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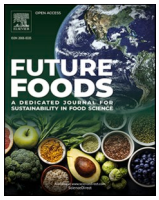

Dietary inclusion of cyanobacteria *Arthrospira (Spirulina platensis)* spp. decreases the aggravating effect of hemin from red meat in a rat colorectal carcinogenesis model

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ABSTRACT

Colorectal cancer (CRC) risk, associated with a high intake of red/processed meat, may be related to hemin. A potential chemopreventive agent, *Arthrospira* (*Spirulina platensis)* spp. (AP*)* is a cyanobacteria employed as a functional food with the potential to reduce malnutrition in developing countries. Thus, we assessed the effects of dietary AP against hemin-promoting effects in a dimethylhydrazine (DMH)-induced colorectal carcinogenesis model. Male Sprague-Dawley rats were allocated into five groups. G1–G4 received four subcutaneous DMH injections (40 mg/kg body weight, twice a week for 2 weeks) and G5 received DMH vehicle. At weeks 4 to 16, groups were fed a balanced diet (BD, G1 and G5), BD containing 2 % AP (20 g powder/kg chow, G2), BD containing hemin (0.32 g/kg chow, G3) or BD containing hemin+2 % AP (G4), respectively. Hemin group (G3) increased aberrant crypt foci (ACF) development, malondialdehyde (MDA), and *Nox-1* levels and reduced glutathione (GSH) and GSH-Px levels in colonic mucosa when compared to DMH group (G1). In contrast, the dietary hemin+2 % AP regimen (G4), reduced both preneoplastic lesion development and MDA levels while increased GSH levels, compared to the DMH+hemin group (G3). Dietary AP may reduce hemin-promoting effects by modulating oxidative stress and ACF lesions.

1. Introduction

Colorectal Cancer (CRC) is the third most common neoplasia and the second-leading cause of human death worldwide (Siegel et al., 2020). According to the World Health Organization (WHO) GLOBOCAN for 2020, 1.9 million new cases and 935,000 deaths by CCR for both sexes and all ages (Siegel et al., 2020). In Brazil, colon cancer or rectal cancer events may increase by 79 % and 66 % in the next 24 years (from 2016 up to 2040), respectively (Martin et al., 2021). Dietary habits such as a Western diet based on a high intake of saturated fats, refined sugars, and red and processed meat are linked to sporadic CRC disease, which is the most prevalent among CRC total cases (Johnson et al., 2013; Keum and Giovannucci, 2019; Sung et al., 2021).

Red meat contains essential proteins and micronutrients such as B vitamins, iron (both free iron and heme iron), and zinc (Giromini and Givens, 2022). Despite its reported beneficial value on human nutrition, several prospective cohort studies have shown a strong association between CRC risk and high intake of red meat, especially processed meat (Norat et al., 2005; Aune et al., 2013; Berstein et al., 2015; Carr et al., 2017). Several factors could explain the positive association between the CRC risk and high red/processed meat consumption. One of these factors is the heme iron, which consists of an iron atom at the center of a heterocyclic ring called porphyrin (Cross et al., 2003; Balder et al., 2006; Bastide et al., 2011). Unbound heme is a potential carcinogen in colonic mucosa, primarily because of induction of lipoperoxidation byproducts, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), both proposed to be genotoxic and cytotoxic on colonocytes as demonstrated in both *in vivo* and *in vitro* assays (Bastide et al., 2011, 2015).

Arthrospira spp. *(Spirulina)* (AP) refers to a few group photosynthetic prokaryotes-known as *cyanobacteria,* including *A.platensis, A.maxima,*

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and *A.fusiformis*, which are no longer considered under the *Spirulina* genera given morphological and molecular criteria (Scheldeman et al., 1999; Komárek et al., 2014). *Arthrospira* spp. *(Spirulina)* is ubiquitously present in alkaline lakes and is employed for nutritional purposes due to its composition, mostly of protein: 64–74 %, whether is dry or fresh matter (Grosshagauer et al., 2020; Bortolini et al., 2022). "*Spirulina platensis*" and commercial-based products have been certified as "Generally Recognized as Safe" (FDA, 2003), indicating that AP dried extract may be safe for animal and human feeding.

This cyanobacteria is regarded as a potential anti-carcinogenic resource given its blocking and suppressing properties against tumorigenesis (Yogianti et al., 2014; Ouhtit et al., 2014; Álvarez-González et al., 2015; Smieszek et al., 2017; Marková et al., 2020; Amadeu et al., 2023). AP contains C-phycocyanin (C-PC), a blue-pigmented water-soluble photosynthetic protein, presents cyclooxygenase 2 (COX-2) suppressive activity (Reddy et al., 2000; Kefayat et al., 2019). Dietary 1 % AP reduced breast tumor incidence and their biomarkers for estrogen receptor-alpha (ER-α) and Ki-67 (i.e., a cell proliferation marker) expression in a mammary carcinogenesis model induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female Sprague-Dawley (SD) rats (Ouhtit et al., 2014). Moreover, in a mouse hepatocarcinogenesis model induced by diethylnitrosamine/carbon tetrachloride (DEN/CCl_4) administrations, AP extract at oral doses of 250 and 500 mg/kg reduced the number and size of hepatic nodules, as well as decreased serum levels of alpha-fetoprotein (AFP) (Mahmoud et al., 2021). In two colon carcinogenesis models induced by 1,2 dimethylhydrazine (DMH) or its metabolite, azoxymethane (AOM), dietary AP (1 % and 2 % in chow, w/w) and *Arthrospira maxima* (Spirulina) (100, 400, and 800 mg/kg), administered during the initiation phase of CRC, reduced the development of preneoplastic aberrant crypt foci (ACF) lesions in rats and mice, respectively (Álvarez-González et al., 2015; Amadeu et al., 2023). However, the modulation of dietary AP on the promotion/progression stages of murine colorectal carcinogenesis has not yet been clarified.

Drawing upon previous research indicating the carcinogenic effects of hemin from red meat in colonic mucosa, as well as the protective properties of this cyanobacteria against the CRC in rodent models, this study investigated the potential of dietary AP to mitigate colorectal carcinogenesis induced by DMH and promoted by hemin. Using DMH-initiated rats, our evaluation focused on preneoplastic ACF and antioxidant levels as endpoints.

2. Materials and methods

2.1. DMH, hemin, and Arthrospira (Spirulina platensis) spp. (AP)

1,2-dimethylhydrazine dihydrochloride (DMH PubChem CID: 1322) and hemin (PubChem CID: 455,658) were purchased from Sigma–Aldrich (St Louis, USA). DMH was dissolved in 1 mM ethylenediaminetetraacetic acid (EDTA) immediately before use. *Arthrospira (Spirulina platensis)* spp. (AP) powder was kindly gifted by Tamanduá® Farm (Paraíba, Brazil) with the following characteristics per gram: proteins (66.7 %), total carbohydrates (20.0 %), total lipids (9.33 %), and fiber (6.0 %) expressed on dry weight (Amadeu et al., 2023). AP powder was incorporated into a cereal-based commercial diet (Nuvilab-CR1, Nuvital - Brazil), reaching the final concentration of 2.0 % (20 g of AP powder/kg chow) while hemin was incorporated at 0.32 g/kg chow.

2.2. Animal housing and study design

Six-week-old male Sprague-Dawley (SD) rats were obtained from the Multidisciplinary Center for Biological Research (CEMIB) at the State University of Campinas (UNICAMP, Campinas, Brazil). Animals were maintained under standard housing conditions of room temperature (22 ± 2 °C), humidity (55 ± 10 %), and 12 h light/12 h dark cycle. After a 2-week acclimation period, fifty-six animals were allocated into four groups (Fig. 1). At weeks 1-2, groups G1-G4 ($N = 14$ each) received a balanced diet (BD) and four subcutaneous injections (s.c.) of DMH [4 × 40 mg/kg body weight (b.wt.), twice a week] for initiation of colorectal carcinogenesis (Caetano et al., 2020; Amadeu et al., 2023) while G5 ($N = 05$) received EDTA solution (Ethylenediaminetetraacetic acid, 37 mg/1000 ml, 4 × 5 ml/kg b.wt., s.c.). Between weeks 3 and 16, groups were fed a BD (G1 and G5), BD containing 2 % AP (20 g powder/kg chow, G2), BD containing hemin (0.32 g/kg chow, G3) or BD containing hemin + 2 % AP (G4), respectively. At the end of week 16, all animals were euthanized under xylazine and ketamine anesthesia (10 mg/kg and 80 mg/kg body weight, respectively), and the large intestine of each animal was collected. Body weight and food consumption were recorded twice a week. All animals received food and drinking water ad libitum throughout the experimental period.

Dietary hemin dosage (0.32 g/kg chow) was selected based on previous studies, representing a diet containing beef at 60 % (Pierre et al., 2003; de Moura et al., 2019). AP at 2.0 % in the diet was chosen due to its safety and demonstrated to reduce CRC when administered before and during DMH injections in male SD rats (Amadeu et al., 2023). As the average recommended intake for AP (tablets/capsules/powder) is between 3 and 10 g/day (~50–166 mg/kg b.wt./day, considering 60 kg adults) (Gogna et al., 2023), we calculated the Human Equivalent Dose (HED) to estimate human consumption of AP applicable to our study, as it follows: HED (mg/kg/day) = Animal dose (mg/kg/day) x (Rat Km /Human KM), where Km = Species-related constant based on body weight and surface area (Reagan-Shaw et al., 2008). Thus, HED = 1100 × 6/37, resulting in 178.4 mg/kg/day, where 1100 mg/kg b.wt. was based on animal food consumption (Table 1).

The animals were handled according to the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and ARRIVE guidelines (Kilkenny et al., 2010). All experimental procedures were approved by the Botucatu Institute of Biosciences's Ethics Review Board (CEUA-Protocol n° 1128).

2.3. Colon processing and histopathological analyses: ACF and colon tumors

Large intestine specimens were removed, opened longitudinally, pinned flat, and washed with 0.9 % NaCl for macroscopic analyses. Then, intestine samples and macroscopic lesions were fixed in 10 % phosphate-buffered formalin for 24 h and kept in ethanol 70 % until further analysis. Fixed colon samples were divided into three segments (proximal, middle, and distal) and then stained with 2.0 % methylene blue dissolved in phosphate-buffered salt solution (PBS) for ACF and aberrant crypts (AC) analyses (Caetano et al., 2020; Amadeu et al., 2023). The total number of ACF, AC, ACF multiplicity (AC/ACF ratio), and ACF size (2–4 AC or > 4 AC) were determined under light microscopy at 40x magnification. ACF was counted on middle and distal segments since these putative preneoplastic lesions are mainly located in these anatomical segments in male rats (Rodrigues et al., 2002; Caetano et al., 2020). The ACF were identified topographically according to Bird's morphological criteria: increased size, thickened epithelial cell lining, increased pericryptal space, and irregular lumens (Bird, 1987; Bird and Good, 2000). Each colon sample was examined by two observers in a double-blinded manner. After ACF analysis, colonic segments and tumors were embedded in paraffin for histological evaluation which was carried after hematoxylin-eosin (H&E) staining. Colonic tumors were diagnosed under H&E staining, according to criteria established by Nolte et al. (2016). The incidence and total number of these neoplasms were calculated for all groups.

2.4. RNA extraction, quantitative and qualitative analyses, and qRT-PCR

Distal colon scrapped samples were collected and stored at −80 °C for gene analysis. Total RNA was isolated from these samples ($n = 5$ for only G1, G3 and G4) using the RNeasy Mini kit (Qiagen, Hilden,

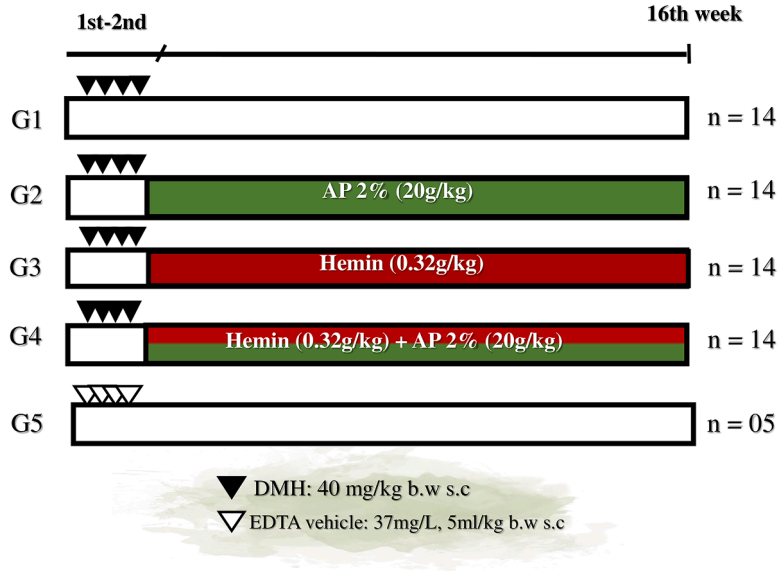

Fig. 1. Experimental design (for details see Material and Methods section). DMH = 1,2-dimethylhydrazine hydrochloride (4 × 40 mg/kg body weight by subcutaneous injections); AP = Arthrospira (Spirulina platensis) spp. added to a balanced diet at 2 % (20 g/kg chow) and hemin = 0.32 g/kg chow added to a balanced diet both for 14 weeks).

Table 1
. General findings evaluated after introduction (week 3) of dietary cyanobacteria *Arthrospira (Spirulina platensis)* spp. and hemin from red meat in the different groups.[1]

Groups/Treatment[2]	DMH (G1, $N = 14$)	DMH+2 %AP (G2, $N = 14$)	DMH+Hemin (G3, $N = 14$)	DMH+Hemin+2 %AP (G4, $N = 13$)	EDTA (G5, $N = 05$)
Initial body weight (g)	296.21 ± 28.14	298.64 ± 19.89	296.00 ± 14.61	295.79 ± 23.61	295.80 ± 10.27
Final body weight (g)	442.64 ± 36.85	439.28 ± 31.77	453.93 ± 26.22	441.57 ± 24.19	438.60 ± 14.13
Body weight gain (g)	146.43 ± 37.60	140.64 ± 30.58	157.93 ± 17.49	145.78 ± 14.92	145.70 ± 17.38
Food consumption (g/rat/day)	24.84 ± 1.34	24.42 ± 1.82	25.87 ± 2.24***	23.10 ± 1.60*,**	24.25 ± 1.90
AP consumption (mg/kg b.wt/day)	0	1108.19 ± 60.01	0	1093.81 ± 84.73	0
Absolute liver weight (g)	13.89 ± 1.58	13.52 ± 1.25	14.15 ± 1.24	14.39 ± 0.77	13.9 ± 1.3
Relative liver weight (%)	3.13 ± 0.19	3.08 ± 0.25	3.12 ± 0.23	3.27 ± 0.22	3.2 ± 0.2

[1] Values are presented as mean ± standard deviation

[2] DMH = 1,2 dimetylhydrazine [4 × 40 mg/Kg body weight (b.wt.) subcutaneously (s.c.)]; ETDA (DMH vehicle)= Ethylenediaminetetraacetic acid, 37 mg/1000 ml, 4 × 5 ml/kg b.wt., s.c.); AP = *Arthrospira (Spirulina platensis)* spp. added to a balanced diet (BD) at 2 % (20 g/kg chow) and hemin = 0.32 g/kg chow added to a BD for 14 weeks; N = number of animals per group. ANOVA followed by post hoc Turkey's test, considering $p < 0.05$. **Food consumption:** *G4 vs. G3 (< 0.001), **G4 vs. G5 ($p = 0.034$), ***G3 vs. G1 ($p = 0.017$).

Germany). The quantity (Optic Density – OD, $Abs_{260/280}$) and quality (RNA integrity number – RIN) of RNA were evaluated using NanoVue Plus (GE HealthCare, Little Chalfont, UK) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), respectively. cDNA was synthesized using SuperScript® VILO cDNA Synthesis Kit and Master Mix (Thermo Scientific, USA). For real-time reverse transcription-polymerase chain reaction (qRT-PCR), a total of 200 µl reaction mixture with 100 µl of cDNA template (422 ng mRNA) was added to 100 µL of TaqMan® Fast Advanced Master Mix (Life Technologies, USA). The cycling protocol included heat activation (50 °C, 1 min), and denaturation (95 °C, 10 min), followed by 40 cycles (95 °C, 15 s, and 60 °C, 1 min). Fluorescence detection was executed on a QuantStudio 12 K Flex Real-Time PCR System (Life Technologies, USA). The relative expression of target genes was analyzed by the comparative Ct method (ExpressionSuite™ software; Life Technologies USA). This study was conducted according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR experiments) guidelines (Bustin et al., 2009). Commercially available Thermo Fisher Scientific primers of *NADPH oxidase 1* (*Nox-1*: Rn00586652_m1) and *8-oxoguanine DNA glycosylase-1* (*Ogg1*:Rn00578409_m1) target genes were used for comparison among groups while *Actin Beta* (*Actb*:Rn00667869_m1) and *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*:Rn01775763_g1) housekeeping genes were used for sample normalization.

2.5. Malonaldehyde (MDA) and glutathione analyses

Colon samples from DMH-initiated were homogenized in 50 mM phosphate buffer (pH 7.4) using a motor–driven Teflon glass Potter Elvehjem (100 × *g*/min) and centrifuged (12,000 × *g*, 4 °C, 15 min). The supernatant was used for malondialdehyde (MDA) (Young and Trimble, 1991), glutathione reduced (GSH) and oxidized (GSSG) (Sedlak and Lindsay, 1968), glutathione peroxidase (GSH-Px) activity (Nakamura et al., 1974). For lipid peroxidation analysis, malondialdehyde (MDA) was measured by high-performance liquid chromatography with fluorometric detection (HPLC, system LC10A, Shimadzu, Japan). Colon tissue homogenate (total protein) was incubated with thiobarbituric acid (TBA 42 mmol/L) and 1 % ortho-phosphoric acid (1.22 mol/L) in a water bath at 100 °C for 60 min to form the fluorescent adduct TBA-MDA. Deproteinization was performed with 2 mmol/L sodium hydroxide and methanol (NaOH: MetOH; 1:1) submitted to centrifugation (2000 × *g*, 5 min). After 50 µL of the supernatant was filtered and injected into an octadecylsilic column (ODS RP18 column VC-250 mm × 4.6 mm). The isocratic mobile phase comprised of the phosphate buffer (10 mmol/L, pH:6.8) and HPLC grade methanol (60:40 v/v), which was run through the system at a flow rate of 0.8 mL/min. The calibration curve was obtained with the preparation of 1,1,3,3 tetra-ethoxy propane solutions. Fluorimetric detection was performed at λ_{exc} 532 nm and λ_{emm}

553 nm, using a detector model RF-10AXL (Shimadzu, Tokyo, Japan). The results were expressed as nanomol per milligram protein (nmol/mg protein). For glutathione analyses, colon homogenates were added to ice-cold 5 % trichloroacetic acid and centrifuged at 7000 x g for 15 min at 4 °C, and the supernatants were used to quantify glutathione content in a spectrophotometer at 412 nm (Biochrom, Libra 522, Berlin, Germany). The results were expressed as reduced GSH and GSSG nmol/mg protein and compared with a standard curve using known concentrations of cysteine. GSH-Px determination followed the oxidation of 0.16 mM NADPH to NADP+ in the presence of glutathione reductase (GR), which catalyzes the reduction of GSSG to produce GSH. Spectrophotometric determinations were performed in a Pharmacia Biotech spectrophotometer with temperature-controlled cuvette chamber (UV/visible Ultrospec 5000 with Swift II applications software for computer system control, Cambridge, UK).

2.6. Statistical analyses

Data were presented as mean ± standard deviation (SD) or median and min-max ranges. Differences among groups were evaluated by One-Way Analysis of Variance (ANOVA) for parametric data or Kruskal-Wallis for nonparametric data, followed by *post hoc* Tukey's and Dunn's tests, respectively. The incidence of colonic tumors (%) was calculated using Fisher Exact's test. Gene expression was analyzed using the Expression Suite Software v1.0.3 (Life Technologies, USA). Statistical significance was established as $p < 0.05$. GraphPad Prism software 9.0 (GraphPad, USA) was used for data analysis.

3. Results

3.1. General findings

The body weight of animals increased gradually during the experimental period in all groups. After 14 weeks of dietary interventions with AP and hemin, final body weight, liver (absolute and relative) weights, as well as body weight gain were similar among groups (Table 1). The hemin-fed group (G3) consumed more chow (~1.5–1.8 g) when compared to the hemin + 2 % AP group (G4) ($p < 0.05$). One animal from the 2 % AP and hemin-fed group (G4) died before the necropsy procedure, hence it was not considered for data analysis given advanced postmortem changes.

3.2. Dietary 2 % AP effects on ACF development

All animals initiated with DMH subcutaneous injections developed preneoplastic lesions in colonic mucosa (Fig. 2). Findings on ACF development including a mean number of ACF and AC per group, AC/ACF ratio (multiplicity), and ACF size (2–4 AC or 4 > AC) as well as the total number and incidence of tumors are presented in Table 2. After 14 weeks, 2 % AP feeding *per se* (G2), administered during post-initiation of colorectal carcinogenesis, significantly reduced ACF multiplicity (AC/ACF ratio) ($p < 0.001$) with a significantly higher number of small ACF (2–4 AC) ($p < 0.05$) in comparison to the DMH-initiated group (G1). No ACF was detected in the mucosa in the control group (EDTA)

Compared with the DMH-initiated group (G1), the hemin fed group (G3) showed a significant increase in the total number of AC ($p < 0.001$) and ACF multiplicity (AC/ACF ratio) ($p < 0.002$) (Table 2). In addition, a significant increase in ACF size (2–4 AC and > 4 AC, $p < 0.035$; < 0.001, respectively) was observed in the hemin fed group (G3) when compared to the DMH-initiated group (G1). On the other hand, hemin and 2 % AP intervention group (G4) significantly reduced the mean number of AC ($p < 0.001$), ACF ($p = 0.002$), AC/ACF ratio ($p < 0.001$) and ACF size (2–4 AC and > 4 AC, $p < 0.026$; < 0.001, respectively) in comparison to the only hemin fed group (G3). Thus, these results confirmed the hemin-promoting effect while a protective action of AP against hemin intervention (G4) on chemically-induced colonic putative preneoplastic lesions was observed.

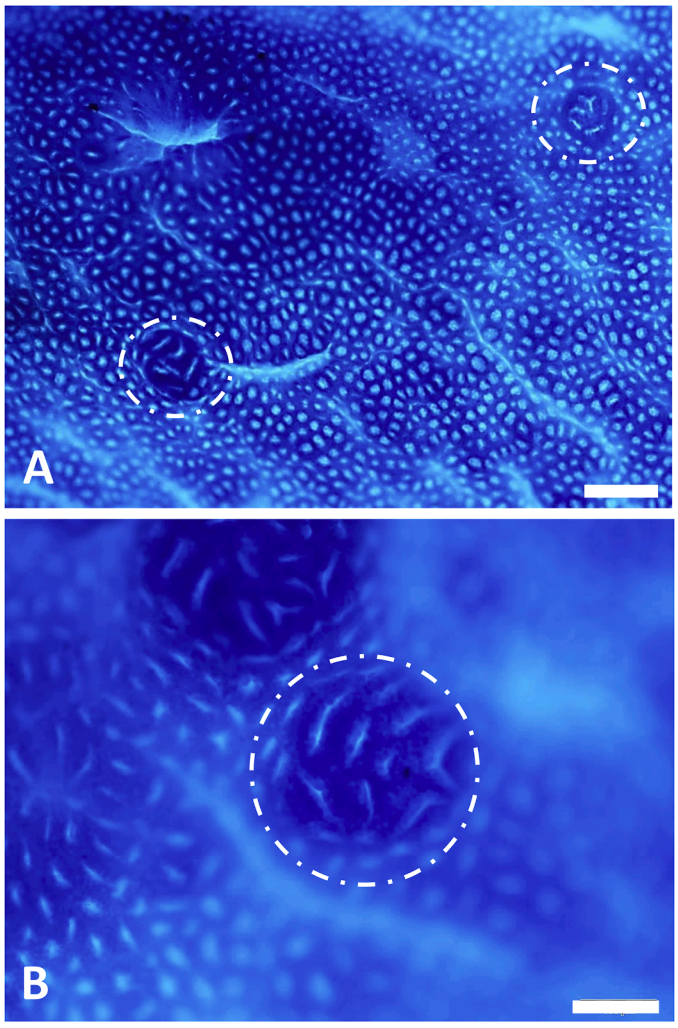

Fig. 2. Representative photomicrographs of methylene blue-stained aberrant crypt foci (ACF, dotted lines) containing > 4 aberrant crypts (AC) detected in colonic mucosa at week 16. A) Bar = 1 mm; B) Bar = 100 μm.

At the end of the 16 weeks, a small number of colorectal tumors were detected in the different DMH-initiated groups (Fig. 3 and Table 2). The total number of tumors and tumor incidence were higher, althought not significant, ($p = 0.054$) in the hemin-fed group (G3) when compared to the DMH-initiated group (G1). Neither 2 % AP *per se* (G2) nor 2 % AP associated with the hemin intervention group (G3) seemed to interfere with the early onset of tumor formation induced by DMH in our medium-term rat bioassay (Table 2).

3.3. Dietary 2 % AP intervention on antioxidant enzymes and gene expression

Dietary hemin intervention during 14 weeks induced an evident oxidative stress condition in the large intestine mucosa in DMH-initiated animals (G3), characterized by a significant increase in MDA levels ($p < 0.0001$) and a significant reduction in glutathione enzymes [GSH ($p < 0.0001$) level and GSH-Px activity ($p = 0.007$)] when compared to the DMH-initiated group (G1) (Fig. 4A–D). On the other hand, hemin and 2 % AP group (G4) significantly reduced colonic MDA levels ($p < 0.0001$) associated with a significant increase in GSH levels ($p < 0.001$) when compared to the only hemin-fed group (G3). As hemin intervention induced oxidative stress in the colonic mucosa (G3) and it was partially attenuated by 2 % AP (G4), we set out to evaluate *Ogg1* and *Nox1*

Table 2
. Effects of dietary cyanobacteria *Arthrospira (Spirulina platensis)* spp. on preneoplastic and neoplastic colonic lesions promoted by hemin from red meat.

Groups/Treatment[1]	Number of rats	ACF size		Number of AC and ACF[3]			Colon Tumor
		2–4 AC	> 4 AC	Total AC	Total ACF	AC/ACF	Total Number
(G1) DMH[2]	14	53.8 ± 11.0	59.8 ± 19.4	625.6 ± 183.0	113.1 ± 26.9	5.5 ± 0.5	5 (29 %)[4]
(G2) DMH + 2 % AP	14	73.1 ± 22.0*	52.2 ± 22.5	604.0 ± 225.3	129.7 ± 39.1	4.6 ± 0.6*	4 (29 %)
(G3) DMH + Hemin	14	63.2 ± 21.6**	79.6 ± 23.6*	838.6 ± 249.9*	144.1 ± 42.2*	5.9 ± 0.4**	13 (64 %)
(G4) DMH + Hemin + 2 % AP	13	42.9 ± 15.6***	40.5 ± 15.7**	420.3 ± 140.1**,***	83.4 ± 26.5**	5.0 ± 0.6***	3 (23 %)
(G5) EDTA	05	0	0	0	0	0	0

[1] Values are presented as mean ± standard deviation.
[2] DMH = 1,2 dimethylhydrazine [4 × 40 mg/Kg body weight (b.wt.) subcutaneously administered (s.c.)]; ETDA (DMH vehicle)= Ethylenediaminetetraacetic acid, 37 mg/1000 ml, 4 × 5 ml/kg b.wt., s.c.); AP = *Arthrospira (Spirulina platensis)* spp. added to a balanced diet at 2 % (20 g powder/kg chow) and hemin = 0.32 g/kg chow added to a balanced diet, both consumed for 14 weeks;[3] AC= aberrant crypt, ACF= aberrant crypt foci.
[4] Tumor incidence (% of rat bearing tumors). One-Way ANOVA followed by Tukey *post hoc.* $0.026 < p < 0.001$ for DMH-initiated groups. **2–4 AC:** *G2 vs. G1 ($p = 0.0355$), **G2 vs. G4 ($p = 0.0005$), ***G4 vs. G3 ($p = 0.0267$). **> 4 AC:** *G3 vs. G1 ($p = 0.006$), **G4 vs. G3 ($p < 0.0001$). **Total AC:** *G3 vs. G1 ($p = 0.0394$), **G4 vs. G3 ($p = 0.0224$), ***G4 vs. G2 ($p < 0.0001$). **Total ACF:** *G3 vs. G1 ($p = 0.0151$), **G4 vs. G3 ($p = 0.0002$). **AC/ACF:** *G2 vs. G1 ($p = 0.0003$) **G3 vs. G1 ($p < 0.0001$), ***G4 vs. G3 ($p = 0.001$).

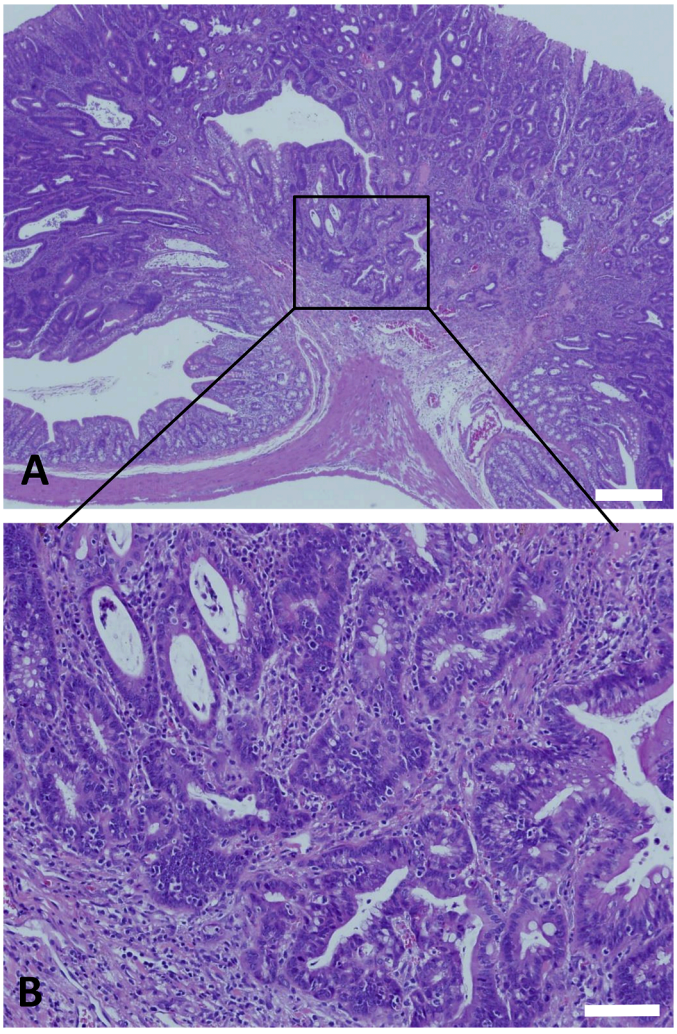

Fig. 3. . Representative photomicrograph of one colonic tumor hematoxylin-eosin-stained section detected at week 16. (A) Bar = 100 μm and (B) Bar = 50 μm.

expression in these two groups compared with DMH-initiated group (G1), both genes associated with protective or promoting effects on oxidative stress, respectively. qRT-PCR analysis showed that hemin fed (G3) induced a significant increase in *Nox1* expression ($p = 0.013$) but did not alter *Ogg1* expression in the colonic mucosa when compared to the DMH-initiated group (G1) (Fig. 4E, F). In addition, hemin and 2 % AP in the group (G4), despite reducing *Nox1* expression, no difference were observed ($p = 0.068$) in colonic mucosa when compared to the only hemin fed group (G3).

4. Discussion

This preclinical study investigated the potential preventive effects of *Arthrospira (Spirulina platensis)* spp. (AP) incorporated into a balanced diet, during the post-initiation phase of colorectal carcinogenesis, against the promoting effects of hemin derived from red meat in a medium-term DMH-induced colorectal rat model, using preneoplastic lesions and antioxidant enzymes levels, as endpoints. To better evaluate the protective effects of AP feeding on hemin-promoting effects on tumor development, a long-term study of the progression of colon carcinogenesis is necessary using a similar DMH administration regimen (Sarmiento-Machado et al., 2024).

Although this cyanobacteria is listed by the US Food and Drug Administration under the category Generally Recognized as Safe (GRAS) (FDA, 2003) and being widely used as an ingredient/supplement, its possible effects on chronic illnesses, such as carcinogenesis, needs to be deeply understood. Furthermore, this animal study drives the knowledge of the broader implications of dietary AP, including potential interactions that could modify its intended functionality or nutritional profile. The current assessment offers insights into the possible interactions between AP and red meat hemin. Understanding these mechanisms is essential for using AP during the initial stages of colorectal carcinogenesis and for predicting the health-promoting benefits linked to its consumption. To our knowledge, this is the first study to assess dietary AP effects on the promotion stage of colorectal carcinogenesis associated with hemin fed intervention.

Our findings indicate that neither dietary hemin (0.32 mg/kg chow) and 2 % AP (20 g powder/kg chow) nor its association (hemin + 2 % AP) affected both body weight and liver absolute and relative weights parameters as observed by others (Pierre et al., 2008; Yang et al., 2011; Ismail et al., 2009; Bigagli et al., 2017; Khafaga et al., 2018; de Moura et al., 2019). Thus, the 2 % AP dosage (178.4 mg/kg b.wt./day) used in this medium-term study did not cause any unintended deleterious nutritional changes, suggesting that our AP dosage is safe for human dose extrapolation, considering the higher human dose as AP supplement is approximately 166 mg/kg b.wt./day (Gogna et al., 2023).

Red meat is a nutritional source of essential amino acids, fatty acids, vitamins, minerals, and bioavailable iron (Pereira and Vicente, 2013; Giromini and Givens, 2022). In addition, red meat curing, smoking, or cooking processes produce pro-carcinogenic agents such as N-nitroso compounds (NOC), heterocyclic amines (HCA), and polycyclic aromatic hydrocarbons (PAH) and, it generally contains eventual incorporation of food additives and preservatives (e.g. nitrates, salt, and others) (Turesky

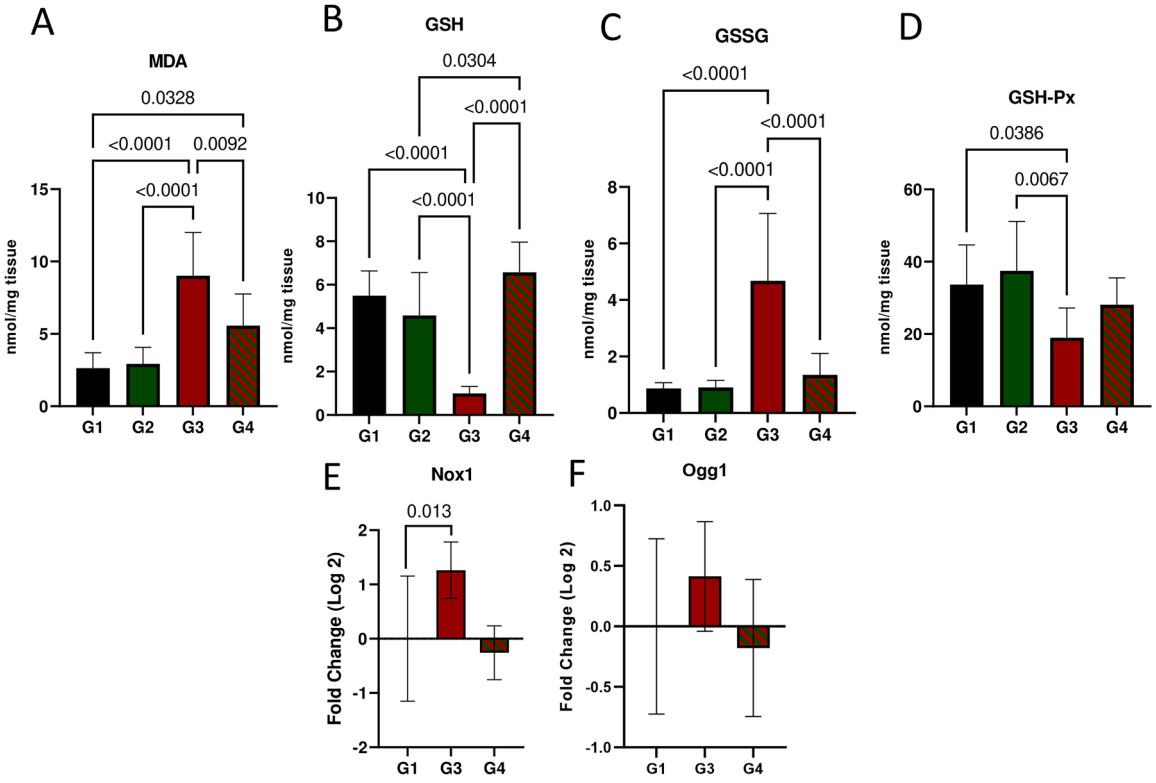

Fig. 4. (A–D) Malondialdehyde (MDA), reduced gluthatione (GSH), oxided gluthatione (GSSG) levels and gluthatione peroxidase (GSH-Px) activity in colonic mucosa. Data are mean and standard deviation (SD) ($N = 10$ for each group). E-F) Relative quantitation of *Nox-1* and *Ogg1* genes differently expressed in colonic mucosa tumors G1, G3, and G4 groups ($N = 5$ samples for each group). Data are expressed as median ± min-max (Fold changes for 1.5). G1= DMH group (black), G2= DMH + 2 % AP fed group (green), G3= DMH + hemin fed group (red) and G4= DMH + hemin 2 % AP fed group (green and diagonal red lines). DMH = 1,2 dimetylhydrazine (4 × 40 mg/kg body weight by subcutaneous injections); AP = *Arthrospira (Spirulina platensis)* spp. added to a balanced diet at 2 % (20 g/kg chow) and hemin = 0.32 g/kg chow added to a balanced diet both for 14 weeks. * Diference from G1 group, $p = 0.013$.

et al., 2018). A communication from the European Commission discussed actions to promote the algae production sector - expected to grow 8.7 % by 2025 and to reach € 9 billion - in Europe for healthiest diets, lower greenhouse gas emissions, and water reuse (EC Document number 52020DC0381, 2020). Thus, microalgae-rich-protein diets associated with less red and processed meat consumption could reduce the risk of CRC development.

ACF is regarded as putative preneoplastic lesions in rodent colon carcinogenesis assays induced by alkylant agents such as DMH and AOM (Li et al., 2019; Lee et al., 2019; Kobaek-Larsen et al., 2019), used as biomarker for cancer prevention studies (Wargovich et al., 2000; Corpet and Taché, 2002; Clapper et al., 2020). Nevertheless, the development of these preneoplastic lesions is a dynamic event, considering that ACF may differ in growth and number at different time points after the initiation of colorectal carcinogenesis (Magnuson et al., 1993; Baijal et al., 1998). Despite hyperplastic ACF may progress into dysplastic ACF (erroneously considered as microdenomas or intraepithelial neoplasia by some authors), these last lesions are regarded as precursors of adenomatous lesions with epithelial β-catenin altered expression (cytoplasm-nucleus accumulations) similarly as in colonic adenomas and adenocarcinomas in rodents (Takahashi et al., 2000; Xiao et al., 2008) and humans (Hardy et al., 2002; Mi et al., 2009).

Hemin is included in so-called hemoproteins, involved in oxygen supply (hemoprotein, myoglobin) and electron transfer reactions (cytochromes) (Ryter and Tyrell, 2000; Correia et al., 2011). Our findings highlighted that dietary hemin induced the development/growth of preneoplastic lesions in DMH-initiated rats, consistent with previous studies (Pierre et al., 2008; Santarelli et al., 2010; de Moura et al., 2019). Noteworthy, diets containing hemin increased mucin-depleted foci (MDF)/dysplastic lesions compared to HCA and NOC associated with red meat in male F344 rats (Bastide et al., 2015). These reported effects indicate a putative hemin-promoting activity on ACF development as also observed in our study. These and other findings underscore the hazardous effects of non-degraded hemin or its byproducts by its toxic/carcinogenic effects on colonic enterocytes (Ijssennagger et al., 2012, 2013). Some studies suggest that the induction of a hyperproliferative response in epithelial cells, achieved by downregulating proliferation inhibitors, could be a potential mechanism underlying the hemin effects. Additionally, hemin has shown to upregulate genes associated with damaged surface cells in mouse colonic mucosa (Ijssennagger et al., 2012).

The meat heme iron concentration is approximately 1.57 mg/100 g meat (Archundia-Herrera et al., 2024). On the assumption that the total estimated meat (poultry, pork, and beef) intake was 340 g/day in 2021 in the US (FAO US, 2023), an average intake of 5.34 mg heme consumed on a daily basis is plausible. In our study, the mean food intake was 24 g chow/rat/day which represents a mean hemin consumption of approximately 7.68 mg hemin/rat/day. In general, the dietary concentrations of hemin used in rodent studies are elevated when compared the amount found in meat or in a mixed meal (Turner and Lloyd, 2017). Thus, effective doses of hemin for humans and rodents are much different when considering body size, weight and metabolic rates (Reagan-Shaw et al., 2008).

Dietary 2 % AP *per se* reduced ACF multiplicity (AC/ACF ratio), while 2 % AP plus hemin intervention group reduced the mean number of AC and ACF and ACF multiplicity compared to corresponding counterparts DMH-initiated and hemin-fed groups, respectively. Thus, AP intake reduced the early post-initiation step of colorectal carcinogenesis, mainly when associated with hemin intervention. *Arthrospira (Spirulina platensis)* spp. has shown antioxidant, antiangiogenic, and anti-

inflammatory properties, as investigated in various *in vitro* and *in vivo* assays (Yogianti et al., 2014; Smieszek et al., 2017; Khafaga et al., 2018; Marková et al., 2020; Mahmoud et al., 2021). This antioxidant activity might be due to its compounds, such as chlorophyll and C-PC content, both are free radical scavengers (De Vogel et al., 2004; Papalia et al., 2019; Bannu et al., 2019). Furthermore, this microalga exerted a suppressive effect on ROS production caused by UVB in the skin 8-oxoG adduct formation in *Ogg*1 KO mice (Yogianti et al., 2014). C-PC, derived from AP, impaired colonic COX-2 activity and subsequent PGE_2 production in male SD rats (Saini et al., 2012; Saini and Sanyal., 2014). Therefore, these previous findings could explain AP preventive effects on the development of colonic preneoplastic lesions in our study, as observed by the suppressive effect of *Arthrospira maxima (Spirulina)* on ACF development in an AOM-induced model in male Swiss mice (Álvarez-González et al., 2015).

The increased production of ROS mediated by NADPH oxidase (NOX1) overexpression on colon enterocytes of animals treated with hemin, compared to the DMH-initiated group (G1), was corroborated by the enhanced MDA levels. NOX1 gene is predominantly expressed in colon tissue and belongs to a group of enzymes known as NADPH oxidases, which are involved in electron transfer and ROS-generating enzymes (Bedard and Krause, 2007; Chen et al., 2023). NOX-1 upregulation has been associated with ROS-mediated cytotoxic effects in colonic enterocytes (Chen et al., 2023) while it is overexpressed in colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP), DMH in male rats (Wang et al., 2011; Sarmiento--Machado et al., 2024) or in CRC patients (Wang et al., 2011).

In this context, we identified hemin as a pro-oxidant compound, which amplifies ROS production through NADPH oxidase overexpression in colon cells. ROS such as superoxide anion ($\bullet O_2^-$) and hydroxyl radicals ($\bullet OH$) trigger the initiation of lipid peroxidation resulting in MDA, which was used here as a marker for oxidative damage in tissues and cells. They are produced from polyunsaturated fatty acids (PUFAs), such as those from cell membranes, producing lipid peroxides via oxidation processes and quantifiable through the measurement of TBARS reactive substances. Lipids peroxides are unstable and break down into various complicated chemicals, some of which are reactive carbonyl compounds such as MDA (Rochetti, 2024). In fact, the impaired effects of hemin are associated with its catalytic effect on lipid peroxidation byproducts, namely MDA and 4-hydroxynonenal (Bastide et al., 2011; 2015). Other studies have reported that hemin, as an iron-containing porphyrin compound, triggers ROS pathways, exerting a fatal impact on carcinogenic effects (Ryter and Tyrrell, 2000; Bastide et al., 2011; 2015). Moreover, hemin has shown to act as a catalyst of NOC and induce the formation of cytotoxic and genotoxic aldehydes by lipid peroxidation, inducing oxidative stress and DNA damage in colonocytes (Bastide et al., 2011; 2015).

In addition to the increase in MDA levels, the colonic mucosa in hemin-fed animals displayed lower GSH levels and GST-Px activity. These effects were partially mitigated by simultaneous AP supplementation. In general, GSH and GSH-Px are important non-enzymatic and enzymatic antioxidant agents, respectively, protecting cells against oxidative damage to protein, lipids, and DNA (Lu, 2013; Brigelius-Flohé and Maiorino, 2013). GSH is a major antioxidant agent that neutralizes ROS. GSH-Px catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water (Lu, 2013; Brigelius-Flohé and Maiorino, 2013). Within this system, the generated ROS may be scavenged through glutathione equivalent consumption by forming a disulfide bond - GSSG, a process-catalyzed reaction orchestrated throughout the enzyme GSH-Px. This landscape is depicted in Fig. 4, wherein animals receiving dietary AP exhibited contrasting behaviors in these biomarkers. When hemin is combined with AP feeding, oxidative stress damage is alleviated by reducing NADPH oxidase activity. Consequently, there was less consumption of GSH as a substrate and reduced generation of its disulfate form - GSSG, leading to their higher and lower levels, respectively. Notably, our results indicate that the decreased production of GSSG attributed to the protective effect of AP is supported by an increase in GSH-Px activity. This enzyme mediates the elimination of H_2O_2 into water and oxygen using reduced glutathione (GSH) as a substrate, which contributes to reducing and neutralizing lipid hydroperoxide radicals into hydroxy fatty acids (Lu, 2013; Brigelius-Flohé and Maiorino, 2013).

In the context of the repair of DNA damage caused by ROS production, it is imperative to delve into the role of OGG1. Specifically, OGG1 gene encodes the repair enzyme DNA glycosylase/AP lyase that removes oxidized base 8-oxoG-DNA, a mutagenic base lesion, from genomic DNA, showing a protective action against oxidative stress (Li et al., 2022). Yogianti et al. (2014) showed a protective effect of 10 % AP in UVB-induced skin tumor development in both Ogg1-KO and wild-type mice (WT). Although in our study, the similarity in Ogg1 gene expressions was observed between DMH, DMH + hemin-fed DMH+hemin+AP-fed groups, the oxidative stress induced in this model was in enough magnitude to trigger similar repair responses in this preneoplasia stage evaluated, therefore the AP and hemin role in modulating Ogg1 in a colon carcinogenesis context is not conclusive, considering that upregulation of *Nox-1* and downregulation of *Ogg1* has been described in colon tumors induced by our DMH regimen (Sarmiento-Machado et al., 2024).

5. Conclusions

This study highlights the potential of dietary *Arthrospira (Spirulina platensis)* spp. (AP), commonly known as Spirulina, to mitigate the promoting effects of hemin, an iron-containing porphyrin found in red and processed meats on a chemically-induced colorectal carcinogenesis model. Dietary 2.0 % AP counteracts hemin-promoting/prooxidant effects on preneoplastic ACF lesions initiated by DMH and reduced lipid peroxidation.These findings indicate a AP protective condition against hemin cytoxicity on colonic mucosa, a deleterious effect described on both *in vitro* and *in vivo* (Ishikawa et al., 2010; Seiwert et al., 2021) Altogether, our findings provide valuable insights into the potential role of dietary AP at preventing colorectal cancer (de Oliveira and Bragotto, 2022).

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Ethical statement - Studies in humans and animals

The animals were handled according to the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and ARRIVE guidelines (Kilkenny et al., 2010). All experimental procedures were approved by the Botucatu Institute of Biosciences's Ethics Review Board (CEUA-Protocol n° 1128).

CRediT authorship contribution statement

Luis Manuel Sarmiento-Machado: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Simone Oliveira Amadeu:** Methodology, Formal analysis. **Nelci Antunes de Moura:** Supervision, Methodology, Formal analysis, Conceptualization. **Luciana Azevedo:** Writing – review & editing. **Luis Fernando Barbisan:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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