

Enhancement of colon carcinogenesis by the combination of indole-3 carbinol and synbiotics in hemin-fed rats



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ABSTRACT

The risk of developing colorectal cancer (CRC) could be associated with red and processed meat intake. Experimental data supports that hemin iron, found abundantly in red meat, promotes CRC in mice and rats, while indole-3 carbinol (I3C) and synbiotics (syn) exert anti-carcinogenic activities in most studies of colon carcinogenesis. This study aimed to investigate the modifying effects of I3C and syn (inulin + *Bifidobacterium lactis*), given separately or together, on dimethylhydrazine (DMH)-induced colon carcinogenesis in hemin-fed rats. All animals were given four subcutaneous DMH injections and then, two weeks after carcinogen exposure, they began a basal diet containing hemin, hemin + I3C, hemin + syn, or hemin + I3C + syn for 23 weeks. The combination of I3C + syn significantly increased fecal water genotoxicity, tumor volume and invasiveness when compared to the hemin-fed control group. The groups fed I3C or syn alone had a significant reduction in the number of preneoplastic aberrant crypt foci (ACF) lesions compared to the hemin-fed group. Dietary I3C also reduced fecal water genotoxicity. Gene expression analysis of colorectal tumors demonstrated that the combination of dietary I3C + syn increased transcript levels for *Raf1* and decreased tumor progression and invasiveness related to the genes *Cdh1* and *Appl1*. This analysis also revealed that the *Tnf* and *Cdh1* genes were significantly up- and down-regulated, respectively, in tumors of rats that received I3C, in comparison with the hemin-fed group. These findings reveal that the joint administration of I3C and syn enhanced the development of colon tumors induced by DMH in hemin-fed rats, while they potentially reduced ACF development when given alone.

1. Introduction

Colorectal cancer (CRC) is the third most common type of human cancer and a leading cause of death in developed countries (Siegel et al., 2016). Epidemiological and experimental studies have linked the risk of CRC in westernized societies to the ingestion of diets rich in fat and red and processed meats associated with reduced consumption of fruits, vegetables, and whole grains (Boada et al., 2016; Dermadi et al., 2017). Over the last 50 years, global red meat consumption rose from 63 g/day to 115 g/day (Sans and Combris, 2015). The World Cancer Research Foundation (WCRF) recommends less than 71 g of red meat per day and to avoid processed meat entirely (WCRF, 2017). Indeed, the International Agency for Research on Cancer (IARC) has classified red meat as probably carcinogenic to humans (Bouvard et al., 2015). However, the effect of meat consumption on cancer risk is still a controversial issue (Corpet, 2011; Turner and Lloyd, 2017).

Experimental data supports that hemin iron, found abundantly in red meat (approximately 1.5 μmol/g dry weight) promotes CRC in mice and rats (Schwartz and Ellefson, 1985; Sesink et al., 1999; Bastide et al., 2011, 2015). Hemin can lead to dietary polyunsaturated fatty acid oxidation, which produces malondialdehyde, a cytotoxic and genotoxic compound (Guéraud et al., 2015). Hemin iron is known to induce fecal cytotoxicity, and thus increases cell proliferation and the risk of developing tumors in the colonic mucosa (Ijssennagger et al., 2012, 2013). However, the procarcinogenic effects of hemin in the colon can be inhibited by certain antioxidant compounds, such as polyphenols, chlorophyll, and vitamin C (Gorelik et al., 2008; Mirvish et al., 2008).

Most studies addressing the relationship between dietary habits and CRC have focused on associations between disease risk and isolated dietary factors/components, disregarding their potential synergistic or additive effects (Liu, 2003, 2004). A number of dietary bioactive agents found in vegetables, fruits and whole grains such as isoflavones, β-

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List of abbreviations

CRC	colorectal cancer	Gusb	glucuronidase, beta
I3C	indole-3-carbinol	IARC	International Agency for Research on Cancer
Syn	synbiotics	PBS	Phosphate Buffered Saline
DMH	dimethylhydrazine	RT-qPCR	Reverse Transcription Polymerase Chain Reaction
<i>B. lactis</i>	<i>Bifidobacterium lactis</i>	TLDA	Taqman Low Density Array
ACF	aberrant crypt foci	DMEM	Dulbecco's Modified Eagle Medium
AC	aberrant crypt	MDA	malondialdehyde
Raf1	Raf-1 proto-oncogene, serine/threonine kinase	MTT	brometo de [3-(4,5-dimetiltiazol-2yl)-2,5-difenil tetrazolium
Cdh1	cadherin 1	DPBS	Dulbecco's Phosphate-Buffered Saline
Appl1	adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1	DMSO	Dimethyl sulfoxide
Tnf	tumor necrosis factor	EDTA	Ethylenediamine tetraacetic acid
Actb	actin, beta	EMT	epithelial–mesenchymal transition
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	AKT	serine/threonine kinase
Hprt1	hypoxanthine phosphoribosyltransferase 1	MEK	MAP kinase-ERK kinase
		ERK	extracellular regulated MAP kinase

carotene, chlorophyll, indole-3-carbinol (I3C), lycopene, and others have been associated with a lower risk of developing CRC when given alone in different *in vitro* and *in vivo* bioassays (Miller and Snyder, 2012; Baena Ruiz and Salinas Hernández, 2016). However, bioactive food compounds derived from plants or animals sources are commonly eaten together and phytochemicals in fruits and vegetables demonstrate synergistic and/or additive interactions that usually result in a higher capacity than individually isolated properties (Wang et al., 2011; Miller and Snyder, 2012; Baena Ruiz and Salinas Hernández, 2016). These interactions can either potentiate or interfere with different bioactive compounds, resulting in opposing or negative effects (Wang et al., 2011).

Dietary bioactive agents found in fruit and vegetables can prevent CRC by modulating the expression of anti-carcinogenic genes (Hou et al., 2016). One such agent is I3C, a compound found in brassica vegetables that has been reported to have anticarcinogenic effects in several rodent models, including induction of cell cycle arrest, apoptosis, immunomodulation, antioxidant activity, and DNA repair (Aggarwal and Ichikawa, 2005; Higdon et al., 2007). Conversely, I3C has also been hypothesized to either reduce or enhance the development of colon tumors when administered chronically after carcinogen exposure (Dashwood, 1998). Similarly, synbiotics, (syn) defined as a formulation of probiotic bacteria and the growth promoting prebiotic, is capable of promoting the selective growth of the endogenous bacteria rods, such as *Bifidobacterium* and *Lactobacillus*, and reducing the number of cancer-promoting bacteria (Chong, 2014; Ambalam et al., 2016). The anti-carcinogenic effects of syn include anti-inflammatory, apoptotic, and immunomodulatory activities on the colonic mucosa (Hammer, 2011; Raman et al., 2013).

Therefore, considering the high global consumption of red meat, particularly in some westernized societies, and the potential interactions between the compounds ingested in foods, this study aimed to

investigate the modifying effects of I3C and syn (inulin + *Bifidobacterium lactis*), given alone or combined, on dimethylhydrazine (DMH)-induced colon carcinogenesis in hemin-fed rats.

2. Material and methods

2.1. Chemicals

1,2-dimethylhydrazine hydrochloride (DMH, 4 × 40 mg/kg body wt), (PubChem CID: 1322); Hemin (0.5 mmol/kg chow), (PubChem CID: 455658) and Indole-3-carbinol (I3C, 0.1% chow), (PubChem CID: 3712) were purchased from Sigma–Aldrich (St. Louis, USA). Inulin (5% chow) and *Bifidobacterium lactis* BB-12 (*B. Lactis*, 2.5 × 10¹⁰ CFU/g chow) were purchased from BENEORafti® (Tienen, BEL) and Chr. Hansen (Hørsholm, DNK), respectively.

2.2. Diets and study design

Hemin (0.32 g/kg), indole-3-carbinol (1 g/kg), inulin (50 g/kg) and *Bifidobacterium lactis* (2.5 × 10¹⁰ CFU/g) were incorporated into the basal diet (NUVILAB-CR-1, Curitiba-PR, Brazil). To ensure stability, the diets were prepared every 4 weeks, stored at 4 °C, and given fresh every 2 days. The dose selection for hemin, I3C, and *Bifidobacterium lactis*/inulin was based on previous studies (Xu et al., 1996; Femia et al., 2002; de Vogel et al., 2004; Bertkova et al., 2010). The animals used in this study were handled in accordance with the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA). The animal study was approved by the institution's Ethics Review Board (CEEA 921/2012).

Four-week-old male Wistar rats weighing approximately 200 g (ANILAB, Paulínia-SP, Brazil) were housed under standard conditions

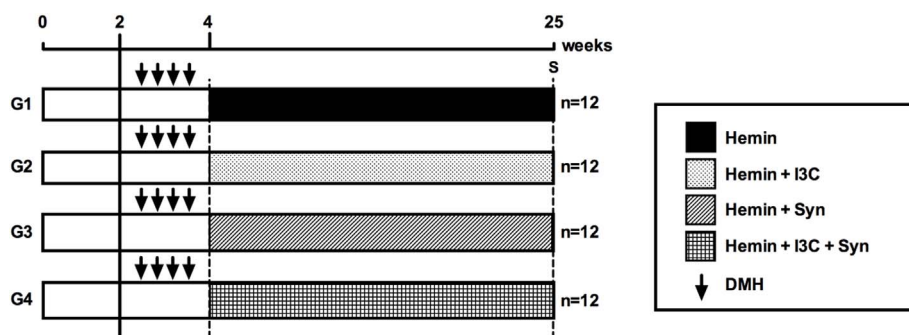


Fig. 1. Experimental design (for details see Material and Methods section). DMH = 1,2-dimethylhydrazine hydrochloride, hemin = 0.5 mmol/kg in chow, I3C = indole-3-carbinol 0.1% in chow, and syn = synbiotics (Inulin 5% plus *Bifidobacterium lactis* BB-12 2.5 × 10¹⁰ CFU/g) in chow. S = sacrifice.

(22 ± 2 °C temperature, 55 ± 10% humidity, 12 h light/12 h dark cycle) with free access to water and commercial chow (NUVILAB-CR-1, Curitiba-PR, Brazil). Following a 2-week acclimation period, the animals were randomly allocated into four groups (12 rats in each): All animals were given four subcutaneous DMH injections twice a week. Two weeks after DMH administration, all groups were given a basal diet containing hemin (G1), hemin + I3C (G2), hemin + syn (G3), or hemin + I3C + syn (G4), for 23 weeks (Fig. 1). Individual body weight and food consumption were recorded twice a week throughout the experimental period. At the end of week 25, the animals were sacrificed under xylazine and ketamine anesthesia.

2.3. Colon processing and histopathological analysis

After sacrifice, colon specimens were opened longitudinally, washed with 0.9% NaCl, pinned flat, fixed in 10% phosphate-buffered formalin for 24 h, and kept in 70% ethanol until analysis. All macroscopic tumors were removed, registered, and their volumes were calculated using the formula for the volume of a prolate spheroid: $\text{volume} = 4/3 \times 3.14 \times (\text{length}/2) \times (\text{width}/2) \times (\text{depth}/2)$, (Chatterjee et al., 2010). Also, samples of macroscopic tumor were collected and fixed in buffered formalin or stored at -80 °C for molecular analysis. Then, segments of distal, medial, and proximal colon from each animal were stained with 0.2% methylene blue and dissolved in phosphate-buffered salt solution (PBS). Aberrant crypt foci (ACF) are clusters of abnormal crypts (AC) displaying oval-like lumens and a thicker epithelial cell lining (Takahashi et al., 2012). The total number of ACF, AC, and the number of ACF with 2–3 AC, 4–9 AC and ≥ 9 AC were counted according to Bird's criteria, under light microscopy at 20× magnification (Bird and Good, 2000). After ACF analysis, the segments of colon and tumor samples were processed and histological sections were obtained. Colon tumors identified in histological sections hematoxylin-eosin-stained were classified into adenoma and adenocarcinoma according to the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice. The colonic lesions exhibiting a papillary or polypoid growth pattern without invasion of the muscularis mucosa were classified as adenomas, and tumors displaying invasion through the muscularis mucosa or deeper layers of the intestinal wall were classified as adenocarcinomas (Nolte et al., 2016). Tumor incidence and multiplicity were estimated for all groups.

2.4. Quantitative analyses of gene expression by real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from frozen colon adenocarcinomas (1 tumor/animal, n = 5, each group showing similar histological grade and size) using Rneasy Mini kit (Qiagen, Hilden, Germany), solubilized in nuclease-free water, and quantified by spectrophotometry (NanoVue Plus; GE Healthcare, Little Chalfont, UK). After determining the total RNA concentration (ng/μl), purity (ensured by 260/280 nm ratio of ~2.0), and integrity (RNA integrity number > 7; RNA 6000 Nano assay; Agilent, Waldbronn, Germany), cDNA was synthesized using SuperScript® VILO cDNA Synthesis Kit and Master Mix (Thermo Scientific, USA) according to the manufacturer's protocols. Gene expression profiling of genes involved in cell proliferation, cell death, cell differentiation, invasiveness, and metastasis were measured by RT-qPCR, using microfluidic TaqMan Low Density Array cards (TLDA, Life Technologies, Carlsbad, CA, USA) (supplementary data). The expression of the reference control genes Actb, Gapdh, and Gusb were used to normalize mRNA data based on geNorm calculations (Bustin et al., 2009). A total of 200 μl reaction mixture with 100 μl of cDNA template (58 ng mRNA) was added to 100 μl of TaqMan® Fast Advanced Master Mix (Life Technologies, USA) and was dispensed into loading wells on the TLDA card. The cards were centrifuged twice at 1200 rpm for 1 min each time, sealed, and run on QuantStudio™ 12K Flex Real-Time PCR System (Thermo Scientific, USA). The following cycling conditions

were used for all TLDA applications: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min. The TLDA data was analyzed using Expression Suite Software v1.0.3 (Life Technologies, USA) and the relative quantification of mRNA expression was evaluated using the $2^{(-\Delta\Delta C_T)}$ Method (Livak and Schmittgen, 2001).

2.5. Fecal water assays

2.5.1. Fecal water and lipid peroxidation assay

Cecal contents from all animals were collected at sacrifice and kept frozen at -20 °C. Fecal water was prepared by direct centrifugation, as described by Klinder et al., with minor modifications (Klinder et al., 2004). Feces samples (5 animals per group) were briefly thawed and 1 g of fecal content was weighed and suspended in 1 mL of PBS on a 2-mL microcentrifuge tube. Fecal debris was removed by centrifugation at 5,000 g for 10 min. The supernatant was sterile, filtered with an Ø 0.22 μm filter unit (Millipore, Germany), aliquoted, and frozen until analysis. Malondialdehyde (MDA) was measured in fecal water using a Lipid Peroxidation (MDA) Assay Kit from Sigma-Aldrich (St. Louis, USA) according to the manufacturer's instructions. The amount of MDA equivalents was determined in the absorbance 532 nm.

2.5.2. Cell culture

Caco-2 human colon adenocarcinoma cells were obtained from the Rio de Janeiro Cell Bank (BCRJ, Brazil) and grown in 25 cm² culture flasks in DMEM high-glucose medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 50 μg/mL penicillin, and 50 μg/mL streptomycin in a humid 5% CO₂ atmosphere at 37 °C. Upon reaching confluence, the cells were harvested with a cell detachment solution (Accutase Sigma Aldrich, St. Louis, USA) and seeded on 75 cm² culture flasks for further assays. Caco-2 cells between passages 29 and 33 were used in the analysis of fecal water cytotoxicity/genotoxicity.

2.5.3. MTT assay

Caco-2 cells were seeded on 96-well microplates at a density of 10⁴ cells per well and incubated for 24 h before fecal water exposure. Colon tumor cells were exposed to fecal water or DPBS (fecal water vehicle) diluted at 10% (v/v), in a complete culture medium. Four hours later, the medium was discarded and replaced by 100 μL of MTT solution (50 mg/mL), diluted in DPBS (Dulbecco's Phosphate Buffer Solution, Gibco 14040). The plates were then incubated for another 4 h. Subsequently, 100 μL of DMSO/propanol solution was added and the formazan crystals formed were solubilized. Absorbance was read at 570 nm using a microplate spectrophotometer (Epoch Microplate Spectrophotometer, Biotek Winooski, VT, USA). Cell viability (%) was calculated as the absorbance percentage ratio between exposed samples and non-exposed controls, arbitrarily defined as 100%.

2.5.4. Comet assay

Single cell gel electrophoresis (comet assay) is a method of DNA strand break detection in individual cells. The comet assay was conducted in duplicate as previously described (Liao et al., 2009). Caco-2 cells were seeded onto 6-well plates (10⁵ cells/well density), exposed to fecal water or DPBS (fecal water vehicle), diluted at 10% (v/v) in a complete culture medium for 4 h, harvested, and centrifuged at 1,200 g for 5 min. The cell pellet was mixed with 100 μL of low-melting-point agarose (0.75% in PBS), spread on slides pre-coated with normal-point agarose (1.5% in PBS), and cover slipped. Following agarose solidification (4 °C for 10 min), the coverslips were carefully removed and the slides were incubated overnight in cold lysis solution (1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na₂EDTA, 10 mM Tris with 10% DMSO, pH 10.0) at 4 °C.

The slides were then washed three times in PBS and immersed in fresh cold alkaline electrophoresis buffer (0.3 M NaOH, 1 mM

Na₂EDTA, pH > 13) for 20 min. Electrophoresis was performed at 30 V (300 mA) for 20 min. The slides were removed from the horizontal electrophoresis box, flooded three times with neutralizing buffer solution (0.4 M Tris-HCl, pH = 7.5), dehydrated in 100% ethanol, and stained with Sybr Gold (Invitrogen, USA). Analysis was performed under an epi-fluorescence microscope (Olympus BX-50, Japan) coupled with a CCD camera. One hundred random nucleoids/sample were scored using a Comet Assay IV Image Analysis System (Perceptive Instruments, UK).

2.6. Statistical analysis

The statistical analysis was performed using Sigma Stat 3.5 software Systat Software Inc (most parameters) and Expression Suite software 1.0.3 (Life Technologies, USA) (gene expression parameter). Data on body weight gain, ACF, tumor multiplicity, Caco-2 cell viability, and DNA damage was compared among the groups using ANOVA or Kruskal-Wallis tests. Tumor volume classes (%) were compared by Fischer exact test. The significant differences in gene expression were compared using Student's t-test. The differences among groups were considered significant when $p < .05$.

3. Results

3.1. General findings

At the end of the experimental period, the survival rate of the animals was 100% for all the dietary interventions. At week 25, the mean values of body-weight gain, final body weight and food consumption did not differ among the groups (Table 1).

3.2. ACF analyses

At the end of the experiment, preneoplastic ACF were observed in all animals subjected to the DMH-induced colon carcinogenesis protocol (Fig. 2B). ACF topographical distribution varied between colon segments, mainly being found in the middle and distal colon, as previously described (Rodrigues et al., 2002; Suzui et al., 2013). The data of ACF number and multiplicity per colon is shown in Table 2. A significant reduction ($p = .03$) in total number of aberrant crypts (AC) was observed in the groups fed hemin + I3C (G2) and hemin + syn (G3) compared to the hemin control group (G1). The number of ACF with 2–3 AC and ≥ 9 AC was significantly lower ($p = .007$ and $p = .005$) in the groups fed hemin + I3C (G2) and hemin + syn (G3), respectively, when compared to the hemin-fed control group (G1). The total number of ACF was also significantly lower ($p = .02$) in the group fed hemin + syn (G3) compared to the hemin-fed control group (G1). The combination of I3C + syn (G4) did not significantly modify the total number of ACF or their multiplicity in comparison with the hemin-fed group (G1).

Table 1

Body weight and food consumption data in the different experimental groups.^a

Groups/treatment ^b	(G1) DMH + hemin	(G2) DMH + hemin + I3C	(G3) DMH + hemin + syn	(G4) DMH + hemin + I3C + syn
Number of rats	12	12	12	12
Initial body weight (g)	237.67 ± 25.31	239.83 ± 19.38	233.33 ± 16.48	248.08 ± 13.22
Final body weight (g)	482.50 ± 55.25	486.08 ± 50.13	475.92 ± 36.55	477.08 ± 37.86
Body-weight gain (g)	254.83 ± 46.71	246.25 ± 43.46	242.58 ± 42.87	229.00 ± 43.28
Food consumption (g/rat/day)	22.06 ± 2.11	23.86 ± 2.50	23.50 ± 2.36	24.48 ± 2.38
Hemin consumption (mg/rat/day)	6.93 ± 0.11	7.49 ± 0.12	7.38 ± 0.11	7.69 ± 0.12
I3C consumption (mg/rat/day)		23.43 ± 3.89		24.04 ± 3.90
Inulin consumption (g/rat/day)			1.15 ± 0.19	1.20 ± 0.19
<i>B. lactis</i> consumption (CFU/rat/day)			5.7 × 10 ⁷	6.0 × 10 ⁷

^a Values are mean ± SD.

^b DMH = 1,2 dimethylhydrazine dihydrochloride (4 × 40 mg/kg b.wt., s.c.); hemin = 0.32 g/kg in chow, I3C = 1 g/kg in chow, Syn = Synbiotic (*Bifidobacterium lactis* Bb 12 at 2.5 × 10¹⁰ CFU/kg plus inulin 5% in the chow) for 23 weeks.

3.3. Tumor size and multiplicity and histopathology analyses

After sacrifice, all macroscopic tumors were measured and classified into three volume categories: small (< 30 mm³), medium (30–60 mm³) and large (> 60 mm³) (Table 3). A significantly higher ($p = .001$) incidence of large tumor volume was observed in the group fed hemin + I3C + syn (G4) combined than in the hemin-fed control group (G1). In addition, the number of adenocarcinomas were significantly higher in the group fed hemin + I3C + syn (G4) compared to the hemin-fed control group (G1) (Fig. 2D), (Table 3).

3.4. Colon tumor gene expression analysis

To measure the mRNA levels of transcripts encoding proteins involved in cell proliferation, cell death, cell differentiation, invasiveness, and metastasis, colon tumors from all of the groups underwent gene expression analysis (Table 4, supplementary data). The results of this study demonstrated that the combination of dietary I3C + syn (G4) increased transcript levels for *Raf1*, while it decreased the expression of tumor progression and invasiveness related genes *Cdh1* and *Appl1*. This analysis also revealed that *Tnf* and *Cdh1* were significantly up- and down-regulated, respectively, in tumors of rats that received the I3C-diet (G2) compared to the hemin-fed control group (G1). Moreover, the expression of *Il15* was increased in tumors from the group fed hemin + syn (G3) in comparison to tumors from the hemin-fed group (G1).

3.5. Fecal water lipid peroxidation, cytotoxicity, and genotoxicity

The analysis of the lipid peroxidation in fecal water samples revealed that the amount of malondialdehyde was significantly lower ($p = .001$) in the group fed hemin + I3C (G2) than in the hemin-fed control group (G1) (Fig. 3A).

Fig. 3B shows that Caco-2 cell viability was reduced after exposure to fecal water obtained from all dietary groups. Fecal water cytotoxicity from the group fed hemin + I3C (G2) was significantly lower ($p = .003$) than fecal water cytotoxicity from the hemin-fed control group (G1). Fecal water obtained from the groups fed hemin + I3C (G2) and hemin + syn (G3) also showed lower genotoxicity on Caco-2 ($p < .001$) compared with fecal water obtained from the hemin-fed control group (G1). However, fecal water obtained from the group fed a combination of hemin + I3C + syn (G4) was significantly higher ($p < .001$) than that obtained from the hemin-fed control group (G1) (Fig. 3C).

4. Discussion

In this study, we evaluated the modifying effects of dietary intervention with indole-3-carbinol (I3C) and synbiotics (syn), given alone or combined, on the promotion stage of DMH-induced colon

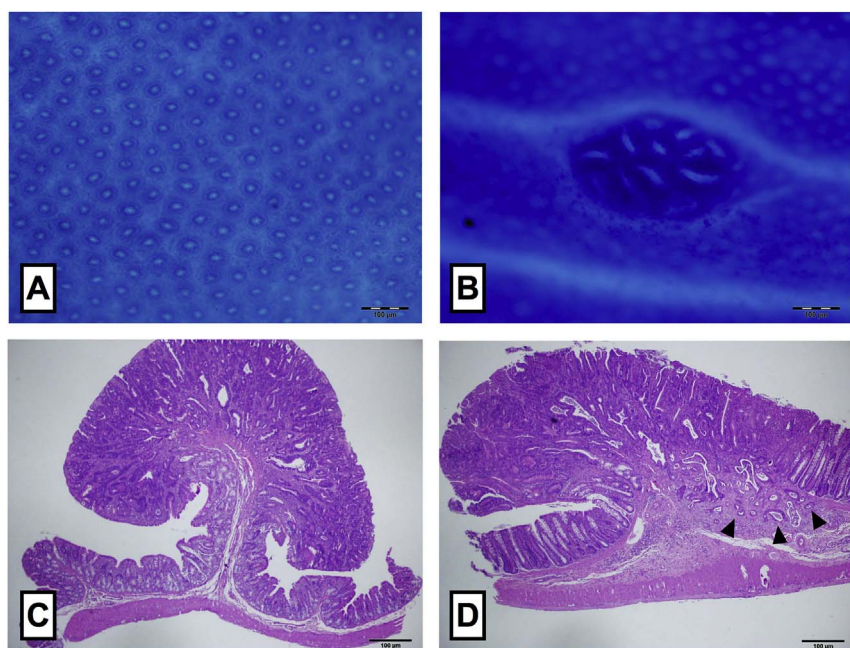


Fig. 2. (A) Normal appearance of the colonic mucosa in whole mount preparation stained with 0.2% methylene blue. (B) One aberrant crypt foci in the colonic mucosa stained with 0.2% methylene blue. (C) Adenoma (D) Adenocarcinoma, arrows indicate invasion through the muscularis mucosa.

carcinogenesis in hemin-fed rats. Our findings show that I3C decreased lipid peroxidation and the cytotoxicity and genotoxicity of fecal water obtained from the hemin-fed rats. Furthermore, dietary I3C or syn, given alone, decreased the number of preneoplastic lesions in the colon. Conversely, a combination of I3C and syn increased the number of large and invasive colon tumors and decreased the expression of genes regulating cell adhesion and migration.

Hemin iron, a putative cancer-promoting agent found in red meat, has been shown to enhance CRC development via lipid peroxidation-induced oxidative stress on colonic mucosa (Santarelli et al., 2008; Bastide et al., 2011). The underlying mechanisms of hemin-mediated cytotoxicity and genotoxicity in the rodent colon mucosa involve protein and DNA modification by oxidized lipids, such as malondialdehyde (Bastide et al., 2011). Malondialdehyde has been considered the most mutagenic product of lipid peroxidation, inducing oxidative stress, organelle dysfunction, and DNA-adducts in a number of *in vitro* and *in vivo* studies (Niedernhofer et al., 2003; Del Rio et al., 2005; Ayala et al., 2014). Attenuation of lipid peroxidation levels may therefore be able to protect the colon mucosa against hemin-induced cytotoxicity/genotoxicity (Bastide et al., 2011). Most experimental studies have used hemin or heme levels above those found in human diets (Turner and Lloyd, 2017). In our study the animals were given a high dietary level of hemin, approximately 7mg/rat/day, when compared to the heme concentration in meat (1.4 mg/100 g) reported in human diets (Valenzuela et al., 2009).

Our results showed that dietary intervention with I3C attenuated lipid peroxidation in the fecal water of hemin-fed rats. As a result, fecal water genotoxicity, cytotoxicity and ACF development were also reduced. These findings are consistent with other studies in that I3C was capable of acting as a free radical scavenger, reducing oxidative stress and stimulating an anti-oxidative response (Arnao et al., 1996; Maruthanila et al., 2014). It has also been hypothesized that I3C is able to block tumor initiation and promotion by inducing detoxification pathways in various enzymatic systems (Benabadji et al., 2004). Some I3C by-products can act as potent enhancers of enzymatic pathways, facilitating the metabolism and elimination of chemical carcinogens (Weng et al., 2008; Maruthanila et al., 2014).

Our findings also show that dietary intervention with synbiotic significantly decreased ACF development, but did not reduce fecal water lipid peroxidation, genotoxicity, and cytotoxicity in hemin-fed rats. The synbiotic formulation has been shown to reduce the risk of developing CRC in rats (Raman et al., 2013, 2016). In this regard, several studies have demonstrated the ability of synbiotics to reduce the incidence and multiplicity of chemically-induced ACF and tumors due to their anti-oxidant potential (Rowland et al., 1998; Pool-Zobel, 2005; Lasrado and Gudipati, 2015).

In our study, dietary intervention with a combination of hemin + I3C increased tumor volume compared to the hemin-fed control group. Additionally, tumor gene expression analysis revealed that the *Tnf* and *Cdh1* genes were significantly up- and down-regulated,

Table 2
Effects of dietary hemin, I3C and synbiotics on development of colonic aberrant crypt foci (ACF).^a

Group/Treatment ^b	Number of rats	Number of ACF			Total number ^c AC	ACF
		1-3 crypts	4-9 crypts	≥9 crypts		
(G1) DMH + hemin	12	22.71 ± 11.60	21.90 ± 16.57	7.08 ± 3.71	7.84 ± 2.05	51.70 ± 18.37
(G2) DMH + hemin + I3C	12	10.45 ± 5.90*	12.64 ± 5.52	3.87 ± 3.89	5.93 ± 1.78*	26.98 ± 9.79*
(G3) DMH + hemin + syn	12	15.51 ± 9.29	13.94 ± 9.10	2.33 ± 3.97*	5.29 ± 1.66 *	35.73 ± 17.52
(G4) DMH + hemin + I3C + syn	12	18.61 ± 5.88	23.98 ± 11.59	6.27 ± 2.99	4.72 ± 1.55	45.16 ± 13.55

^aDifferent from G1, .01 < p < .005.

^b Values are mean ± SD.

^c DMH = 1,2 dimethylhydrazine dihydrochloride (4 × 40 mg/kg b.wt., s.c.); hemin = 0.32 g/kg in chow, I3C = 1 g/kg in chow, Syn = Synbiotic (*Bifidobacterium lactis* Bb 12 at 2.5×10^{10} CFU/kg plus inulin 5% in the chow) for 23 weeks.

^c AC = aberrant crypt.

Table 3
Effects of dietary hemin, I3C and synbiotics on development of colon tumors (tumor volume, incidence and multiplicity).^a

Group/Treatment ^b	Number of rats	Incidence	Tumor volume			Multiplicity		
			Large	Medium	Small	Adenoma	Adenocarcinoma	^c Both
(G1) DMH + hemin	12	10 (83)	6	5	7	1.3 ± 0.48	1.25 ± 0.5	2.6 ± 1.07 (18)
(G2) DMH + hemin + I3C	12	11 (92)	14	1	5	1.83 ± 1.32	2.00 ± 1.54	3.1 ± 2.66 (27)
(G3) DMH + hemin + syn	12	12 (100)	4	4	6	1.25 ± 0.70	1.00 ± 0.00	1.8 ± 1.03 (23)
(G4) DMH + hemin + I3C + syn	12	12 (100)	35*	7	3	1.20 ± 0.44	4.90 ± 3.30*	6.2 ± 3.56 (61)

*Different from hemin control group, $p < .001$.

^a Values are mean ± SD.

^b DMH = 1.2 dimethylhydrazine dihydrochloride (4×40 mg/kg b.wt., s.c.); hemin = 0.32 g/kg in chow, I3C = 1 g/kg in chow, Syn = Synbiotic (*Bifidobacterium lactis* Bb 12 at 2.5×10^{10} CFU/kg plus inulin 5% in the chow) for 23 weeks.

^c Total number of colon tumors.

Table 4
Differentially expressed genes among the dietary hemin, I3C and synbiotics in tumor samples.

Group/treatment	Gene	log2 fold change	p-value
G2/hemin + I3C	<i>Cdh1</i>	-0.54	.02
	<i>Tnf</i>	0.79	.004
G3/hemin + syn	<i>Il15</i>	1.3	.006
G4/hemin + I3C + syn	<i>Cdh1</i>	-0.91	.04
	<i>Appl1</i>	-1.17	.003
	<i>Raf1</i>	0.79	.02

Data from gene expression (log2) in relation to DMH + hemin, ²DMH = 1.2 dimethylhydrazine dihydrochloride (4×40 mg/kg b.wt., s.c.); hemin = 0.32 g/kg in chow, I3C = 1 g/kg in chow, Syn = Synbiotic (*Bifidobacterium lactis* Bb 12 at 2.5×10^{10} CFU/kg plus inulin 5% in the chow) for 23 weeks. p -value < .05 statistically different from hemin control group (G1). *Cdh1*: Cadherin-1, *Tnf*: Tumor Necrosis Factor, *Il15*: interleukin 15, *Cdh1*: Cadherin-1, *Appl1*: Adaptor protein phosphotyrosine interacting with PH domain and leucine zipper 1, *Raf1*: RAF proto-oncogene serine/threonine-protein kinase.

respectively, in the tumors of rats fed I3C compared to the hemin-fed group. The *Cdh1* gene encodes E-cadherin, a transmembrane glycoprotein that mediates cell-cell adhesion in epithelial tissues (Bexx and van Roy, 2009). The loss of E-cadherin expression leads to the dissociation of cellular adhesion and has been associated with the acquisition of invasive phenotype (Onder et al., 2008; Kourtidis et al., 2017). E-cadherin downregulation is considered a hallmark of epithelial–mesenchymal transition (EMT) and has been shown to play a critical role in increasing tumor cell motility and promoting metastasis (Lamouille et al., 2014). The *Tnf* gene encodes a multifunctional, pro-inflammatory cytokine that belongs to the tumor necrosis factor family (TNF) (Horiuchi et al., 2010). TNF has been associated with a wide range of biological activities, including inflammation and cell differentiation (Sedger and McDermott, 2014). Studies have demonstrated that over expression of TNF in CRC can be associated with tumor progression, invasion, and metastasis (Al Obeed et al., 2014). Thus, aberrant E-cadherin and TNF gene expression is consistent with the increased tumor invasiveness observed in our study in the group fed hemin + I3C.

Although the protective effects of indole-3-carbinol (I3C) on colon carcinogenesis have long been recognized, it has been demonstrated that I3C can also act as a promoting agent on colon tumors in animals (Bailey et al., 1987; Dashwood, 1998). Pence et al. demonstrated synergistic and antagonistic interactions among dietary initiators and/or promoters of colon carcinogenesis, showing I3C as a significant factor in the development of DMH-induced tumors in rats (Pence et al., 1986). Similar tumor-promoting effects of I3C have also been reported by Suzui et al. using male F344 rats and male Swiss mice (Suzui et al., 2005).

Our study showed that dietary intervention with hemin + syn increased tumor expression of Il-15 in comparison to the hemin-fed

group. Il-15 gene encodes a cytokine that has been shown to stimulate T cell proliferation inside the tumor, increasing the infiltration of local T and B cells and providing a powerful anti-tumor immune-mediated response (Pilipow et al., 2015). The increased expression of the Il-15 gene induced by synbiotics may therefore suppress ACF development promoted by dietary hemin.

Furthermore, we observed increased fecal water genotoxicity in the group fed hemin + I3C + syn. Biomonitoring the genotoxic potential of fecal water can be considered a useful biomarker to assess the impact of nutritional components associated with the risk of or protection against CRC development (Pearson et al., 2009). This result is therefore consistent with the increased tumor invasiveness observed in the group fed hemin + I3C + syn. Moreover, tumor gene expression analysis revealed that the *Cdh1* gene was also down-regulated in this group. We also found a decreased expression of the *Appl1* gene, which is involved in cell migration. The adaptor protein APPL1 has been shown to regulate both activation and function of the serine/threonine kinase Akt, a central regulator of cell migration (Broussard et al., 2012). Cell migration is a highly complex process consisting of changes in the cell phenotype, including membrane protrusion and decreased adhesion (Bexx and van Roy, 2009; Broussard et al., 2012).

Additionally, the molecular analysis showed that dietary intervention with hemin, I3C, and syn increased expression of the *Raf1* gene. The *Raf1* gene encodes cytoplasmic protein serine-threonine with kinase activity. When activated, Raf serine-phosphorylates MEK1 and MEK2, which in turn phosphorylate ERK. MEK1 and MEK2 are dual-specificity protein kinases that function in the MAPK cascade, controlling cell growth and differentiation. The Ras/Raf/MEK/ERK cascade controls growth signals, cell survival, and cell invasion in cancer. This pathway is deregulated in about 30% of all cancers (Nandan and Yang, 2011; Pylayeva-Gupta et al., 2011).

Taken as a whole, our findings suggest that dietary I3C or synbiotics, given alone, exerted a positive impact on the suppression of hemin-promoting effects by decreasing fecal water lipid peroxidation, cytotoxicity, and genotoxicity, and reducing the number of pre-neoplastic lesions during the promotion stage of DMH-induced colon carcinogenesis. In contrast, the combination of dietary I3C and synbiotics had an opposite effect, elevating fecal water genotoxicity and increasing the volume and invasiveness of tumors in hemin-fed rats. The study of an isolated compound does not consider the complex interactions of a proposed chemopreventive agent in the presence of other bioactive molecules in the diet, as these interactions may exert synergistic, additive, or antagonist effects. Taken as a whole, our results shed light on possible synergistic food interactions of known anti-carcinogenic and promoting agents that may modify colon carcinogenesis.

5. Conclusion

The results of the present study revealed that the combination of indole-3 carbinol and synbiotics enhanced the development of colon

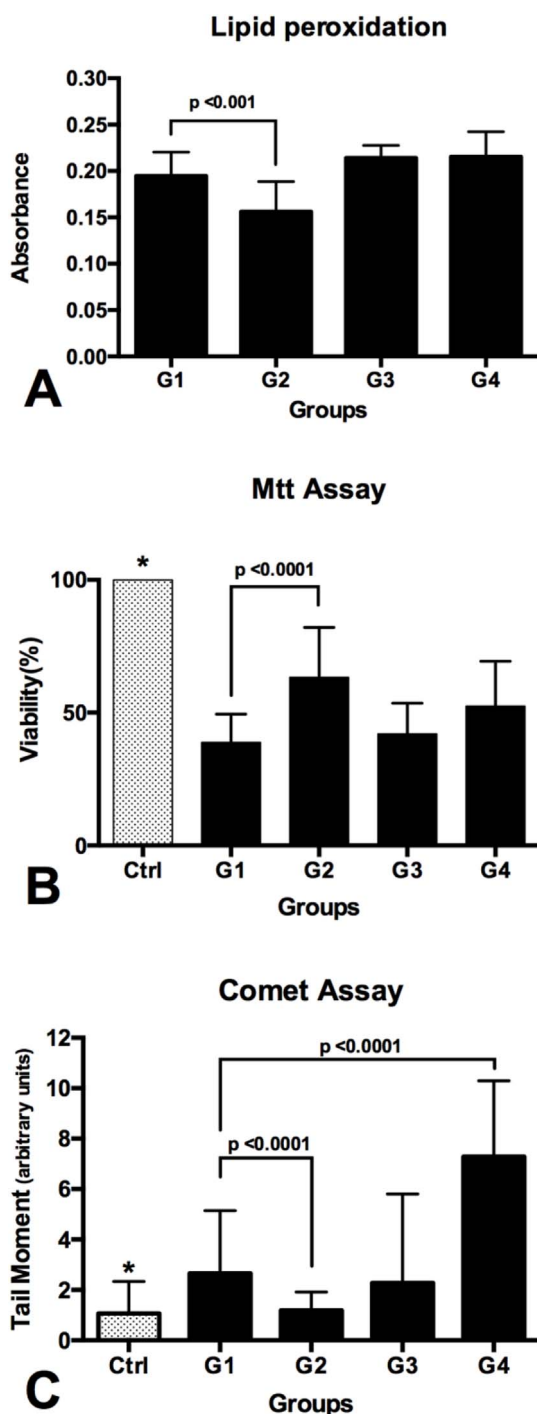


Fig. 3. Fecal water analyses on experimental groups (n = 5). (A) Effect of experimental diets on concentration of lipid peroxidation measured by thiobarbituric acid reactive species (TBARS) in the fecal water. (B) Cytotoxic effect of fecal water from different experimental diets on Caco-2 cell viability as determined by MTT colorimetric assay. (C) DNA damage induced by fecal water from different groups on Caco-2 cells, as assessed by the Comet assay. Ctrl: DPBS exposure control (fecal water vehicle); G1 = hemin; G2 = hemin + I3C; G3 = hemin + synbiotic; G4 = hemin + I3C + synbiotic. DMH = 1,2-dimethylhydrazine hydrochloride (4 × 40 mg/kg, s.c.), hemin = 0.5 mmol/kg in chow, I3C = indole-3-carbinol 0.1% in chow, and syn = synbiotics (Inulin 5% plus *Bifidobacterium lactis* BB-12 2.5 × 10¹⁰ CFU/g in chow). Data are mean ± Standard Deviation (SD), and analysis were conducted in duplicate. *Significantly different from that in the fecal-water exposed groups, p < .0001.

tumors induced by dimethylhydrazine in hemin-fed rats, while when given alone they had a preventive influence on ACF development. These findings highlight the complex interactions among bioactive

molecules in the diet on the development of colon tumors and further mechanism studies in this field are need.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fct.2017.12.029>.

Transparency document

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