Arthrospira (Spirulina) platensis feeding reduces the early stage of chemically-induced rat colon carcinogenesis

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Abstract

Colorectal cancer is the third most diagnosed cancer worldwide and linked to dietary/lifestyle factors. *Arthrospira* (*Spirulina) platensis* (AP) contains bioactive compounds with beneficial effects *in vivo*/*in vitro*. Thus, we evaluated the preventive effects of AP feeding against 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis. Male Sprague Dawley rats were given subcutaneous injections of DMH (4×40 mg/kg body weight) (G1-G3) or vehicle (G4-G5) twice a week (weeks 3-4). During weeks 1-4, animals were fed a diet containing 1% (G2) or 2% (G3-G4) AP powder (w/w) as a chemopreventive agent. After this period, all groups received a balanced diet until week 12. Some animals were euthanized after the last DMH injection (week 4) for histological, immunohistochemical (Ki-67, γ-H2AX and caspase-3) and molecular analyses (RT-PCR for 91 genes), while other animals were euthanized at week 12 for preneoplastic aberrant crypt foci (ACF) analysis. Both AP treatments (G2-G3) significantly decreased the DMH-induced increase in γ-H2AX (DNA damage) and caspase 3 (DNA damage–induced cell death) in colonic crypts at week 4. In addition, *Cyp2e1* (Drug metabolism), *Notch1*, *Notch2*, and *Jag1* genes (Notch pathway) *and Atm*, *Wee1*, *Chek2*, *Mgmt*, *Ogg1* and *Xrcc6* genes (DNA repair) were also down-regulated by 2% AP feeding (G3) at week 4. A significant reduction in ACF development was observed in both AP-treated groups (G2-G3) at week 12. In conclusion, findings indicate that AP feeding reduced acute colonic damage after DMH, resulting in fewer preneoplastic lesions. Our study provided mechanistic insights on dietary AP-preventive effects against early colon carcinogenesis.

Keywords: *Arthrospira (Spirulina) platensis*; tumor initiation; Notch and DNA repair genes; colonic preneoplastic lesions; colon cancer prevention.

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed gastrointestinal malignancies in western industrialized countries in both men and women aged > 50, but a dramatic increase among those younger people (20 to 49) has been reported (Siegel et al., 2020; Sung et al., 2021). Globally, CRC is the third most commonly diagnosed neoplasia, and the second leading cause cancer-related deaths (Siegel et al., 2020; Sung et al., 2021). About 55–70% of cases are classified as sporadic, and more than 50% of these cases are related to modifiable risk factors. In fact, epidemiological and animal evidence indicate that a sedentary lifestyle and "westernized" dietary habits, including a high intake of red and processed meat, saturated fats and refined starches, associated with a low consumption of fresh fruit/vegetables and fiber, are risk factors for CRC development (Lofano et al. 2013; Bouvard et al. 2015; Pan et al., Wang, 2018; Keum & Giovannucci 2019; Siegel et al. 2020; Sung et al. 2021). In order to identify potential dietary agents against sporadic CRC development, carcinogen-induced preclinical models in rodents have been widely used for the screening of new preventive strategies (Corpet & Pierre, 2005; Raju, 2008; Fleet, 2014). In these translational rodent models, 1,2-dimethylhydrazine (DMH) hydrochloride and its main metabolite azoxymethane (AOM) are potent genotoxic agents to induce colonic preneoplastic (*i.e.*, aberrant crypt foci, ACF) and neoplastic (*i.e.*, adenomas and adenocarcinomas) lesions that resemble human CRC in most morphological and molecular aspects (Rosenberg et al., 2009; Perše & Cerar, 2011; Ward & Treuting 2014).

In this context, the photosynthetic cyanobacteria *Arthrospira spp.*, an edible blue-green microalga, has been widely studied and commercialized worldwide. This cyanobacteria contains high protein levels that can reach between 60-70% of the its dry-weight biomass (Lupatini et al., 2017; Lafarga et al., 2020; Barros de Medeiros et al., 2021). Other bioactive compounds have been described including antioxidants [β-carotene and C-Phycocyanin (C-PC)], minerals (P, K, Na, Ca, Mg, Fe, Zn), vitamins (E, B1, B2, B3, B9), essential amino acids, PUFAs [especially γ-linolenic acid (ALA, 18:3 n-6)] and some phenolic compounds (Lupatini et al., 2017; Papalia et al., 2019; Lafarga et al., 2020; Barros de Medeiros et al., 2021). In special, C-PC is a natural light-blue pigment used in food, cosmetic and pharmaceuticals products or formulations (Park et al., 2018, Pez et al., 2021). Nowadays, *Arthrospira spp.* powder is used as a nutraceutical and functional food supplement, and is clinically recommended for patients with higher risk factors for cardiovascular diseases or

metabolic syndrome, including arterial hypertension, insulin resistance, hypercholesterolemia and obesity (Martínez-Sámano et al., 2018; Moradi et al., 2019; Ramos-Romero et al., 2021).

Some *in vivo* studies have demonstrated that *Arthrospira spp.* has preventive properties against oral, liver, skin and breast carcinogenesis, exerting anti-inflammatory, antioxidant and pro-apoptotic properties (Grawish et al., 2010; Yogianti et al., 2014; Ouhtit et al., 2014; Mahmoud et al., 2021). Álvarez-González et al. (2015) demonstrated a potential preventive effect of oral administration of high concentrations of *Arthrospira maxima* (100, 400, and 800 mg/kg) on the initial stages of AOM-induced colon carcinogenesis. Nonetheless, the mechanistic landscape of this preventive effect is not fully unveiled. Thus, in the light of the potential beneficial properties of *Arthrospira (Spirulina) platensis* (AP) (Wu et al., 2016), we evaluated the protective effects of AP feeding, before and during DMH administrations, against carcinogen-induced colonic mucosal damage and preneoplastic lesion development in rodents.

2. Materials and Methods

2.1 – Chemicals, AP composition, and chow preparation

1,2-dimethylhydrazine hydrochloride (PubChem CID: 1322) was obtained from Sigma–Aldrich (USA) and AP powder was originated and generously donated by Fazenda Tamanduá (Brazil) with the following composition per gram: proteins (66.7%), total carbohydrates (20.0%), total lipids (9.33%) and fiber (6.0%) expressed on dry weight. According to Pereira et al. (2020) findings, C-PC levels in AP powder are 0.492 (± 0.086) mg/g (in triplicate). AP powder was incorporated into a standard rodent chow (Nuvilab-CR1, Nuvital - Brazil), achieving the final concentration of 1 or 2% (10 or 20 g of AP powder/kg chow). Thus, C-PC concentrations in chows were estimated: 4.92 (AP 1%) and 9.84 (AP 2%) mg/kg. The balanced diet was standardized according to the National Research Council's recommendation affording the rodent nutritional necessities (National Research Council, 1995). The chow was complete homogenized and humidified into an industrial mixer (model M60, CAF, Brazil), then pelleted (7.5 CV model, Chavantes, Brazil), dried trough ventilation, stored into identified plastic bags, and finally kept under refrigeration (−4°C). Representative photos of each chow are displayed in **Figure 1**. The nutritional composition of the experimental chow can be found in **Table 1**.

2.2 - Study design and sampling

For the sample size calculation, the total number of animals (n = 68) was estimated using the G*Power 3.1118 software, considering a significance level (α) of 5%, power (1-β) close to 95% and effect (f) corresponding to 0.55 (large effect), as previously recommended (Charan & Kantharia, 2013). The calculation can be found in **Supplementary Data 1.** Following a 2-week acclimation period, seventy-six four-week-old male Sprague Dawley rats - obtained from Multidisciplinary Center for Biological Research (CEMIB) at the State University of Campinas (UNICAMP, Campinas, SP, Brazil) - were randomly distributed into five groups (G1 – G5, n = 10 or 16 rats/group) (**Figure 1**). Chows were offered to the groups as it follows: standard chow (G1 and G5), or standard chow containing AP at 1 (G2) or 2% (G3 and G4) for four weeks. AP powder concentrations in chow were based on European Food and Safety Authority Scientific Committee's recommendations, indicating that feeding interventions should not exceed 5% in order to avoid nutritional imbalances in rodents (EFSA, 2011). Indeed, AP was safely consumed - considering body weight evolution and histopathological endpoints - up to 5% supplemental level in both male and female mice (Yang et al., 2011). Injections of DMH (G1 – G3; 40 mg/kg body weight) or DMH vehicle (G4 and G5; Na_2EDTA, 37 mg/L, 5 mL/kg body weight) were subcutaneously applied twice a week at weeks 3 and 4 (Figure 1), as previously established by our research group for male rats (Caetano et al., 2020). At the week 4, 24 hours after the last DMH administration, five animals of each group were euthanized. The remaining rats had their AP-supplemented chows (G2 – G4) replaced for standard chow (as G1 and G5) for more 8 weeks, until they were euthanized at week 12 (**Figure 1**). In both procedures, rats were euthanized by exsanguination under ketamine/xylazine anesthesia (160 and 10 mg/kg body weight, respectively).

At necropsy (weeks 4 and 12), colon samples were opened longitudinally, cleaned with 0.9% NaCl, pinned flat, fixed in 10% phosphate-buffered formalin during 24 hours, and then kept in 70% ethanol for histological analysis. Distal portions of the colon mucosa (week 4) were also sampled, snap frozen in nitrogen, and stored at -80°C for molecular analysis. Body weight, water and chow consumption were weekly registered during the experiment. Animals were accommodated in polypropylene cages under accepted conditions (22 ± 2°C temperature, 55 ± 10% humidity, 12h light/12h dark cycle) with free access to drinking water and chow. The study was developed according to the principles of the institution's Ethics Committee Board under the protocol CEUA-1128/2019.

2.3- Immunohistochemistry for Ki-67, caspase-3, and γ-H2AX and at week 4

Ki-67 (*i.e.*, proliferation), active caspase-3 (*i.e.*, apoptosis) and γ-H2AX (*i.e.*, DNA damage) markers were detected in colon sections. Briefly, deparaffinated 5 μm colon histological sections were placed onto silanized slides and sequentially treated with 0.01 M citrate buffer (pH 6.0) at 120ºC for 5 min in a Pascal Pressure Chamber (Dako Cytomation Denmark A/S), 10% H_2O_2 in phosphate-buffered saline (PBS) for 10 min, skim milk for 60 min, anti-Ki-67 (1:200 dilution, MA5-14520, Thermo Fisher Scientific, USA) anti-active caspase-3 (1:100 dilution, ab179517, 1:200, Abcam, UK) and anti-γ-H2AX (1:100 dilution, MA5-27753, Thermo Fisher Scientific, USA) antibodies overnight at 4°C, a one-step horseradish peroxidase (HRP)-polymer (EasyPath - Erviegas, Brazil) for 20 min at room temperature. Chromogen color was accomplished with 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich, USA). The slides were counterstained with Harris hematoxylin for 1 min. For each group (n = 5 each group), 20 randomly entire colonic crypts were scored per animal. Ki-67, caspase-3 and γ-H2AX labeling indexes (LI%) were scored by the number of positive-stained cells/number of cells per crypt ratio.

2.4- Low Density Gene Array, functional and network analysis at week 4

Total RNA was isolated from distal colon samples (n = 5 each group) with RNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. The quantification of RNA was performed by NanoVue Plus (GE HealthCare, UK) and the quality was evaluated using Bioanalyzer 2100 plataform (Agilent Technologies, USA). The cDNA synthesis was performed using SuperScript VILO cDNA Synthesis Kit and Master Mix (Thermo Scientific, USA) following the manufacturer's instructions. TaqMan Low Density Array cards (TLDA, Life Technologies, USA) were used for quantitative real-time polymerase chain reaction (RT-qPCR) following the manufacturer's instructions.

TLDA cards comprised 94 genes involved in DNA repair, anti/pro-oxidant metabolism, cell proliferation, death, and differentiation (**Supplementary Data 2**). Briefly, 100 μl of cDNA template (422 ng mRNA) was added to 100 μL of TaqMan Fast Advanced Master Mix (Life Technologies, USA) and dispensed into loading wells on the TLDA card. The cycling protocol included heat activation at 50°C for 1 min and denaturation at 95°C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min. Fluorescence detection was performed on a QuantStudio 12 K Flex Real-Time PCR System (Life Technologies, USA). Relative quantitation was calculated based on the $2^{-(\Delta\Delta Ct)}$ method (Livak &

Schmittgen, 2001), using Expression Suite Software v1.1 (Life Technologies, USA). *Actb, Gapdh, Gusb and Hprt* housekeeping genes were used for normalization.

The output list of differentially expressed mRNA (≥1.5fold change) was analyzed on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) database via the DAVID tools website (https://david.ncifcrf.gov/) in order to identify significantly enriched biological pathways [P<0.05; enrichment score = -log (p value)] (Huang et al., 2009). Network confidence analysis was carried out using the STRING database (https://string-db.org/).

2.5- Screening of colonic preneoplastic lesions at week 12

All the segments (distal, medial, and proximal) from each animal were stained with 0.2% methylene blue solution. Aberrant crypt foci (ACF) are clusters of abnormal crypts (AC) displaying oval-like lumens and a thicker epithelial cell lining (Bird & Good, 2000). The total number of ACF, AC, and the number of ACF with ≤ 4 AC or > 4 AC were counted according to Bird's criteria, under light microscopy at 20× magnification (Bird & Good, 2000).

2.6- Statistical analysis

Data from body weight gain, water and chow consumption, immunohistochemistry and ACF analysis were compared among the groups using one-way analysis of variance (ANOVA) or Kruskal Wallis followed by *post hoc* Tukey test. The analyses were performed using the GraphPad Prism software (V4.03, GraphPad, USA). The pairwise comparisons of gene expression data were performed and analyzed by using the Student *t*-test. Correlations were performed using Pearson's coefficient (r). The differences among groups were considered significant when $p < 0.05$.

3. Results

3.1 – Both weight, water and chow consumption at weeks 4 and 12

During the 4-week AP feeding period, no significant alteration in body weight, body weight gain or chow and water consumption was observed with exception of absolute liver weight that was lower in DMH-initiated groups (G1-G3) when compared to the non-initiated groups (G5 and G6) (**Table 2**). The average consumption of AP powder during the 4 weeks was 236.4± 25.5 (G2), 489.4 ± 52.1 (G3) and 470.1 ± 42.8 (G4) mg/rat/day (data are mean ± standard deviation). Based on average AP intake, the estimated C-PC consumption was 0.11

± 0.01 (G2), 0.24 ± 0.02 (G3), and 0.23 ± 0.02 (G4) mg/rat/day (data are mean ± standard deviation). In addition, the mean body weight and liver absolute and relative weights did not differ among groups from weeks 5 to 12 (**Supplementary Data 3**). At week 12, survival rates were 100% in both DMH and vehicle groups.

3.2- Colonic cell proliferation, apoptosis and DNA damage at week 4

Twenty-four hours after the last DMH administration, the γ-H2Ax and caspase-3-associated to DNA insult LI%, but not Ki-67 LI%, were significantly increased in the colonic mucosa from DMH-initiated groups (G1-G3, n=8 each) in comparison to non-initiated groups (G4 and G5, n=5 each) (**Figures 2, 3 and 4**). Noteworthy, a significant ($p < 0.001$, for both) reductions in γ-H2AX and caspase-3 LI% were observed in the colonic mucosa from DMH-initiated fed with AP at 1% and 2% groups (G2 and G3) when compared to only control DMH-initiated group (G1) (**Figures 2 and 3**). However, colonic cell proliferation LI% did not significantly differ among groups (**Figure 3**).

3.3 - Gene expression analysis at week 4

Twenty-four hours after the last DMH administration, the levels of mRNA encoding target proteins involved in the cell cycle, DNA repair and apoptosis pathways were measured in colonic mucosa samples at week 4 (Caetano et al., 2020). Gene expression analysis revealed 16 downregulated and 1 upregulated gene in the colonic mucosa of DMH-initiated and AP 2%-fed rats (G3) compared to the DMH counterpart (G1) (**Table 3**). Genes involved in the Notch pathway were decreased – as *Notch 1*, *Notch 2* and *Jag1* – as well as cell cycle/DNA repair-related ones – including *Atm*, *Wee1*, Chek2, *Mgmt*, *Ogg1* and *Xrcc6*. Of note, *Cyp2e1* gene encoding DMH metabolizing cytochrome P450 CYP2E1 enzyme was downregulated in AP 2%-fed rats. Raw data of the gene expression profile from and DMH-initiated fed 2% AP group (G3) was included in **Supplementary Data 2**.

As expected, functional enrichment analyses of the differentially expressed genes in AP 2%-fed mice (G3) indicated a significant correlation between the downregulation of these genes and "Notch signaling pathway", "Cell cycle" and, "Base excision repair" functional annotations (**Figure 5**). STRING network confidence analysis was in keeping with these functional correlations, as *Notch1*, *Notch2* and *Jag1* network nodes are strongly functionally correlated, a featured also observed among *Xrcc6*, *Atm*, *Chek2* and *Wee1*.

3.4- Colonic preneoplastic lesion analysis at week 12

Classical ACF were evaluated in methylene blue-stained whole-mount colon samples (Bird & Good, 2000) at the end of week 12 (**Supplementary Data 4**). No ACF were observed in non-initiated groups (G4 and G5). Data from the number and multiplicity (AC/ACF) of stereoscopically-analyzed ACF in the different DMH-initiated groups are summarized in **Table 4**. The mean number of ACF with ≤4 crypts, and > 4 AC, and the total number of ACF and AC were significantly ($0.008 < p < 0.01$) lower in the group fed AP at 1.0 or 2.0% (G2 and G3) when compared to the DMH-initiated group (G1).

3.5 Correlations

We evaluated whether the 2% AP-mediated alterations in DNA damage and Notch pathway at week 4 are correlated to AC/ACF development at week 12. The reduction of γ-H2AX was positively correlated to the reductions in AC ($p=0.047$, $r=0.63$) and ACF ($p=0.007$, $r=0.77$) (**Figure 6**). Only the downregulation of Notch 1 was positively correlated to the reductions in AC ($p=0.089$, $r=0.56$) and ACF ($p=0.04$, $r=0.64$) (**Figure 6**), not Notch2.

4. Discussion

In this feeding study, we investigated the beneficial effects of dietary AP interventions, before and during carcinogen administration, on the colonic mucosal acute damage as well as on the late development of putative preneoplastic lesions using a medium-term bioassay for rat colon carcinogenesis. Twenty-four hours after the last DMH administration, the results showed that both dietary AP interventions significantly reduced carcinogen-induced DNA damage and apoptosis in epithelial cells of the colonic crypts. In addition, AP at 2% feeding significantly reduced expression of *Notch 1*, *Notch 2* and *Jag1* genes (Notch pathway), *Atm*, *Wee1*, *Chek2*, *Mgmt*, *Ogg1* and *Xrcc6* genes (DNA repair pathway) and *Cyp2e1* (Metabolism) in the colonic mucosa in comparison to the only DMH-initiated group. As AP feeding reduced the noxious effect of DMH on the colonic mucosa, a significant reduction in mean number of AC and ACF development was also detected for both AP interventions groups at the end of week 12. The estimated AP intake was 236 (G2) and ~470-490 (G3 and G4) mg/rat/day, corresponding to a daily dose of 0.81 (G2) and 1.6 (G3 and G4) g/kg/day (**Supplementary Data 5**). Using the Human Equivalent Dose (HED) allometric dose translation formula (Reagan-Shaw et al., 2008), the estimated animal dose used herein corresponds to a dose of 130 (AP at 1%) and 250 (AP at 2%) mg/kg/day in humans (**Supplementary Data 5**). Although there are no clinical studies on the

chemopreventive effects of AP in humans, our preclinical intervention converted by HED approach is higher – while safe - than previous human studies showing antihypertensive (~28 mg/kg/day) or anticoagulant (~32 mg/kg/day) activities in high dose interventions (Jensen et al. 2016; Ghaem Far et al., 2021).

Several animal studies have demonstrated potential beneficial effects of AP intervention in different chemically-induced carcinogenesis models. Dietary 1% SP powder resulted in a lower incidence of tumors, tumor Ki-67 and estrogen receptor-positive labelling indexes in a classical 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary cancer model in female Sprague-Dawley rat strain (Ouhtit et al., 2014), when *Arthrospira spp.* was administered after a single carcinogenic exposure. In a DMBA-induced hamster buccal pouch carcinogenesis, an AP extract (10 mg/day) fed during the DMBA painting regimen suppressed the development of epithelial dysplasia and squamous cell carcinoma (SCC) accomplished by a reduction in cell proliferation indexes (Grawish et al., 2010).

Álvarez-González et al. (2015) showed that a *Spirulina maxima* powder suspension, administered before, during and after AOM administrations, inhibited ACF development by 66.4%, 46.2%, and 42.3% for 200, 400, and 800 mg/kg doses in relation to the AOM counterpart. These 4-week oral treatments also decreased lipid and DNA oxidation levels as evaluated by malondialdehyde and 8-hydroxy-2'-deoxyguanosine (8-oxoG) adducts markers. In an hepatocarcinogenesis mouse model induced by a specific diethylnitrosamine and carbon tetrachloride regimen, a SP powder suspension (250 and 500 mg/kg body weight), administered by gavage during weeks 25-28, reduced the number and size of macroscopic liver nodules and serum tumor biomarkers such as alpha fetoprotein, MDA and total antioxidant capacity while it increased serum total protein and animal survival (Mahmoud et al., 2021). Yogianti et al. (2014), using an ultraviolet radiation B (UVB)-induced skin mouse tumor development model, demonstrated that dietary 10% SP powder attenuated tumor induction and development with a reduction in 8-oxoG adducts in the skin after UVB exposure in both Ogg1 knockout-(KO) and wild-type (WT) male mice. In addition, dietary SP suppressed the phosphorylation of biomarkers p38 mitogen-activated protein kinase (MAPK), stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and extracellular signal–regulated kinase (ERK) proteins in the skin after ultraviolet radiation exposure, especially in Ogg1-KO male mice. In general, these findings are in keeping with our investigation, reinforcing the protective effect of AP treatment on different preclinical models of carcinogenesis.

Regarding the chemically-induced model used, DMH and its main metabolite AOM are procarcinogens that undergo metabolic activation by a hepatic CYP2E1 (enconded by *Cyp2e1)*, and later also by gut bacterial enzymes to produce reactive metabolites that induces DNA insults in colon epithelial cells (Rosenberg et al., 2009; Megaraj et al., 2014; Venkatachalam et al., 2020). These reactive ions alkylate specific genomic DNA bases, resulting in specific DNA adducts, such as O^6-methylguanine (O^6-mG) and N^7-methylguanine (N^7-mG) in both liver and colon targets. If not removed, these DNA adducts can lead to genomic instability and after a replication cycle and significantly contribute to the initiation of rodent colon carcinogenesis (Rosenberg et al., 2009; Megaraj et al., 2014; Venkatachalam et al., 2020).

In this rat study we demonstrated that AP at 2.0% -treated rats showed decreased expression of *Cyp2e1* gene, an important key target also involved in drug metabolism, including DMH bioactivation on colonic mucosa (Zanger & Schwab, 2013; Reed et al., 2018). An increase or decrease in CYP2E1 activity may affect the DMH/AOM metabolism, which in turn could influence tumor initiation induced by these two classical colon carcinogens (Megaraj et al., 2014). Sohn et al. (2001) demonstrated that metabolic activation of AOM is affected differently in *Cyp2e1*-KOmice in comparison to the *Cyp2e1*-WT mice. The metabolic activation of AOM in *Cyp2e1*-KO mice leads to a significant reduction in DNA guanine alkylation on epithelial colonic cells when compared to the counterpart mice. In concordance, our results revealed a significant reduction in γ-H2Ax (*i.e.*, a DNA damage biomarker) and in acute apoptotic response *(i.e.*, active caspase 3) in epithelial cells of the colonic crypts in both dietary AP interventions groups in comparison to the counterpart group. Therefore, our finding suggests that AP feeding could inhibit CYP2E1 activity in the colon and, potentially in the liver, reducing the genotoxic and apoptotic impact of DMH on the colonic mucosa. In accordance to our findings, Savranoglu & Tumer (2013) have demonstrated that SP oral treatment resulted in inhibition of the specific cytochrome P450 hepatic isozymes in male Wistar rats. In this animal study, the authors observed a significant reduction in hepatic expression levels and inhibition in enzymatic activities of CYP1A2 and CYP2E1.

In addition, our findings showed that the AP at 2% feeding results in a reduced expression in *Mgmt*, *Ogg1* and *Xrcc6* on the colonic mucosa in comparison to the counterpart group. MGMT encodes a specific repair enzyme that removes the O^6-mG adducts through covalent transfer of the alkyl group to the conserved active site, cysteine, restoring the normal structure of guanine. The OGG1 (8-oxoguanine DNA glycosylase) gene encodes a DNA

glycosylase/AP lyase that removes 8-OH-G lesions from genomic DNA while the *Xrcc6* (X-ray repair cross complementing 6) gene is associated with DNA recombination and repair events (Jia et al., 2015; Sampath & Lloyd, 2019; Yu et al., 2020). According to this finding, we suggest that these repair genes are downregulated due to lower DNA damage induced by DMH, probably due to the failure to activate this carcinogen and consequently the lower O^6-mG and free radical formation, since DMH, as a prototype alkylating agent, also inducing extensive DNA oxidative damage in both liver and colon (Rosenberg et al., 2009; Perše & Cerar, 2011). Herein, authors understand that decreased DMH-induced impact may be associated to the direct radical scavenging of AP compounds, as C-PC and β-carotene. Its photosynthetic pigment C-PC scavenges free radicals, suppresses iNOS expression and nitrite production, and inhibits lipid peroxidation while β-carotene reduces singlet oxygen-mediated lipid peroxidation, intracellular accumulation of reactive oxygen species (ROS), and expression of several inflammatory genes (Fiedor & Burda, 2014).

In our feeding study, it was demonstrated that dietary 2% AP inhibited the expression of genes in the Notch pathway. The Notch pathway was more significantly enriched, according to the DAVID functional analysis, with enriched Gene Ontology (GO) terms in the colonic mucosa from 2% AP feeding animals. Notch signaling is commonly deregulated in CRC and increased Notch-1 expression has been associated with tumor progression, tumor grade and metastasis (Vinson et al., 2016). It is known that Notch signaling is a potential therapeutic target against tumor initiation and progression, as it plays a major role in the colonic crypt homeostasis via the fine regulation of stem cell self-renew, behavior and controlled differentiation (Miyamoto & Rosenberg, 2011). As such, bioactive compounds contained in AP modulating Notch signaling could be used as a strategy to prevent CRC initiation since this pathway is mediated by a highly conserved ligand-receptor apparatus that plays key roles in the regulation of cellular homeostasis, including proliferation, survival, apoptosis, differentiation and other (Platonova et al., 2017). There are no *in vitro* or *in vivo* findings regarding C-PC modulation of Notch pathway, but β-carotene, another common AP bioactive compound, reduced the protein levels of many proteins involved in Notch pathway during BALB/c mouse smoking model of gastric epithelial-mesenchymal transition (Lu et al., 2018). Therefore, both downregulation of Notch pathway and reduced DMH-induced impact (apoptosis, DNA damage and repair) may contribute to the decreased ACF burden at week 12. Indeed, we observed a statistical positive correlation between the decrease in DNA damage (γ-H2AX) or the downregulation of Notch1 and AC/ACF development. As these lesions are considered putative biomarkers of the early stages of colon carcinogenesis (Bird

& Good, 2000), our results elicit a chemopreventive effect of AP – in special the higher intervention – on this widely-applied chemically induced models.

The present investigation lacked a dose-response effect in AP 1% and 2% treatments. The authors theorize that this point may be associated with the DMH protocol chosen (Caetano et al., 2020). High DMH doses (40 mg) may cause increased deleterious impact in the colonic mucosa (higher DNA damage and apoptotic response) compared to lower doses this colon carcinogen (Karthikkumar et al., 2015; Ganaie et al., 2019), thus demanding a more pronounced protective response by AP, and implying in the absence of a dose-response effect. Further experiments using higher AP concentrations or other DMH regimens are warranted.

5. Conclusion

The findings of this animal study allow us to conclude that AP feeding, before and during DMH administrations, significantly reduces carcinogen-induced colonic crypt insult, contributing for a lower ACF development. This protective effect could be due to a reduction in DMH-induced mucosa damage (gene expression/DNA damage and apoptosis biomarkers) so suggesting that AP feeding may positively influence DNA damage, mutation and tumor initiation induced by a specific colon carcinogen used in a classic rat colon carcinogenesis model. Since AP have been widely studies as a food additive (in similar concentrations applied herein) (Fradinho et al., 2020; Niccolai et al., 2021), our findings could inspire future clinical investigations on the role of this photosynthetic cyanobacteria.

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Author Contributions

Luis F Barbisan, Guilherme R Romualdo and Nelci Antunes de Moura: Conceptualization, Methodology, Writing- Reviewing and Editing Writing; Simone Oliveira Amadeu, Luis Manuel Sarmiento-Machado and Ariane Rocha Bartolomeu: Methodology, Analysis and Interpretation; María Angel García Chaves: Analyses and Writing- Reviewing and Editing.

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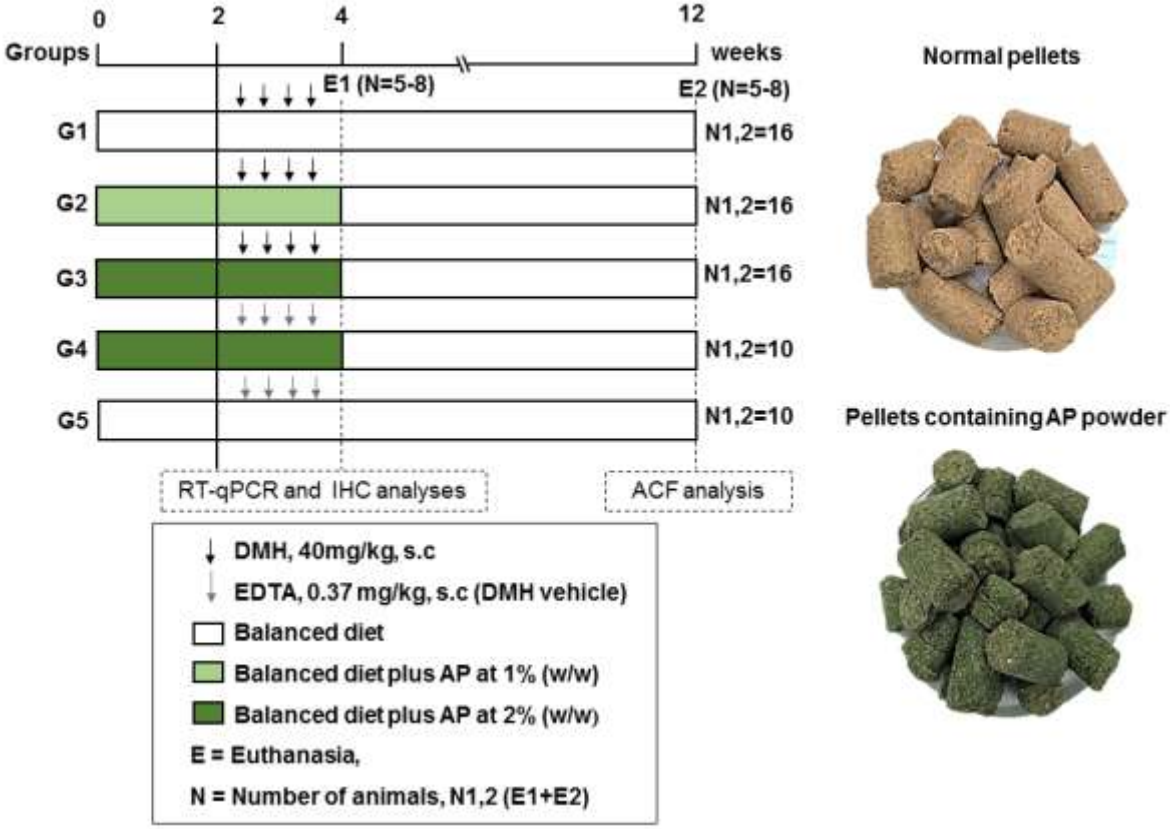

Figure 1. Experimental design (for details see Material and Methods section). DMH=1,2-dimethylhydrazine hydrochloride, EDTA= Ethylenediamine tetraacetic acid, ACF= Aberrant crypt foci, RT-qPCR= Quantitative Real Time, IHC= Immunohistochemistry and E= euthanasia.

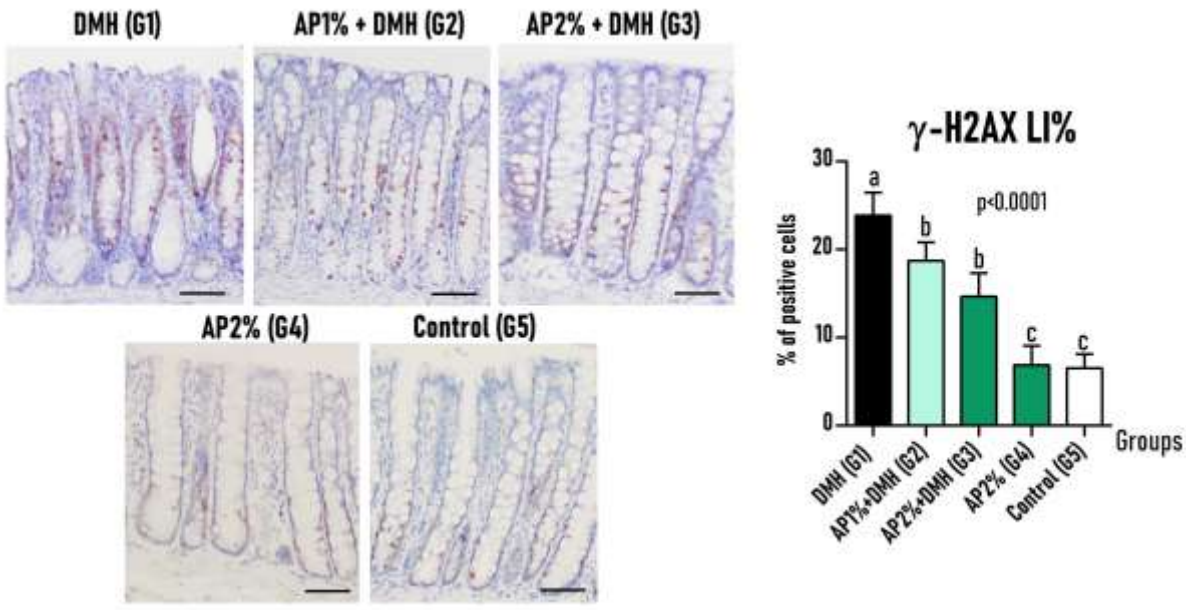

Figure 2. Effects of *Arthrospira (Spirulina) platensis* feeding on γ-H2AX labeling index (LI%) in the different experimental groups at week 4. Representative photomicrographs of phospho-H2A.X-immunostained sections of colonic crypts are presented (scale bar: 50 µm). Data are mean + standard deviation (n= 5-8 rats each group). Different letters correspond to statistical difference by ANOVA followed by Tukey's test ($p < 0.05$). AP= *Arthrospira (Spirulina) platensis* powder at 1% or 2% in the chow (w/w), DMH= 1,2 dimethylhydrazine dihydrochloride (4×40mg/kg body weight by subcutaneous injections).

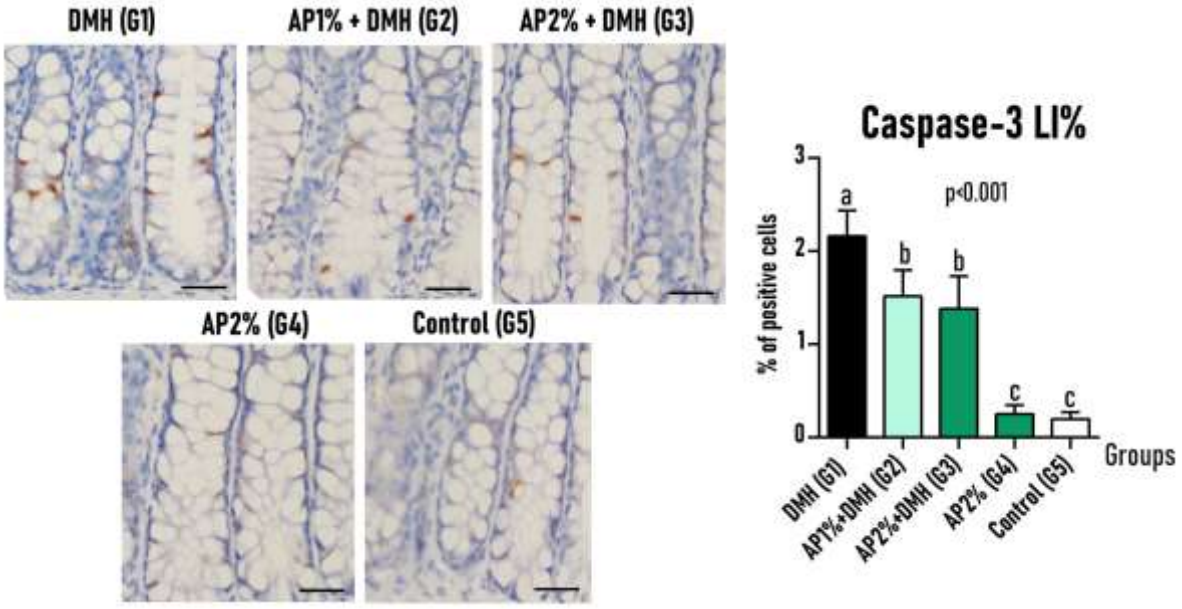

Figure 3. Effects of *Arthrospira (Spirulina) platensis* feeding on caspase-3 labeling index (LI%) in the different experimental groups at week 4. Representative photomicrographs of γ-H2AX-immunostained sections of colonic crypts are presented (scale bar: 25 μm). Data are mean + standard deviation (n= 5 rats each group). Different letters correspond to statistical difference by ANOVA followed by Tukey's test ($p < 0.05$). AP= *Arthrospira (Spirulina) platensis* powder at 1% or 2% in the chow (w/w), DMH= 1,2 dimethylhydrazine dihydrochloride (4×40mg/kg body weight by subcutaneous injections).

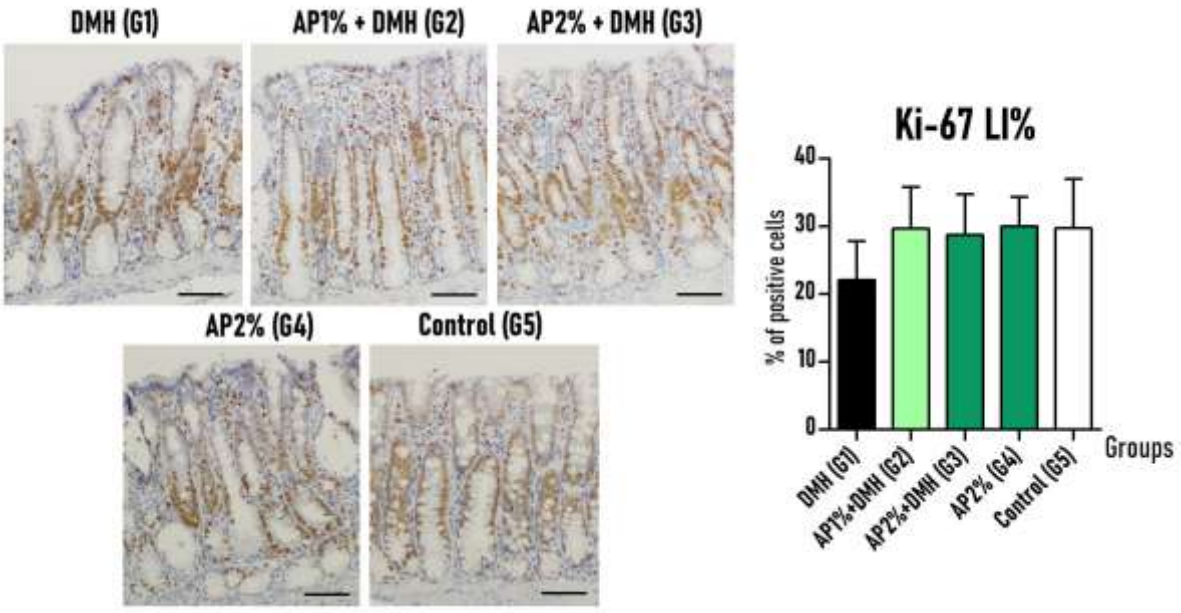

Figure 4. Effects of *Arthrospira (Spirulina) platensis* feeding on Ki-67 labeling index (LI%) in the different experimental groups at week 4. Representative photomicrographs of Ki-67-immunostained sections of colonic crypts are presented (scale bar: 50 µm). Data are mean + standard deviation (n= 5 rats each group). Different letters correspond to statistical difference by ANOVA followed by Tukey's test ($p < 0.05$). AP= *Arthrospira (Spirulina) platensis* powder at 1% or 2% in the chow (w/w), DMH= 1,2 dimethylhydrazine dihydrochloride (4 x 40mg/kg body weight by subcutaneous injections).

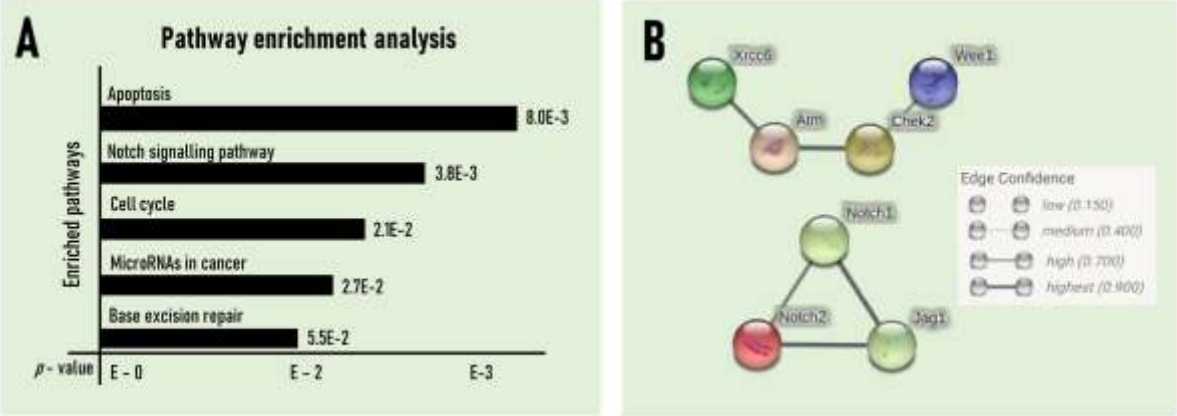

Figure 5. (A) Effects of AP2% treatment on the enrichment of pathways ranked by –log10 (*p value*). The mRNAs expressed differentially are associated to apoptosis (p=0.008) and Notch signaling pathways (p=0.0038). **(B)** STRING confidence network analysis. Nodes of the correlated proteins are presented (with 3D structure inside). Edges correspond to the confidence of functional correlation (caption).

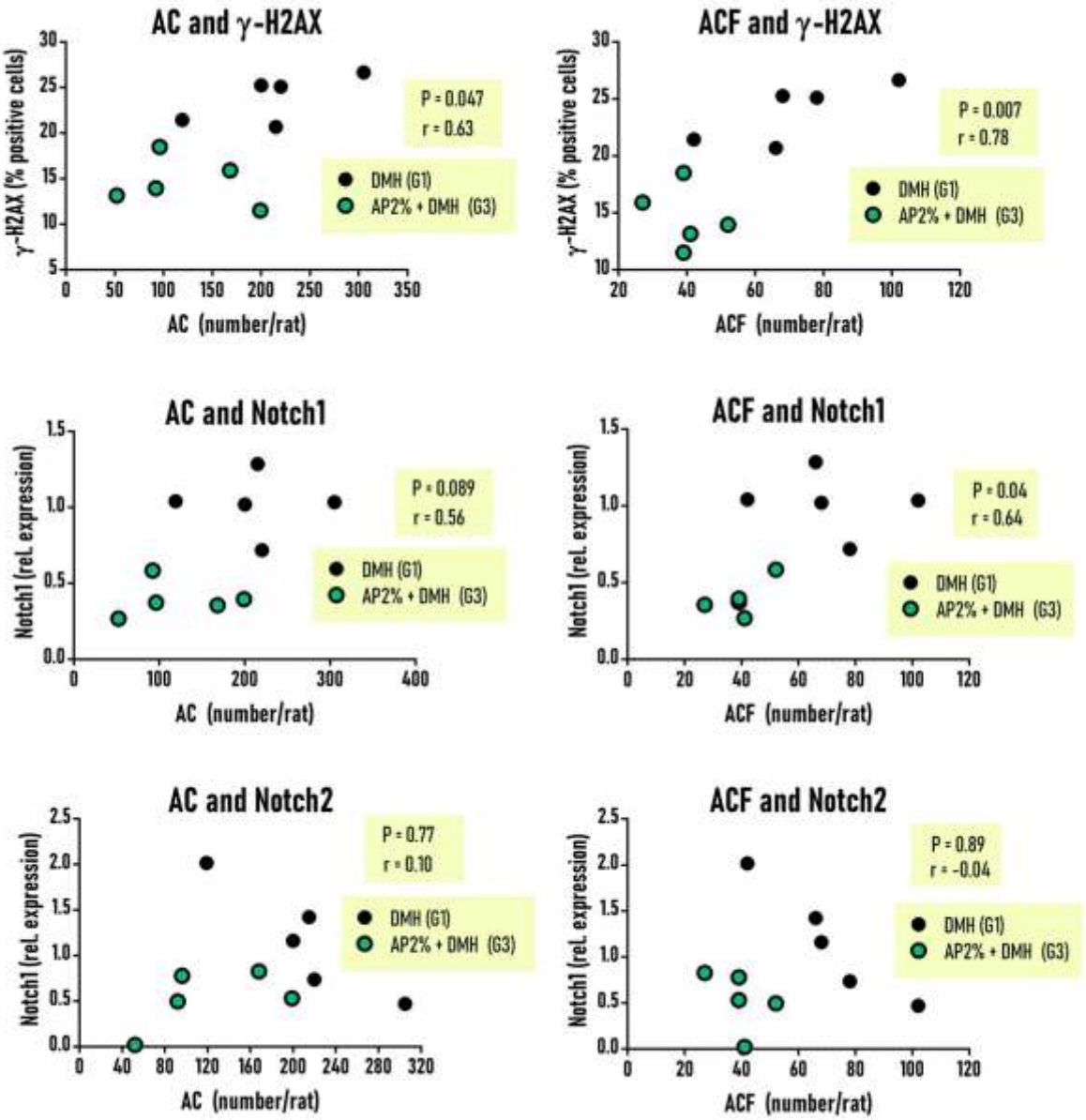

Figure 6. Correlation between DNA damage marker or Notch1 and Notch2 genes at week 4 and preneoplastic ACF development at week 12. Correlations were performed using Pearson's coefficient (r), and were considered significant when $p < 0.05$.

Table 1 : Nutritional composition of experimental chows (in g/g).–

Nutrients (g)	**Chow**	**Chow + AP 1%**	**Chow + AP 2%**
Protein	220	220	220
Corn starch	554	554	554
Sucrose	-	-	-
Soy oil	40	40	40
Corn oil	-	-	-
Fats	-	-	-
Fibers	70	70	70
Mineral mix	90	90	90
Vitamin mix	10	10	10
AP powder	**-**	**10**	**20**
Total (g)	~1000	~1000	~1000

AP = *Arthrospira platensis*; F-PC = C-Phycocyanin.

Table 2 - Effects of *Arthrospira (Spirulina) platensis* feeding on body weight (g), food (g/rat/day) and water (ml/rat/day) consumption and liver weights (absolute and relative) in the different experimental groups at week 4.

Groups/treatments[1]	N	Consumption		Body weight (g)			Absolute liver weight (g)	Relative liver weight (%)
		Food	Water	Initial	Final	Gain		
(G1) DMH	8	23.6 ± 2.7	38.2 ± 4.9	190.6 ± 17.5	296.5 ± 26.4	97.6 ± 29.9	8.80 ± 0.86*	3.45 ± 0.44
(G2) AP1% +DMH	8	23.6 ± 2.5	38.0 ± 4.4	189.8 ± 14.9	291.9 ± 29.6	94.5 ± 28.3	9.01 ± 0.93*	3.49 ± 0.47
(G3) AP2% +DMH	8	24.5 ± 2.6	38.9 ± 4.7	192.4 ± 17.5	298.5 ± 23.4	99.8 ± 24.4	9.66 ± 0.76*	3.48 ± 0.25
(G4) AP2%	5	23.5 ± 2.1	39.8 ± 4.7	188.1 ± 17.2	306.0 ± 21.0	109.0 ± 13.3	11.42 ± 1.31	3.76 ± 0.31
(G5) Control	5	24.8 ± 1.8	39.7 ± 4.0	188.9 ± 16.3	304.2 ± 18.0	104.8 ± 17.7	11.67 ± 1.01	3.80 ± 0.25

Values are mean ± standard deviation; [1]AP= *Arthrospira (Spirulina) platensis* at 1% or 2% in the chow and DMH= 1,2-dimethylhydrazine dihydrochloride (4×40mg/kg body weight, subcutaneous.); N = number of rats/group. Data were analyzed using one-way ANOVA followed by Tukey's test. * Different from groups G4 and G5, $p < 0.001$.

Table 3 - Differentially expressed genes in the colonic mucosa from the animals fed *Arthrospira* (*Spirulina platensis*) at 2% and DMH-initiated (group G3) compared to only DMH-initiated group (G1)[1].

Gene	Gene name	*Fold change*	*p* value
Atm	ATM serine/threonine kinase	0.415	0.004
Casp4	Caspase 4	1.819	0.028
Chek2	Checkpoint kinase 2	0.609	0.008
Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1	0.145	0.023
Dffb	DNA fragmentation factor subunit beta	0.637	0.017
Igf1r	Insulin-like growth factor 1 receptor	0.562	0.007
Jag1	Jagged canonical Notch ligand 1	0.506	0.022
Mgmt	O-6-methylguanine-DNA methyltransferase	0.346	0.021
Msh2	MutS homolog 2	0.480	0.030
Notch1	Notch receptor 1	0.438	0.000
Notch2	Notch receptor 2	0.337	0.031
Nthl1	Noth-like DNA glycosylase 1	0.481	0.003
Ogg1	8-oxoguanine DNA glycosylase	0.525	0.005
Sod1	Superoxide dismutase 1	0.573	0.023
Wee1	WEE1 G2 checkpoint kinase	0.595	0.011
Wnt2b	Wnt family member 2B	0.249	0.000
Xrcc6	X-ray repair cross complementing 6	0.617	0.004

N = 5 rats/group. [1] AP= *Arthrospira (Spirulina) platensis)* at 2% in the chow and DMH= 1,2-dimethylhydrazine dihydrochloride (4x 40mg/kg b.wt., s.c.). The groups were compared using Student's *t*-test. Fold change boundary of 1.5 and a $p < 0.05$ were used.

Table 4 - Effects of *Arthrospira* (*Spirulina*) *platensis* feeding on the development of colonic aberrant crypt foci (ACF) in the different groups at the end of week 12[1]

Groups/treatments[1]	Number of rats	Number of ACF		Total number		Multiplicity
		≤4 AC	> 4 AC	AC[3]	ACF	AC/ACF
(G1) DMH[2]	08	58.25 ± 16.35	8.37 ± 3.77	198.63 ± 60.50	66.50 ± 18.60	2.96 ± 0.29
(G2) AP1%+DMH	08	34.80 ± 9.57*	5.00 ± 3.80	125.9 ± 43.20**	39.60 ± 12.10**	3.04 ± 0.25
(G3) AP2%+DMH	08	35.00 ± 15.70*	2.25 ± 3.10*	121.0 ± 48.80**	40.50 ± 16.10**	3.00 ± 0.53
(G4) AP2%	05	0	0	0	0	0
(G5) Control	05	0	0	0	0	0

[1]Values are mean ± standard deviation; [2] AP= *Arthrospira (Spirulina) platensis* powder at 1% or 2% in the chow (w/w) and DMH= 1,2-dimethylhydrazine dihydrochloride (4x 40mg/kg b.wt., s.c.); [3]AC= aberrant crypts. Data were analyzed using one-way ANOVA followed by Tukey's test. *,**,*** Different from group G1, $p< 0.05$, $p= 0.008$ and $p <0.01$, respectively.