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Composition and Chemopreventive Effect of Polysaccharides from Common Beans (*Phaseolus vulgaris* L.) on Azoxymethane-Induced Colon Cancer

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Common beans (*Phaseolus vulgaris* L.) contain a high proportion of undigested carbohydrates (NDC) that can be fermented in the large intestine to produce short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate. The objective of the present study was to evaluate the composition and chemopreventive effect of a polysaccharide extract (PE) from cooked common beans (*P. vulgaris* L) cv. Negro 8025 on azoxymethane (AOM) induced colon cancer in rats. The PE induced SCFA production with the highest butyrate concentrated in the cecum zone: 6.7 ± 0.06 mmol/g of sample for PE treatment and 5.29 ± 0.24 mmol/g of sample for PE + AOM treatment. The number of aberrant crypt foci (ACF) and the transcriptional expression of *bax* and *caspase-3* were increased, and *rb* expression was decreased. The data suggest that PE decreased ACF and had an influence on the expression of genes involved in colon cancer for the action of butyrate concentration.

KEYWORDS: Polysaccharide; *Phaseolus vulgaris*; short-chain fatty acid; aberrant crypt foci

INTRODUCTION

Considerable variability exists in common beans for seed characteristics, and consumers have developed specific preferences for various combinations of size, shape, and color of the dry seeds (*1*). Common beans are a good source of protein, some vitamins, minerals, and complex carbohydrates (*2*). In addition to these nutritional compounds, common bean seeds contain a number of bioactive substances including enzyme inhibitors, lectins, phytates, polyphenolic compounds (such as condensed tannins and flavonoids), and polysaccharides. Polysaccharides reaching the colon include mainly resistant starch (RS), soluble and insoluble fiber, and nondigestible oligosaccharides (NDO) that play metabolic roles in humans or animals that frequently consume these foods (*3*). For example, polysaccharides can be fermented in the colon to produce short-chain fatty acids (SCFA) such as acetic, propionic, and butyric acids, and these SCFA have been shown to decrease the incidence of colon cancer induced by azoxymethane (AOM) (*4*). Aberrant crypt foci (ACF) are being used as a short-term assay to identify modulators of colon carcinogenesis (*5*). Several studies reported that SCFA, mainly butyrate, inhibited colon cancer through modulation of genes involved in cellular proliferation and induced cell cycle arrest or even apoptosis of colonocytes (*6*). Butyrate up-regulates *B-catenin* signaling (*7*), increases the expression of the *p21* cell cycle independent of the expression of *p53* (*8*), and up-regulates expression of the active subunits of *caspase-3*. Butyrate also causes a concomitant decrease in *bcl-2* expression and an increase in *bax* gene expression via mitochondrial protein (*9*). In addition, many researchers have reported that polyphenols from dry beans may act as antioxidants to inhibit the formation of damaging free radicals (*10*). Furthermore, flavonoids such as condensed tannins and anthocyanins have been reported as antioxidant and antimutagenic agents (*10, 11*). The presence of these bioactive compounds in common beans (*Phaseolus vulgaris*) is related to the decrease of chronically degenerative diseases.

The objective of this research was to determine the composition of bioactive substances including nondigested carbohydrates and polyphenolics by a rapid method adapted to a 96-well microplate and to evaluate the chemopreventive effect of a

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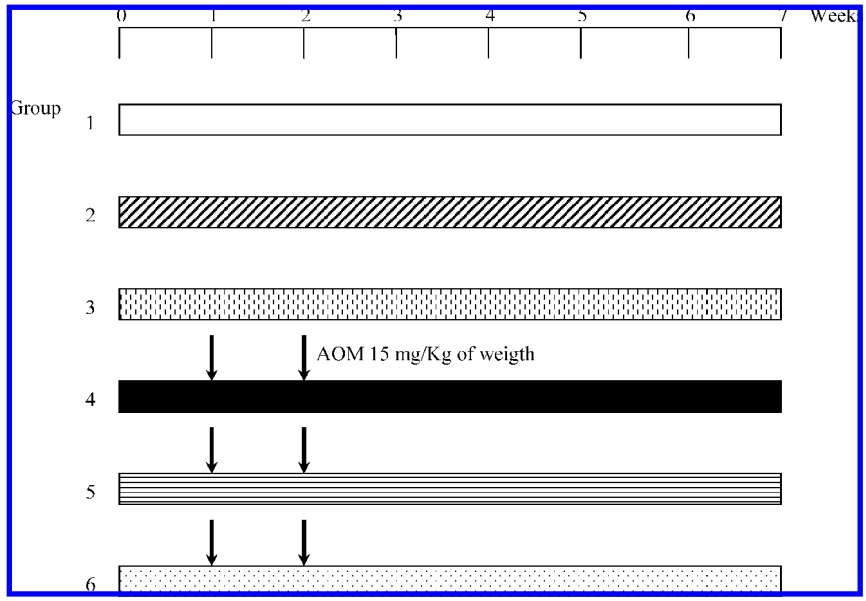

Figure 1. Experimental protocol for animal treatment. The animals were sacrificed 5 weeks after the last injection. Groups: (1) basal diet; (2, 3) basal diet cooked common bean (3.2 g) and PE (1.84 g) per kg of weight, respectively; (4) basal diet plus AOM; (5, 6) basal diet plus AOM with common bean and PE, respectively.

Table 1. Sequence of Primer Sets Used during This Study

gene	sense and antisense	amplified fragment size (bp)
caspase-3	5′-TTCATCATTCAGACCTGC-3′ 3′-ACGGGTAAGGATGTGCATGAAT-5′	247
p21	5′-GGACAGTGAGCAGTTGAGC-3′ 3′-GAGTGCAAGACAGCGACAAGG-5′	288
p53	5′-GCAGCACAGGAACCTGGAACT-3′ 3′-ATGATGGTAAGGATGGGC-5′	582
bcl-2	5′-GGGAGAACAGGGTATGATAAC-3′ 3′-GCAGATGCCGGTTCAGGTACT-5′	540
bax	5′-GGCCCACCAGCTCTGAACAGT-3′ 3′-TCAGCTCGGGCACTTTAG-5′	370
rb	5′-CAGATTTCATACCTCAGACCCTCT-3′ 3′-ACACTTGTATGAGGGAAAAC-5′	364
β-catenina	5′-GCTGACGTCGTACTCAGGCA-3′ 3′-TCCACATCCTCTTCCTCAGG-5′	211
gadph	5′-GCTCCTACCATTCATGCTGACA-3′ 3′-TCAAGACGAATAAACTCATCG-5′	291

polysaccharide extract (PE) from cooked common beans (*P. vulgaris* L.) cv. Negro 8025 on colon cancer induced by azoxymethane.

MATERIALS AND METHODS

Plant Material and Standards. Common beans (*P. vulgaris* L.) cv. Negro 8025, grown and harvested in 2005, were kindly provided by the Bean Project of the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP, Mexico). The beans were stored at room temperature until processing. (+)-Catechin, vanillin, D-(+)-raffinose, rutin, tannic acid, and AOM were obtained from Sigma Chemical Co. (St. Louis, MO).

Sample Processing. Beans were cooked according to the method of Aparicio-Fernández et al. (*10*). Fifty grams of beans was placed in a beaker with 250 mL of HPLC-grade water and boiled for 2.5 h. Cooked beans were lyophilized and stored in amber flasks at 4 °C for further analyses.

Table 2. Protein, Carbohydrate, Dietary Fiber, Polysaccharides, Oligosaccharides, Fat, and Ash Contents of Raw and Cooked Common Beans (*P. vulgaris* L.) Cv. Negro 8025[a]

	raw bean	cooked bean
fat	1.54 ± 0.06 a	1.41 ± 0.01 a
ash	4.32 ± 0.005 a	4.14 ± 0.15 b
protein[b]	20.99 ± 0.43 a	23.15 ± 0.07 a
carbohydrate[b]	59.83 ± 0.27 b	71.85 ± 0.22 a
polysaccharides[b]	63.7 ± 0.02 a	57.5 ± 0.05 b
dietary fiber[b]		
total fiber[b]	28.41 ± 0.31 a	26 ± 0.4 b
soluble fiber[b]	3.34 ± 0.29 b	6.00 ± 0.01 a
insoluble fiber[b]	25.07 ± 0.03 a	20 ± 0.03 b
resistant starch	33.0 ± 0.01 a	30.8 ± 0.02 a
oligosaccharides[c]	5.00 ± 0.06 b	6.8 ± 0.48 a

[a] The results represent the average of two independent experiments by triplicate ± standard error, expressed as mg/g of lyophilized sample. Different letters by row express significant differences $\alpha = 0.05$, Tukey test. [b] Percentage per gram of sample lyophilized. [c] Milligram equivalent of raffinose per gram of sample lyophilized.

Analytical Methods. Moisture, protein, fat, carbohydrate, sodium, crude fiber, dietary fiber, and ash contents of raw and cooked beans were analyzed according to methods of the Association of Official Analytical Chemists (*12*). All measurements were performed in triplicate.

Extraction of Polysaccharides. The extraction of polysaccharides was performed following the method of Kurtzman and Halbrook (*13*). Three hundred grams of the cooked beans (beans and cooking water) was ground and sieved through a 0.05 cm mesh screen, 1.5 L of water was added, and this was shaken for 1 min and centrifuged (Hermel Z323K USA) at 9000*g* for 10 min. The pellet from the first centrifugation was dissolved in 100 mL of 10% tannic acid, and the pH was adjusted to 4.0; then the sample was centrifuged at 9000*g* for 10 min, and the pellet was washed three times with 100 mL of acetone, centrifuged for 10 min between each of the washings to obtain the polysaccharide extract (PE). PE samples were lyophilized and stored in amber flasks at 4 °C until further analyses.

Extraction and Quantification of Oligosaccharides. The extraction and quantification were done according to the methods of Gulewicz et al. (*14*) and Brenes et al. (*15*) with modifications. Briefly, 10 mg of raw and cooked beans was extracted with 80% v/v aqueous ethanol (10 mL) for 5 min using a magnetic stirrer at room temperature and centrifuged (Hermel Z323K USA) at 50003*g* for 5 min. The recovered

Table 3. Quantification and Validation of Condensed Tannins and Total Flavonoids in Different Extracts from Common Bean (*P. vulgaris*) Cv. Negro 8025[a]

	condensed tannins			total flavonoids[b]		
sample[c]	not fortified	fortified	recovery (%)	not fortified	fortified	recovery (%)
raw bean[1]	21.04 ± 1.89 b	48.18 ± 2.51 b	80 c	1.9 ± 0.5 a	35.44 ± 1.6 a	94.0 a
cooked bean[2]	15.15 ± 1.53 c	42.78 ± 1.31 c	79 d	1.8 ± 0.1 a	35.38 ± 1.7 a	94.1 a
PE[3]	9.11 ± 0.27 d	14.43 ± 2.32 d	80.4 b	1.6 ± 0.4 a	34.63 ± 1.2 a	92.6 a
ME[4]	858.25 ± 37.63 a	887.82 ± 4.89 a	94.79 a	ND	ND	ND

[a] PE, polysaccharides extract; ME, methanolic extract of common bean; ND, not determined. Each value is the mean of four independent experiments in triplicate ± standard error. Different letters in each column express significant difference ($\alpha = 0.05$) by Tukey test. [b] Samples were fortified with 35.8 mg of rutin. Total flavonoids are expressed as mg equiv of rutin/g of lyophilized sample. [c] Samples were fortified with [1]39.18, [2]39.01, [3]8.84, or [4]78.37 mg of (+)-catechin. The condensed tannins are expressed as mg equiv of (+)-catechin/g of lyophilized sample.

Table 4. SCFA in Cecum, Colon, and Feces[a]

	SCFA (mM/g of sample)								
	cecum			colonic			feces		
group	acetate	propionate	butyrate	acetate	propionate	butyrate	acetate	propionate	butyrate
control	29.54 ± 1.1 d	6.34 ± 0.1 ab	6.52 ± 0.1 a	32.31 ± 1.3 a	6.33 ± 0.09 bc	5.45 ± 0.03 a	35.24 ± 0.4 ab	3.07 ± 0.01 d	3.56 ± 0.01 e
bean	37.10 ± 0.71 ab	6.36 ± 0.3 ab	6.20 ± 0.07 ab	31.70 ± 1.6 a	5.80 ± 0.14 d	4.73 ± 0.05 c	33.14 ± 0.4 bc	4.89 ± 0.03 b	3.80 ± 0.01 cd
PE	35.25 ± 0.1 bc	6.92 ± 0.08 a	6.70 ± 0.09 a	31.15 ± 0.4 a	4.92 ± 0.11 e	5.30 ± 0.09 ab	32.84 ± 0.3 bc	3.92 ± 0.18 c	3.99 ± 0.05 bc
AOM	35.97 ± 1.2 abc	6.69 ± 0.1 ab	6.12 ± 0.1 ab	33.85 ± 1.8 a	6.15 ± 0.02 cd	4.87 ± 0.03 bc	32.91 ± 0.7 bc	4.97 ± 0.03 b	3.66 ± 0.04 de
bean + AOM	40.36 ± 1.80 a	5.61 ± 0.1 b	5.40 ± 0.3 c	34.17 ± 1.1 a	7.96 ± 0.03 a	4.60 ± 0.03 c	30.80 ± 1.8 c	5.13 ± 0.14 b	4.02 ± 0.02 b
PE + AOM	32.11 ± 1.77 cd	6.68 ± 0.4 ab	5.29 ± 0.34 c	36.55 ± 0.9 a	4.90 ± 0.10 e	4.72 ± 0.3 c	38.01 ± 0.2 a	7.02 ± 0.14 a	5.49 ± 0.07 a

[a] PE, polysaccharides extract; AOM, azoxymethane. Each value is the mean of four independent experiments in triplicate ± standard error. Different letters in each column express significant difference ($\alpha = 0.05$) by Tukey's test.

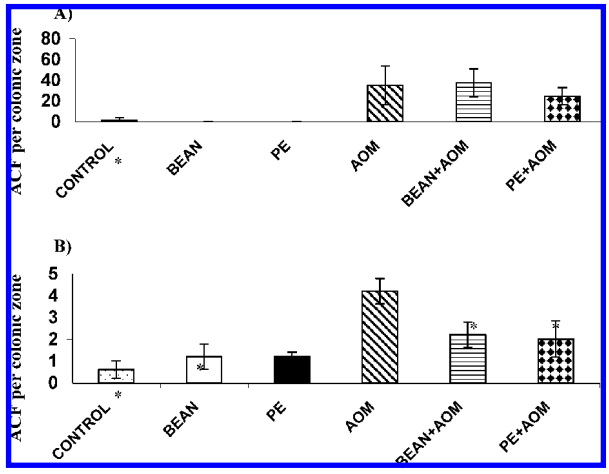

Figure 2. Analysis of aberrant crypt foci (ACF) with methylene blue and Dapi: (**A**) distal zone stained with methylene blue; (**B**) distal zone stained with Dapi.

supernatant was stored in the dark at −20 °C until further analyses were performed. One milliliter of the supernatant was added to 25 μL of phenol (80% v/v) and mixed. This was followed by the addition of 2.5 mL of concentrated H_2SO_4 and vigorous mixing by shaking (Vortex-2 Genie) for 1 min. The sample was left for 10 min at room temperature and incubated in a water bath (25−35 °C) for 15 min. The absorbance of the solution was monitored at 490 nm with a spectrophotometer and compared to a standard curve (1−100 mg of raffinose). Results are expressed as milligrams of raffinose equivalents per gram of sample.

Extraction of Resistant Starch. The resistant starch was extracted according to the procedure described in Shiga et al. (*16*) and Saura-Calixto et al. (*17*) with modifications. Six milliliters of 2 M KOH was added to 100 mg of insoluble fiber from lyophilized raw or cooked beans or PE with continuous agitation (HAAKE model SWB20 at 40 rpm) for 30 min at 25 °C. Three milliliters of acetate buffer (0.4 M, pH 4.75) was added, and the pH was adjusted to 4.7 with 5 mL of HCl (2 N). Sixty microliters of amyloglucosidase was then added, and the mixture was homogenized and incubated for 30 min at 60 °C with continuous agitation (HAAKE model SWB20 at 40 rpm). The samples were centrifuged (Hermel Z323K USA) at 3000*g* for 15 min. The pellet was resuspended in 10 mL of distilled water, and the centrifugation was repeated to obtain the resistant starch. The resulting samples were lyophilized, and the amount was calculated by difference with respect to the insoluble fiber of the samples.

Extraction of Condensed Tannins. The extraction of phenolic compounds from raw bean seed coats, cooked beans, and cooking water was performed as previously described by Cardador-Martínez et al. (*11*). A lyophilized sample (200 mg) was placed in a 50 mL flask and mixed with 10 mL of methanol. The flask was protected from light and shaken (HAAKE model SWB20) at 40 rpm for 24 h at 25 °C. After incubation, the samples were centrifuged at 4000*g* for 10 min, the methanol was removed by rotary evaporation, and the samples were lyophilized and stored in the dark at −70 °C until analysis.

Quantification of Condensed Tannins. Condensed tannins expressed as milligrams of (+)-catechin equivalents per gram of sample (mg of condensed tannins/g) were quantified according to the procedure of Deshpande and Cheryan (*18*) modified for use with a 96-well plate (microplate). Briefly, 200 μL of vanillin reagent (0.5% vanillin, 4% HCl in methanol) was added to 50 μL of methanolic extract and placed in a 96-well plate; each sample was tested in triplicate. Condensed tannins were quantified at 495 and 540 nm in a microplate reader (Thermo Electron Corp., Multiskan Ascent, model 51118307) using (+)-catechin (up to 0.2 mg/mL) as a reference standard. To correct for potential interference from natural pigments in bean, a blank sample was prepared by subjecting the original extract to the same conditions of reaction without the vanillin reagent.

Flavonoid Content. The spectrophotometric assay for the determination of flavonoid content was quantified according to the method suggested by Oomah et al. (*19*). Briefly, the method consisted of mixing 50 μL of the methanolic extract with 180 μL of distilled water and 20 μL of a solution of 10 g/L 2-aminoethyldiphenylborate in a 96-well plate. The absorbance of the solution was monitored at 404 nm with a microplate reader (Thermo Electron Corp., Multiskan Ascent model 51118307). A rutin standard was prepared in 80% methanol. Extract absorption was compared with that of a rutin standard curve (0−50 μg mL^{-1}). Flavonoid content was expressed as milligrams of rutin equivalent per gram of sample.

Recovery and Reproducibility Test. The validation of the microplate assay methods was performed using both recovery and reproducibility tests. For the recovery test, the internal standard [(+)-catechin or rutin] was added to the samples from condensed tannins and

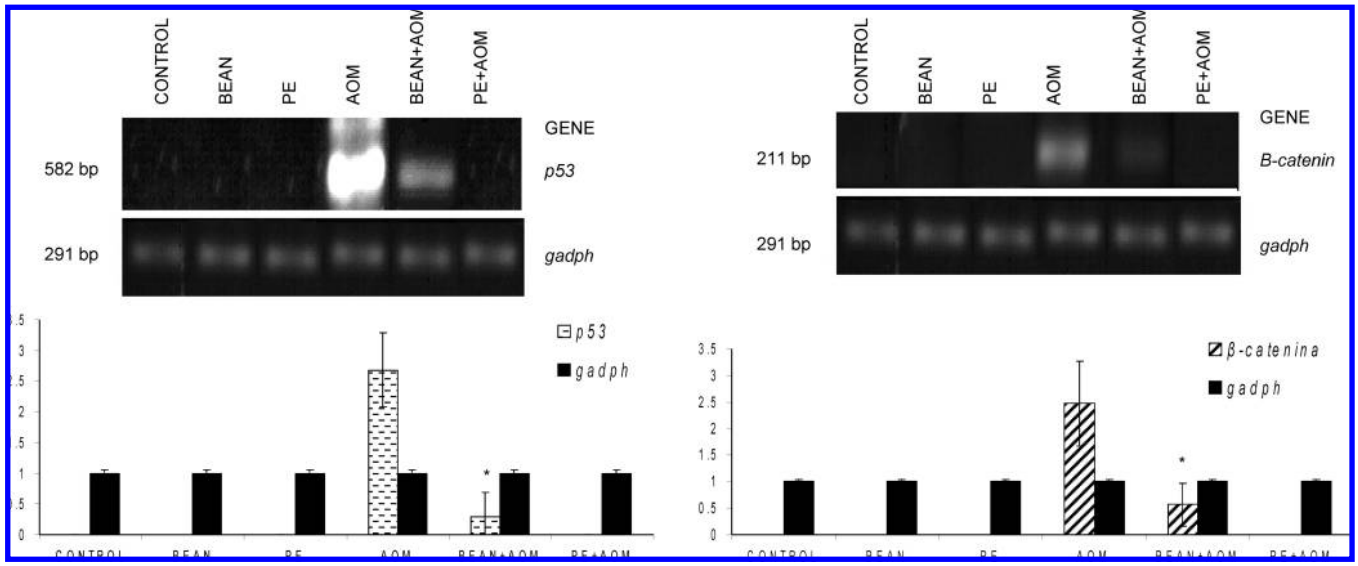

Figure 3. Expression and normalization of genes involved in cellular arrest and apoptosis in distal zone.

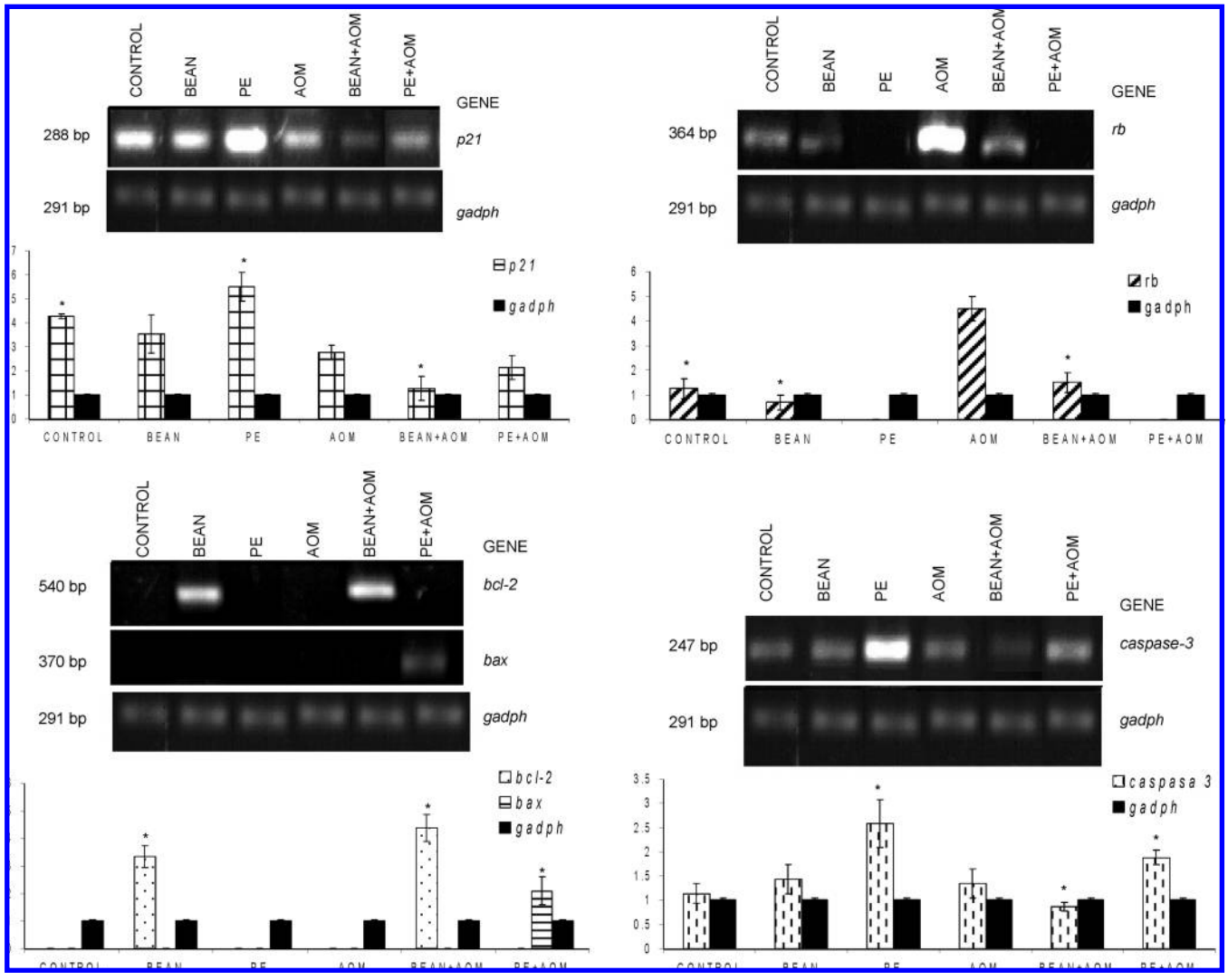

Figure 4. Expression and normalization of genes involved in apoptosis in distal zone.

flavonoid, respectively. The absorbance was monitored in each sample test and determined as the percentage of recuperation of the standard [(+)-catechin or rutin]. For the reproduction tests, both intra-assay and interassay reproducibility of standard and sample were determined. The intra-assay reproducibility was obtained by analyzing the same sample four times in the same day. The interassay test results were determined by analyzing different samples up to four times. Raw bean, cooked bean, PE samples, and ME were fortified with 39.18, 39.01, 8.84, and 78.37 mg of (+)-catechin, respectively. For the total flavonoid determination, all samples were fortified with 35.8 mg of rutin.

Animal Studies. Sprague−Dawley rats with an initial weight of 79 ± 5 g at 4 weeks of age were used in this study. The rats were

maintained in an air-conditioned animal room with an ambient temperature of 21 °C, 55% humidity, and 12−12 h light−dark cycle. The animals were given free access to a basal diet (2018S Harlan Tekland) and regular tap water. Both cooked beans and PE were administered intragastrically once a day during the experimental period. One week after the acclimation, the rats were randomly placed into six groups ($n = 10$): (1) control, basal diet and water ad libitum; (2) bean, basal diet plus cooked bean (3.2 g/kg of body weight); (3) PE, basal diet plus PE (1.84 g/kg of body weight); (4) AOM, basal diet plus subcutaneous injection of AOM (15 mg/kg of body weight, dissolved in 1 mL of physiological saline) once a week for 2 weeks; (5) bean + AOM, basal diet plus AOM and common bean; and (6) PE + AOM, basal diet plus AOM and PE (**Figure 1**). All of the animals were sacrificed 5 weeks after the last injection, and the colons were removed from the cecum to the anus. Cecal and colonic contents (midcolon and distal colon) and feces samples were collected at the time the animals were sacrificed, immediately frozen, and stored at −20 °C until analysis. The colon was divided into ascending (proximal) and descending (distal) zones. The distal colon was fixed in phosphate-buffered formalin for further analyses.

SCFA Analysis. One gram of cecal or colon contents or feces samples was suspended in 5 mL of water and homogenized for about 3 min. Each sample was centrifuged (Hermel Z323K USA) at 8000*g* for 10 min at 4 °C. Eight hundred microliters of the supernatant was adjusted to pH 2.9−3.1 with 1 M HCl, and a 750 μL portion was transferred to a flask and mixed with 120 μL of formic acid. A 1.5 μL aliquot of the resulting material was injected into a gas chromatograph (HP 6890 Plus GC) system coupled to a flame detector. A chromatography column HP (30M), Carbowax 30M, capillary 25.0 m × 320 μm × 0.30 μm nominal, was used. Helium was supplied as the carrier gas at a flow rate of 1.0 mL/min. The initial oven temperature was 95 °C, maintained for 2 min, increased to 20 °C, held for 1 min, then increased to 140 °C, and held for 20 min. The amounts of acetic, propionic, and butyric acid were calculated by using standard curves of acetic, propionic, and butyric acid standards, respectively.

ACF Analysis. The colon was removed, washed with PBS, opened longitudinally, and fixed in 10% buffered formalin for at least 24 h. After fixation, the colons were stained with 0.2% methylene blue and examined under a stereomicroscope. ACF were distinguished from adjacent normal crypts by their larger sizes, greater staining intensity, elongated pericryptal area, and thickened layer of epithelial cells according to the method used by Bird (*5*). Briefly, the distal colon was dissected and embedded in paraffin (*20*). Paraffin-embedded sections (5 μm) parallel to the mucosal surface were mounted on slides. The sections were deparaffinized in xylenes and stained with Dapi (*20*) to identify the ACF. The numbers of ACF were counted and reported as the mean per colon zone.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). The RT-PCR analysis consisted of obtaining the first chain of cDNA according the conditions recommended by the manufacturer (SuperScript One-Step RT-PCR System; Invitrogen, Carlsbad, CA), and the second chain of cDNA was obtained using the following conditions: 95 °C 1 min and then 27 cycles of 95 °C−5 s/65 °C−5 s/68 °C−6 min. Furthermore, specific PCR amplifications were carried out using 1 μL of each sample obtained above and amplified with specific primers for each evaluated gene in the study (**Table 1**). RT-PCR reaction mixtures (50 μL) contained 1 μL (61.17 ng) of total RNA, 1 μL of gene-specific sense and antisense primers (10 μM), 1 μL of SuperScript II RT/Platinum *Taq* Mix, 25 μL of 2× Reaction Mix buffer, and 21 μL of sterile deionized water. In each case, a RT-PCR analysis was carried out for each gene from samples of colonic tissues from rats either treated or untreated with azoxymethane.

Statistical Analysis. Results are expressed as mean ± standard error. Statistical significance was determined using Tukey's procedure ($\alpha = 0.05$) (*21*).

RESULTS AND DISCUSSION

Composition of Raw and Cooked Beans. Table 2 shows the protein, carbohydrate, fiber, polysaccharides, oligosaccharides, fat, and ash contents in raw and cooked beans (*P. vulgaris* L.) cv. Negro 8025. Protein content increased with the cooking process from 20.99 to 23.15%; these levels were in the ranges previously reported (6.25 and 26%) (*2, 22, 23*). The fat contents were similar in raw (1.54%) and cooked beans (1.41%), and these levels were lower than those in common beans cv. Flor de Mayo as reported by Costa de Oliveira et al.(*22*) (2.12%) and González de Mejía et al.(*1*) (2.56%).

Common beans are an important source of carbohydrates and have also been reported to be a good source of dietary fiber. Carbohydrate content increased with cooking (from 59.83 to 71.85%). Our results agree with those reported by Ratnayake et al. (*24*), Osório-Diaz et al. (*25*), and Tharanathan and Mahadevamma (*26*), who demonstrated that the thermal process increased total carbohydrate content including resistant starch. The milling bean process before curing or dehydrating can increase the physical exhibition of the amylose and the amylopectin to interact with other components, thus increasing the content of carbohydrate.

The content of crude fiber in lyophilized beans was 5.95%, in agreement with Costa de Oliveira et al. (*22*), at 5.7%, and De Almeida Costa et al. (*23*), at 6.26%. The small variations in fiber content could be explained by the four different cultivars used in those studies.

The concentrations of oligosaccharides in the raw and cooked bean were 5.0 and 6.8 mg/g equiv of raffinose/g of lyophilized sample, respectively. Guillon and Champ (*27*) showed an interval of 2.6−6.6 mg/g of dry matter, whereas Granito et al. (*28*) reported 1.7 mg/g of dry matter in a black bean cultivar. The variations could also be explained by genetic differences and environmental growing conditions.

The amounts of total polysaccharides, resistant starch, oligosaccharides, and fiber contents are important due to their implications as health benefits, through the fermentation of these components by the colon bacteria to produce SCFA (*29*).

Total fiber was statistically different in raw and cooked beans (28.41 ± 0.31 and 26.0 ± 0.4%, respectively) (**Table 2**). Our results are in agreement with those reported by Granito et al. (*28*) and Alfonso-González (*30*), whereas De Almeida Costa et al. (*23*) reported a range of 19.9−22.6%.

Resistant starch (RS) in raw and cooked bean (33 and 30%, respectively) was not statistically different (**Table 2**) and agrees with data reported by Shiga et al. (*16*), whereas Guillon and Champ (*27*) reported higher RS with a range of 33−35% in raw and cooked bean, respectively. Rehman et al. (*31*) reported 30.9 and 31.8% RS for two varieties of cooked kidney beans (red and white, respectively). Osorio-Díaz et al. (*25*), using the same extraction method (*17*), reported a lower RS content for black (23.5%) and Flor de Mayo beans (24.3%). The differences could be attributed to the different cultivars and growing conditions used in these studies.

In the PE, the RS content was 39.32%, and to our knowledge, this is the first time that RS content has been reported in PE from black beans. RS levels in raw and cooked beans are statistically different, and this could be due to retrogradation of the starch, as was reported by Osorio Díaz et al. (*25*). Guillon and Champ (*27*) showed that RS concentration increases with the boiling processes over time or after a cooking−cooling cycle. Additionally, changes in granule structure were measured by X-ray diffraction after soaking, prolonged cooking, and pressure cooking of common white and red kidney beans, where the starch granules from beans were classified as C by X-ray diffraction pattern with 33−35% of starch.

Condensed Tannins and Total Flavonoids. To validate the method of quantification of total phenolics and flavonoids in a

microplate assay, the raw beans, cooked beans, and polysaccharide extracts were fortified with (+)-catechin (in the range 8.84−78.37 mg) and rutin (35.8 mg). Samples were quantified by interpolation of their absorptions in standard curves of (+)-catechin and rutin.

The condensed tannins and total flavonoids in raw beans, cooked beans, and PE are displayed in **Table 3**. A methanolic extract was included as a reference, because the concentration of condensed tannins in cooked bean ME was determined previously (*10*). In general, the recovery (percent) was acceptable and >70%, being superior for the total flavonoid assay (92.6−94.1%).

Condensed tannins were statistically different in the raw beans, cooked beans, and PE [21.04, 15.15, and 9.11 mg of (+)-catechin, respectively]. Previous studies in the literature demonstrate that total phenolic compounds are dependent on the growing and storage conditions as well as thermal treatment (*10, 11*). Barroga et al. (*32*) reported that thermal treatment induced changes in the solubility of phenolic compounds, thus affecting their determinations.

Our results agree with those of other researchers. For example, Guzmán-Maldonado et al. (*33*) reported 26.5 mg equiv of (+)-catechin for whole bean seeds in the cv. FEB174. Aparicio-Fernández et al. (*10*) found lower values for the black beans cv. Jamapa [13.76 ± 1.2 and 5.20 ± 0.69 mg equiv of (+)-catechin/g in stored and newly harvested beans 1999 and 2003, respectively]. Oomah et al. (*19*) reported a concentration of 3.38 mg equiv of (+)-catechin for a black bean cultivar. Those differences can be attributed to the climatic conditions during the growing season, or the type of soil used where the beans were grown, as well as the bean genotype and the storage conditions. To our knowledge, this is the first report on tannins condensed in a PE of common bean.

Total flavonoids were similar among raw and cooked beans and polysaccharide samples (1.9, 1.8, and 1.6 mg equiv of rutin, respectively). Nevertheless, total flavonoid content in raw beans was higher than the content reported by Oomah et al. (*19*) for a black bean cultivar (0.86 mg of rutin). This difference can also be attributed to the genotype, as well as to the time of storage.

SCFA in Cecal, Colon, and Feces Contents. SCFA concentrations in the colon contents and feces samples from the animals of the different treatments are shown in **Table 4**. Overall, acetate, propionate, and butyrate concentrations were higher in cecal content, followed by colon content and feces except for the PE + AOM group, for which a higher concentration of the these SCFAs was found in feces > colonic content > cecal content. In cecum, the control group had the lowest acetate concentration, whereas the bean and bean + AOM groups had the highest acetate concentrations; the bean + AOM group presented the lowest propionate and butyrate concentrations. Similar significantly elevated SCFA levels were observed in cecum and feces of rats fed a diet with high-amylose corn starch (*34*). Nakanishi et al. (*35*) demonstrated that rats fed high-amylose maize starch (HAS) and *Clostridium butyracum* spores strain MIYAIR1588 (CBM588) showed an increase in butyrate concentration in the cecum, indicating that HAS and CBM588 changed the metabolism of colonic microbiota.

Propionate (6.92 mM/g of sample) and butyrate (6.70 mM/g of sample) concentrations in the cecum of the PE group were higher than that of the other treatments, suggesting that NDC increase the butyrate production. The increase in butyrate was also noted by other researchers, who showed that butyric acid production was increased as a result of a diet rich in nondigestible compounds (*36, 37*).

The acetate concentration in colon contents show no statistical differences, whereas the highest propionate production (7.96 mM/g of sample) was obtained in the bean + AOM group; this result is lower than that reported by Nakanishi et al. (*35*) (10.9 mM/g), who evaluated the addition of high-amylose maize starch into the diet. The difference would be attributable to the RS composition of diets.

In feces, we observed a lower acetic acid production in the bean + AOM group (30.8 mM/g of sample). Group PE + AOM presents the highest propionic acid production (7.02 mM/g sample), whereas the control group presented the lowest production of propionic acid (3.07 mM/g of sample). The RS content in the PE was higher (39.32%) than in whole cooked beans (30%). Furthermore, PE + AOM treatment showed the highest butyric acid concentration (5.49 mM/g of sample). Le Leu et al. (*34*) reported that rats fed RS (10% high-amylose maize starch) showed a significant increase in butyrate concentration in the distal colon zone (feces). To our knowledge, this is the first report on the production of SCFA from polysaccharides of common beans.

ACF Analysis. The number of ACF obtained from both histological analyses (methylene blue or Dapi stain) is shown in **Figure 2**. For the methylene blue stain, bean and PE groups did not show positive ACF, whereas PE + AOM treatment decreased the ACF number (24.5 ± 8.2 per colonic zone) compared to AOM treatment (35.0 ± 18.9 per colonic zone). On the other hand, in the histological analysis by Dapi, the bean + AOM and PE + AOM groups showed lower ACF numbers (2.2 ± 0.6 and 2.0 ± 0.8 ACF per colonic zone, respectively) compared with AOM (4.2 ± 0.6 ACF per colonic zone). Our results indicate that cooked beans and PE are rich in insoluble fiber, RS, and oligosaccharides, which, after fermentation by the colonic bacteria, produce SCFA in cecal and colonic contents and feces, where the butyrate concentration was higher in feces after PE + AOM treatment. Therefore, these results suggest that SCFA, mainly butyrate, are involved in the chemoprotection of PE against AOM.

Butyrate is an energy source for large intestinal epithelial cells. It has been demonstrated that butyrate also induces cell cycle arrest and apoptosis (*6*). Several researchers have demonstrated that diets rich in nondigestible compounds increase butyrate concentration (*36, 37*), such as soluble and insoluble fiber, RS, and nondigestible oligosaccharides (NDO) (*4*). Le Leu et al. (*34*) reported that rats fed RS (10 and 20% of high-amylose maize starch in the diet) showed reduced numbers of ACF and significantly increased total SCFA concentration, mainly butyrate, in the distal colon. Nakanishi et al. (*35*) demonstrated that rats fed high-amylose starch showed decreased numbers of ACF and that the coadministration of *C. butyracum* spores (CBM588) as probiotics helped to decrease the number of ACF (from 69.1 ± 31.3 to 53.9 ± 28.2 ACF per colonic zone, respectively). To our knowledge this the first report of the chemopreventive effect of a PE from cooked beans on ACF numbers.

Gene Expression Analysis. The gene expression in the distal colon is shown in **Figures 3** and **4**. The PE + AOM group did not show *β-catenin* and *p53* expression compared with the AOM group. Interestingly, a higher *p21* expression independent of *p53* and an increased expression of *bax* and *caspase-3* as well as a decreased expression of *rb* and *bcl-2* was found in the colon of animals treated with PE + AOM. Our data suggest that polysaccharides from common beans can induce gene expression

involved in proliferation, cell cycle arrest, and apoptosis in vivo in a similar way to that of in vitro studies.

Butyrate is a SCFA that has been related to the decrease in ACF in in vivo models and acts as a physiological regulator of colonic epithelial cell differentiation cell cycle arrest and apoptosis. Recently, microarray studies have been used to investigate the anticancer effects of butyrate on *p53*, *p21*, *rb*, different *cyclins*, *E2F*, and *histones*, demonstrating a complex cascade of reprogramming in SW620 colonic epithelial cells upon treatment with butyrate. The reprogramming was also characterized by the progressive recruitment of gene sets as a function of time (*7*). Butyrate responsive elements have been identified in the promoters of a number of genes and up-regulate the *β-catenin* signaling (*38*). Therefore, altered accessibility of cis acting elements to trans acting factors by changes in extent of histone acetylation may all contribute to modulation of transcription by butyrate, thus reducing the expression of *β-catenin*. Furthermore, butyrate has been related to the up-regulation of the cyclin-dependent kinase inhibitor, $p21^{waf1/Clip1}$, which induces cell cycle arrest (*8*). Other researchers have shown that *p21* expression can be stimulated independently of *p53*. Mahyar-Roemer and Roemer (*8*) demonstrated that butyrate can induce the expression of *p21* with immediate-early kinetics and independent of *p53* to levels comparable to those obtained with adriamycin treatment, the topoisomerase II inhibitor etoposide, and the DNA- and RNA-damaging agent 5-FU [Fluoracil (Adrucil)].

Butyrate has also been related to the induction of apoptosis via mitochondria mechanisms, by inhibiting *bcl-2* expression and increasing the expression of *bax*. Hass et al. (*39*) showed that colonocytes incubated with butyrate showed a difference that was lower in the transcript levels of *bcl-2* and an increased expression of *bax* in colonic mucosa. Other studies demonstrated that several SCFA-producing *Propionibacteria* induced typical signs of apoptosis including a loss of mitochondria transmembrane potential, generation of reactive oxygen species, *caspase-3* processing, and nuclear chromatin condensation (*9*). Here it has been demonstrated that PE + AOM treatment decreased the expression of *bcl-2* and increased the expression of *bax* and *caspase-3* compared to AOM treatment. Consequently, the results suggest an apoptotic process was induced by butyrate via mitochondria mechanisms.

Conclusions. The common bean (*P. vulgaris* L.) cv. Negro 8025 can be a reliable source of polysaccharides and phenolic compounds. Total phenolic compounds and total flavonoids were determined by a scaled-down method. The recovery was >70%, validating the utility of this method.

PE contains considerable amounts of nondigested carbohydrates that are fermented in the large intestine to produce SCFA, mainly butyrate, which was related to the decrease in ACF and to the induction of the expression of genes involved in proliferation, cellular arrest, and apoptosis (*b-catenin*, *p53*, *p21*, *rb*, *bax*, and *caspase-3*).

LITERATURE CITED

(1) González de Mejía, E.; Valdez-Vega, M. C.; Reynoso-Camacho, R.; Loarca-Pïna, G. Tannins, trypsin inhibitors and lectin cytotoxicity in tepary (*Phaseolus acutifolius*) and common (*Phaseolus vulgaris*) beans. *Plant Foods Hum. Nutr.* **2005**, *60*, 137–145.

(2) Reyes-Moreno, C.; Paredes-López, O. Hard-to-coock phenomenon in common beans, a review. *Crit. Rev. Food Sci. Nutr.* **1993**, *33* (3), 227–286.

(3) Champ, M. M. Non-nutrient bioactive substances of pulses. *Br. J. Nutr.* **2002**, *88* (3), 307–319.

(4) Hangen, L.; Bennik, M. R. Consumption of black beans and navy beans (*Phaseolus vulgaris*) reduced azoxymethane-induced colon cancer in rats. *Nutr. Cancer* **2002**, *44* (1), 60–65.

(5) Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.* **1987**, *30*, 147–151.

(6) Mentschel, J.; Claus, R. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. *Metabolism* **2003**, *52* (11), 1400–1405.

(7) Mariadason, J. M.; Georgia, A. C.; Augenlicht, L. H. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemeprevention of colon cancer. *Cancer Res.* **2000**, *60*, 4561–4572.

(8) Mahyar-Roemer, M.; Roemer, K. p21Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. *Oncogene* **2001**, *20*, 3387–3398.

(9) Jan, G.; Belzacq, A. S.; Haouzi, D.; Rouault, A.; Métivier, D.; Kroemer, G.; Brenner, C. Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell. Death Differ.* **2002**, *9* (2), 179–188.

(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) Cardador-Martínez, A.; Castaño-Tostado, E.; Loarca-Piña, G. Antimutagenic activity of natural phenolic compounds in the common bean (*Phaseolus vulgaris*) against aflatoxin B_1. *Food Addit. Contam.* **2002**, *19* (1), 62–69.

(12) Association of Official Analytical Chemists. *Official Methods of Analysis*, 17th ed.; AOAC: Arlington, VA, 2002; Methods 920.105, 925.23, 945.46, 985.29, 991.43.

(13) Kurtzman, R. H. J. R.; Halbrook, W. U. Polysaccharides from dry Navy beans *Phaseolus vulgaris*: its isolation and stimulation of *Clostridium perfringens*. *Appl. Microbiol.* **1970**, *20* (5), 715–719.

(14) Gulewicz, P.; Ciesiolka, D.; Frias, J.; Vidal-Valverde, C.; Frejnagel, S.; Trojanowska, K.; Gulewicz, K. Simple method of isolation and purification of α-galactoside from legumes. *J. Agric. Food. Chem.* **2000**, *48*, 3120–3123.

(15) Brenes, A.; Slominski, B. A.; Marquardt, R. R.; Guenter, W.; Viveros, A. Effect of enzyme addition on the digestibilities of cell wall polysaccharides and oligosaccharides from whole, dehulled, and ethanol-extracted white lupins in chickens. *Poult. Sci.* **2003**, *82*, 1716–1725.

(16) Shiga, M. T.; Lajolo, M. F.; Filisetti, M. T. Cell wall polysaccharide of common beans (*Phaseolus vulgaris* L). *Cienc. Tecnol. Aliment. Campinas* **2003**, *23* (2), 141–148.

(17) Saura-Calixto, F.; Goñi, I.; Bravo, L.; Mañas, E. Resistant starch in foods: modified method for dietary fiber residues. *J. Food Sci.* **1993**, *58* (3), 642–643.

(18) Deshpande, S. S.; Cheyran, M. Determination of phenolic compounds of dry beans using vanillin, redox and precipitation assays. *J. Food Sci.* **1987**, *52*, 332–334.

(19) Oomah, B. D.; Cardador-Martínez, A.; Loarca-Piña, G. Phenolics and antioxidative activities in common beans (*Phaseolus vulgaris* L). *J. Sci. Food Agric.* **2005**, *85*, 935–942.

(20) Castro, J.; Heiden, T.; Wang, N.; Tribukait, B. Preparation of cell nuclei from fresh tissues for high-quality DNA flow cytometry. *Cytometry* **1993**, *14*, 793–804.

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

(22) Costa de Oliveira, A.; Silva-Queiroz, K.; Machado, S.; Carraro, F. O processamento domestico do feijao-comun ocasionau uma reducao nos fatores antinutricionais fitato e taninos, no teor de amido e em fatores de flatulencia rafinose, estaquiose e verbascose. *Arch. Latinoam. Nutr.* **2001**, *51*, 276–283.

(23) De Almeida-Costa, G. E.; da Silva Queiroz-Monici, K.; Pissini-Machado, R. S. M. P.; Costa de Oliveira, A. Chemical composition, dietary fiber and resistant starch contents of raw and cooked pea, common bean, chickpea and lentil legumes. *Food Chem.* **2006**, *94*, 327–330.

(24) Ratnayake, W. S.; Hoover, R.; Shahidi, F.; Perera, C.; Jane, J. Composition, molecular structure, and physicochemical properties of starches from four field pea (*Pisum sativum* L.) cultivars. *Food Chem.* **2001**, *74*, 189–202.

(25) Osório-Díaz, P.; Bello-Perez, L. A.; Sayago-Ayerdi, S. G.; Benitez-Reyes, M. D. P.; Tovar, J.; Paredes-López, O. Effect of processing and storage time on in vitro digestibility and resistant starch content of two bean (*Phaseolus vulgaris* L.) varieties. *J. Sci. Food Agric.* **2003**, *83* (12), 1283–1288.

(26) Tharanathan, R. N.; Mahadevamma, S. Grain dry pulses—a boom to human nutrition. *Trends Food Sci. Technol.* **2003**, *14* (12), 507–518.

(27) Guillon, F.; Champ, M. M.-J. Carbohydrate fractions of legumes: uses in human nutrition and potential for health. *Br. J. Nutr.* **2002**, *88* (Suppl. 3), S293–S306.

(28) Granito, M.; Champ, M.; David, A.; Bonnet, C.; Guerra, M. Identification of gas-producing components in different varieties of *Phaseolus vulgaris* by in vitro fermentation. *J. Sci. Food Agric.* **2001**, *81*, 543–550.

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

(30) Alfonzo-González, G. C. Efecto del tratamiento térmico sobre el contenido de fibra dietética total, soluble e insoluble en algunas leguminosas. *Arch. Latinoam. Nutr.* **2000**, *50* (3), 281–285.

(31) Rehman, Z.; Salariya, A. M.; Zafar, S. I. Effect of processing on available carbohydrate content and starch digestibility of kidney beans (*Phaseolus vulgaris* L). *Food Chem.* **2001**, *73*, 351–355.

(32) Barroga, C. F.; Laurena, A. C.; Mendoza, E. N. Polyphenols in mung bean (*Vigna radiata* (L.) Wilczek). Determination and removal. *J. Agric. Food Chem.* **1985**, *36*, 1157–1159.

(33) Guzmán-Maldonado, S. H.; Castellanos, J.; González de Mejía, E. Relationship between theoretical and experimentally detected tannin content of common beans (*Phaseolus vulgaris* L.). *Food Chem.* **1996**, *55*, 333–335.

(34) Le Leu, R. K.; Brown, I. L.; Hu, Y.; Morita, T.; Esterman, A.; Young, G. P. Effect of dietary resistant starch and protein on colonic fermentation and intestine tumourigenesis in rats. *Carcinogenesis* **2007**, *28* (2), 240–245.

(35) Nakanishi, S.; Kataoka, K.; Kuwahara, T.; Ohnishi, Y. Effects of high amylose maize starch and *Clostridium butyricum* on metabolism in colonic microbiota and formation of azoxymethane induced aberrant crypt foci in the rat colon. *Microbiol. Immunol.* **2003**, *47* (12), 951–958.

(36) McBain, A. J.; Macfalane, G. T. Modulation of genotoxic enzyme activities by non-digestible oligosaccharides metabolism in *in vitro* human gut bacterial ecosystems. *J. Med. Microbiol.* **2001**, *50*, 833–842.

(37) Roy, C. C.; Kien, C. L.; Bouthiller, L.; Levy, E. Short-chain fatty acids: ready for prime time. *Nutr. Clin. Pract.* **2006**, *21* (4), 351–366.

(38) Bordonaro, M.; Mariadason, J. M.; Aslam, F.; Heerdt, B. G.; Augenlicht, L. H. Butyrate induced cell cycle arrest and apoptotic cascade in colonic carcinoma cells: modulation of the B-catenin-Tcf pathway, and concordance with effects of sulindac and trichostatin, but not curcumin. *Cell. Growth. Differ.* **1999**, *10*, 713–720.

(39) Hass, R.; Busche, R.; Luciano, L.; Reale, E.; Engelhardt, W. V. Lack of butyrate is associated with induction of Bax and subsequent apoptosis in the proximal colon of guinea pig. *Gastroenterology* **1997**, *112* (3), 875–881.

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