; Takahashi, N.; Shima, Y.; Umezawa, K.; Murai, M. Suppression of hormone-refractory prostate cancer by a novel nuclear factor B inhibitor in nude mice. *Cancer Res.* **2003**, *63*, 107−110.

(48) Izzo, J. G.; Malhotra, U.; Wu, T. T.; Ensor, J.; Luthra, R.; Lee, J. H.; Swisher, S. G.; Liao, Z.; Chao, K. S.; Hittelman, W. N.; Aggarwal, B. B.; Ajani, J. A. Association of activated factor nuclear factor κB with chemoradiation resistance and poor outcome in esophageal carcinoma. *J. Clin. Oncol.* **2006**, *24*, 748−754.

(49) Tedelin, S.; Westberg, F.; Kjerrulf, M.; Vidal, A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World J. Gastroenterol.* **2007**, *13*, 2826−2832.

(50) Hodin, R. Maintaining gut homeostasis: the butyrate−NF-κB connection. *Gastroenterology* **2000**, *118* (4), 798−801.

(51) Baldwin, A. S. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. *J. Clin. Invest* **2001**, *107* (3), 241−246.

(52) Weichert, W.; Röske, A.; Niesporek, S.; Noske, A.; Buckendah, A. Class I histone deacetylase expression has independent prognostic impact in human colorectal cancer: specific role of class I histone deacetylases *in vitro* and *in vivo*. *Clin. Cancer Res.* **2008**, *14*, 1669−1677.

(53) Hinnebusch, B. F.; Meng, S.; Wu, J. T.; Archer, S. Y.; Hodin, R. A. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J. Nutr.* **2002**, *132*, 1012−1017.

(54) Iacomino, G.; Tecce, M. F.; Grimaldi, C.; Tosto, M.; Russo, G. L. Transcriptional response of a human colon adenocarcinoma cell line to sodium butyrate. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 1280−1289.

(55) Corfe, B. M. Hypothesis: butyrate is not an HDAC inhibitor, but a product inhibitor of deacetylation. *Mol. Biol. Syst.* **2012**, *8*, 1609−1612.