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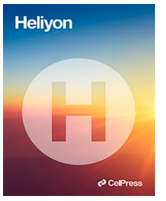

Research article

Amino acid HPLC-FLD analysis of spirulina and its protective mechanism against the combination of obesity and colitis in wistar rats

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ABSTRACT

Objective: The cafeteria diet (CD), designed as an experimental diet mimicking the obesogenic diet, may contribute to the pathogenesis of inflammatory bowel diseases (IBD). This study delves into the influence of spirulina (SP) on obesity associated with colitis in Wistar rats.

Methods: The amino acids composition of SP was analyzed using HPLC-FLD. Animals were equally separated into eight groups, each containing seven animals and treated daily for eight weeks as follows: Control diet (SD), cafeteria diet (CD) group, CD + SP (500 mg/kg) and SD + SP. Ulcerative colitis was provoked by rectal injection of acetic acid (AA) (3 % v/v, 5 ml/kg b.*w.*) on the last day of treatment in the following groups: SD + AA, SD + AA + SP, CD + AA, and CD + AA + SP.

Results: Findings revealed that UC and/or CD increased the abdominal fat, weights gain, and colons. Moreover, severe colonic alteration, perturbations in the serum metabolic parameters associated with an oxidative stress state in the colonic mucosa, defined by overproduction of reactive oxygen species (ROS) and increased levels of plasma scavenging activity (PSA). Additionally, obesity exacerbated the severity of AA-induced UC promoting inflammation marked by the overexpression of pro-inflammatory cytokines. Significantly, treatment with SP provided notable protection against inflammation severity, reduced histopathological alterations, attenuated lipid peroxidation (MDA), and enhanced antioxidant enzyme activities (CAT, SOD, and GPX) along with non-enzymatic antioxidants (GSH and SH-G).

Conclusions: Thus, the antioxidant effects and anti-inflammatory proprieties of SP could be attributed to its richness in amino acids, which could potentially mitigate inflammation severity in obese subjects suffering from ulcerative colitis. These results imply that SP hold promise as a therapeutic agent for managing of UC, particularly in individuals with concomitant obesity. Understanding SP's mechanisms of action may lead novel treatment strategies for inflammatory bowel diseases and hyperlipidemia in medical research.

Abbreviations: SP, Spirulina; CAT, Catalase; CD, Cafeteria diet; SD, Standard diet; GPx, Glutathione peroxidase; H_2O_2, Hydrogen peroxide; AA, Acetic acid; UC, Ulcerative colitis; ROS, Reactive oxygen species; GSH, Reduced glutathione; SOD, Superoxide dismutase; MDA, Malondialdehyde.

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1. Introduction

Hyperlipidemia is a multifatctorial condition influenced by genetic, endocrine, and behavioral factors [1]. The cafeteria diet is a meticulously crafted experimental dietary protocol developed to mimic western dietary habits and their pathophysiological effects in rodents [2,3]. Although, this diet composition, usually contains high-calorie and high-fat highly appetizing human nutrition, it is also appealing the rodents. This diet causes an hyperphagia, resulting in elevated energy intake due on excessive consumption of fat and carbohydrates. Compared to the high fat diet, the cafeteria diet more accurately mirrors the pathophysiological features of human obesity. This conditions is characterized by high energy intake resulting from unrestricted dietetic choices and insufficient physical activity [4]. Obesity is characterized by a chronic low-grade systemic inflammation, which has been considered and maintained as a key factor in the metabolic syndrome, particularly when accompanied by elevated levels of circulating cytokines and inflammatory markers [5–7]. Indeed, adipose tissue secretes several inflammatory mediators, including cytokines and chemokines (IL-1, TNFα, IL-6, resistin, and leptin) [8], which may exacerbate the inflammatory response and contribute to inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD). These are prolonged inflammatory disorders affecting the gastrointestinal tract [9]. Meanwhile, Crohn's pathology can alter any segment of the gastrointestinal tract, UC is confined to the colon, manifesting as superficial inflammation that is symmetrical and continuous [10]. Hyperlipidemia escalates intracellular ROS expression and initiates mitochondrial dysfunction [11,12]. Previous research has shown that corpulence exacerbates the diseases of experimental ulcerative colitis [13]. Furthermore, the progression of colonic inflammation following HFD correlates with the development of hyperhagia and a hyperlipidimic phenotype in rats [14]. Obesity constitutes a recognized risk factor for various benign and malignant gastro-intestinal disorders, including colorectal carcinoma [15]. Indeed, acetic acid-induced colonic inflammation causes numerous clinical, biochemical, and histological alterations [16,17]. Traditional therapeutic remedies prevailed for centuries until the advent synthetic medicaments in the nineteenth century [18]. Subsequently, in recent decades, significant emphasis has been placed on harnessing the potential of various microalgua due to their robust pharmacological activities, minimal side impact, and cost-effectiveness [19]. Spirulina, a spiral blue-green microalgae, and filamentous structural, has a rich culinary heritage dating back centuries [20,21]. Characterized by its richness in various chemical compounds including phytochemicals compounds, vitamins, fiber, amino acids, and minerals. Spirulina may be regarded as a treatment against various diseases, owing to its anti-inflammatory, antitumor, antioxidant, antiviral, and antibacterial properties [22]. Several research suggest that microalgae could play a beneficial role in hyperlipidemia management, as they appear to have a favorable impact on appetite regulation, insulin sensitivity, imbalance of pro/antioxidant, food absorption, and infection [23–25]. Spirulina exhibits regulatory effects on specific lipid and carbohydrate metabolisms due to its richness in phenolic elements like chlorogenic acid, syringic acid, and sinapic acid [26]. Human studies have demonstrated that this micro-alga reduces total cholesterol, triglycerides, and LDL-cholesterol levels [27]. Hence, this research was carried out to assessed the effect of spirulina on the severity of colonic inflammation linked to obesity induced by cafeteria diet and colitis induced by acetic acid (AA) in Wistar rats.

2. Materiel and methods

2.1. Reagents

Acetic acid, NaCl, Hematoxylin, Eosine, Formaldehyde, Epeniphrine, Bovine catalase, Butulated hydroxytoluene (BHT), trichloroacetic acid (TCA), 2-Thio-barbituric acid (TBA) NaOH and methanol, were from sigma chemicals Co (Germany). All other chemicals employed were of analytical grade.

2.2. Amino acids extraction

Amino acids (AA) extraction from the spirulina (SP) was carried out by mixing 0.5 g of SP powder in the flask with 4 ml of HCl (6 N). Spirulina sample was then maintained for 24 h in the oven at 105 °C to facilitate the hydrolysis of AA chains, including peptides and protein into individual amino acids. Eventually, 6 ml NaOH (6 N) was added to 2 ml of the hydrolyzed HCl sample [28]. The resulting extracts underwent centrifugation at 3500 rpm and 1 ml of supernatants were evaporated using a rotary evaporator, with 3 washes performed using distilled water to remove residual HCl [29]. The final product was then suspended in 2 ml of distilled water and filtered through paper, then through a syringe filter, and collect in vials. A standard mix of 18 amino acids was prepared, containing alanine, glutamine, leucine, arginine, lysine, isoleucine, threonine, methionine, aspartic acid, phenylalanine, glutamic acid, tyrosine, serine, histidine, glycine, valine, cystine, tryptophane and asparagine.

2.3. HPLC-FLD analysis

The composition of amino acids in the spirulina powder was studied by high-performance chromatography with fluorescence detector (HPLC-FLD) using pre-column derivatization, OPA 10 mg/ml, and the liquid chromatography Agilent 1200. The chromatographic conditions were conducted with C18 column(250 × 4.6 mm; 5 μm bead size),a 43 °C column temperature, a fluorescence detector (λ EX = 340 nm; λ EM = 440 nm), a 1ml/minflow rate, mobile phase A (ACN –MeOH –H_2O (45/45/10, v/v/v) and mobile phase B ((Na_2HPO_4 2,75 g/l pH = 6,5).

2.4. Preparation of spurilina (SP)

The spirulina flakes was obtained from the company Eden Life (Kettana, Tunisia), powdered using an electric blender, and then transferred into bottles for storage in a dark, dry environment. On the treatment day, we prepared the mixture 1 h prior to application. Specifically, 5g of spirulina powder was added to 50 ml of distilled water with gentle stirring.

2.5. Cafeteria-diet preparation

Obesity is induced in rats by the cafeteria diet, which is a high caloric diet consisting of 50 % standard diet and 50 % pâté mix containing cheese, salami, cookies, chips, peanuts, and chocolate in a ratio of 2:2:2:1:1:1 by weight (standard diet/mix, *w/w*) given for two months [30,31]. This diet, rich in calories and fat, triggers hyperphagia followed by obesity. The cafeteria diet composition (420 kJ/100g) includes 23 % energy from protein, 35 % energy from sugar, and 42 % energy from fatty acids [31].

2.6. Animals and diet

Male rats (weighing 220 ± 20g) were employed and housed five per cage. Rats were purchased from the Society of Pharmaceutical Industries of Tunisia (SIPHAT, Ben Arous Tunisia). Rats were kept in animal facility with 12/12h light/dark cycle at a temperature of 22 ± 2 °C and fed a standard diet (Badr, Utique, TN) and water ad libitum. They were employed in accordance with the recommendation of the local ethics committee of Tunisia University concerning on the use and welfare of animals and adhered to NIH guidelines. The treatments and experiments were received approval from the Biomedical Ethical Committee of (CEBM) for the care and use of animals in Tunisia (reference: JORT472003/2020). The minimum total sample size required was established as 56 rats and calculated utilzing Epidat 4.2; 7 males rats were designed to every group. This sample size was selected to achieve a power of 90 %, a confidence level of 95 %, allowing for the detection of a difference of means = 0.015 and a mean standard deviation = 0.001.

Obesity was induced by the consumption of cafeteria diet (360g/days for 8 weeks) at 09:00 a.m., and ulcerative colitis was provoked by acetic acid (3 % v/v, 5 ml/kg b.*w*.) on the last day of the treatment. Rats were separated into eight groups of seven rats each. The standard diet groups (SD) as follows: 1, 2, 3, and 4 to denote control, SP, AA, and SP + AA respectively while 5, 6, 7, and 8 was represented the cafeteria diet (CD) groups similarly as control, SP, AA, and SP + AA respectively. The eights treatment groups were as outlined bellow.

1 Control group (SD): was fed a standard diet and received distilled water.
2 SD + SP:rats were fed a standard diet and received spirulina (500 mg/kg)
3 SD + AA: Male rats were fed a standard diet, received distilled water and treated with acetic acid (3 % v/v, 5 ml/kg*)*.
4 SD + AA + SP: Male rats was given a standard diet, administrated spirulina (SP) and treated with AA (3 % v/v, 5 ml/kg b.*w)*.
5 CD groups: was fed a cafeteria diet and received distilled water.
6 CD + SP: Male rats were fed a cafeteria diet and received spirulina (500 mg/kg b.*w.p.o.*)
7 CD + AA: Male rats were fed a cafeteria diet, received distilled water and treated with AA (3 % v/v, 5 ml/kg b.*w)*.
8 CD + AA + SP: Male rats was given a cafeteria diet, then subsequent treatment of spirulina (SP) and treated with AA (3 % v/v, 5 ml/kg*)*.

The spirulina was orally received by using oral gavages every morning at the same time. A preliminary experiment indicated that 500 mg/kg was the most reliable dose that gave a meaningful defensive and protective impact. Groups 3, 4,7, and 8 and will be intoxicated by AA 3 % v/v, 5 ml/kg b.*w*. on the 59th day by intra-rectal administration. The animal's body weight was monitored every 3 days.

2.7. Induction of ulcerative colitis

On the 59th day of our experiment, all rats underwent an overnight fat. Ulcerative colitis (UC) was induced in the four groups (3, 4, 7, and 8) by the intra-rectal infusion of acetic acid (3 % v/v, 5 ml/kg b.*w*.) for 30s utilizing a polyethylene tube incorporated through the rectum into the colon up to a depth of eight cm. To prevent acetic acid leakage, animals were positioned in a Trendelenburg position for 30 s. After 24 h, rats were euthanized, and colonic segments was promptly removed and macroscopically evaluated [32, 33]. Colonic segments weighing 1g were homogenized in 2 ml of phosphate buffer saline to determine biochemical parameters including calcium, free iron, protein, MDA levels, reduced glutathione (GSH) and –SH groups, along with the antioxidant enzymes activities (CAT, SOD, and GPx), and ROS levels. The remaining part of the colonic fragments was fixed in 10 % (v/v) formalin for histopathological examination. Blood was drawn in heparinized tubes and centrifuged at 3000 g for 15 min, and the plasma was kept at −20 °C for subsequent metabolic parameters analysis.

2.8. Assessments of ulcerative colitis

The distal segments of the colon were excised and scrubbed with saline solution to eliminate fecal debris. Colons were longitudinally cut for macroscopic examination of colon inflammation. Inflammation scores were determined using an arbitrary scale ranging from 0 to 4 [34], as follows: 0 (no macroscopic changes), 1 (mucosal erythema only), 2 (mild mucosal edema, slight bleeding, or small

erosions), 3 (moderate edema, slight bleeding ulcers, or erosion) and 4 (severe ulceration, edema, and tissue necrosis).

2.9. Histopathological study

Following sacrifice, colonic fragments were immediately fixed in 10 % paraformaldehyde and then embedded in paraffin. Afterward, the samples underwent slicing into 5 mm sections, followed by deparaffinization, rehydrated in different ethyl-alcohol concentrations (70–100 %), and colored with hematoxylin and eosin (H&E) following to standard histological protocols [35]. The histological assessment of colonic sections was founded on a semi-quantitative scoring system, considering five features: mucosal architecture, muscle thickness, presence and degree of cellular infiltration, crypt abscesses, and goblet cell mucus depletion [36].

2.10. Oxidative stress assessment

MDA levels was measured following the method outlined by Draper and Hadleey [37] which involves the linking of MDA with thiobarbituric acid. Protein content was assayed utilizing the Hartree method, a minor modification of the Lowry protocol [38].Thiol group (-SH) estimation was conducted in accordance with Ellman's method [39], while GSH levels were evaluted using the Sedlak and Lindsay method [40]. SOD activity was assessed following to the procedure depicted by Misra and Fridovich utilizing the epinephrin/adenochrome system [41]. The protocol outlined by Flohé and Günzler was employed to examine the GPx activity [42], and CAT activity was determined using the method described by Aebi [43].

2.11. Reactive oxygen species measurement

The levels of H_2O_2 in the colon samples were determined using Dingeon's method [44].

In this procedure, hydrogen peroxide undergoes with *p*-hydroxybenzoic acid and 4-aminoantipyrine catalyzed by peroxidase, resulting in the gneration of quinoneimine. The optical density was measured at 505 nm.The hydroxyl radicals level was determined according to Paya et al. method. Briefly, after oxidation of deoxyribose by hydroxyl radical generated by the Fe^{3+} ascorbate-EDTA-H_2O_2 pathway, followed by incubation with colon homogenate at 37 °C for 1 h [45]. The reaction was halted by adding TCA (2.8 %) and TBA (1 %), and then boiled at 100 °C for 20 min. Changes in absorbance were recorded at 532 nm against a blank containing desoxyribose and buffer.

The levels of superoxide radicals were measured employing Marklund and Marklund protocol [46], with slight changes. In brief, samples were incubated in Tris-HCl buffer, and pyrogallol was added to the reaction mixture, followed by incubated at 25 °C for 4 min. The reaction was halted by HCl addition, and optic density was read at 420 nm vs the blank.

2.12. Plasma scavenging activity

The plasma scavenging activity in the different groups was determined employing the DPPH radical protocol [47]. A mixture of 100 μL of plasma sample and 2 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a methanol solvent (100 mM). After incubation of the solution at 37 °C for 30 min, 1 ml of chloroform was incorporated, and the resulting mixture was centrifuged at 3000 g for 10 min. The optical density of the supernatant was then measured at 517 nm utilizing a spectrophotometer with a DPPH solvent serving as a control. The plasma scavenging activity was presented as a percentage and calculated using the following equation:

$$\text{PSA}\ (\%) = 100\ \text{x}\ (A_{517}(\text{control})\ \text{x}\ A_{517}\ (\text{sample})\ /\ A_{517}\ (\text{control}))$$

2.13. Biochemical analysis

2.13.1. Assessment of lipid profil

Total cholesterol (TC) (cat. no. 21014, Biomaghreb, Ariana, Tunisia), triglyceride (TG) (cat. no. 23025, Biomaghreb, Tunisia), low-density choleserol (LDL) (cat. no. 24022, Biomaghreb, Tunisia), high-density cholesterol (HDL) (cat. no. 29010, Biomaghreb, Ariana, A, Tunisia).

2.13.2. Measurement of glycemia levels and inflammation markers

glycemia, free iron, and calcium amounts in both serum and colonic samples. Additionally, C-reactive protein (CRP), alkaline phosphatase (ALP) in serum were measured employing commercially available diagnostic kits obtained from Biomaghreb, Ariana, TN (ISO 9001 certificate).

2.14. Assessment of IL-6, IL-1β, IL-4 and TNFα in colonic mucosa

The interleukin levels in the colonic mucosa were evaluated employing a standard sandwich enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. CRS-B002 and CEA-C010, Bioscience, San Diego, CA, USA) following the manufacturer's protocol and presented in pg/mL.

2.15. Statistical analysis

Statistical analysis was carried out utilizing Statistica 13.0 data analysis software (TIBCO Software Inc., Palo Alto, CA, USA). Before analysis, all results were rigorously examined for normality and homoscedasticity. The influence of various alimentation on weight gain, body weight, food intake, total abdominal fat and biochemical parameters were assessed through a one-way ANOVA followed by a post hoc least significant difference (LSD) test. For multi-treatment comparisons, analysis of variance (ANOVA) was employed, while the independent samples for two-treatment comparisons were determined by a Student'st-test. Antioxidant parameters and lipid peroxidation in every treatment group were examined using the non-parametric Kruskal–Wallis test, followed by Mann–Whitney post-hoc analysis, in accordance with the applied dietary experiments.

Statistical significance was denoted by $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, indicated as *, **, *** in comparison with SD, ¥, ¥¥, ¥¥¥ compared with CD, #, ##, ### compared with SD + AA, and £, ££, £££ compared with CD + AA respectively.**3. Results**.

2.16. Amino acid characterization of SP

The HPLC-FLD analysis of spirulina facilitated for the identification of 17 amino acid compounds, classified into two groups: essential amino acids and non-essential amino acids, with the exception of glutamic acid at 159.72 mg/kg. The most abundant amino acids were alanine (118.55 mg/kg), leucine (99.41 mg/kg), and aspartic acid (90.78 mg/kg). Furthermore, as shown in Table 1, sulfur amino acids such as cysteine (4.23 mg/kg) were among the least abundant.

2.17. Effect of SP, CD and/or AA on body, abdominal adipose weights, food intake and weight gain

As indicated in Table 2, the initial mean body mass of rats were nearly identical across all groups at the beginning of the feeding period. However, after eight weeks dietary exposure, the high-calorie diet notably elevated the body weights of animals, leading to a significant weight gain in the CD-fed group in comparison with the control (SD-fed). This increase in weight was in addition associated with a significant rise in food intake and total abdominal fat weights in the CD groups in comparison with standard diet-fed rats. At the same time, SP pre-treatment mitigated all CD-provoked disruptions, bringing them closer to control levels, thus demonstrating a potent anti-obesity effect.

2.18. Qualitative and quantitative gross evaluation of the colonic injuries and colon wet weight

The effect of spirulina, CD and acetic acid on colon morphology is illustrated in Fig. 1 (A-H). Consumption of CD over an 8-weeks period resulted in mild colonic mucosal damage. Acetic acid administration induced severe colon lesions and macroscopic edematous colon injuries in rats fed a SD, leading to high scores of colonic injuries and increased colon wet weigh (Table 3). More importantly, all these alterations and lesions were more pronounced in cafeteria diet-fed rats with colitis, while treatment with the SP (500 mg/kg b.*w*.) significantly reduced colon wet weight in comparison with the colitis or colitis/obese groups. Similarly the macroscopic injuries revealed that SP treatment (500 mg/kg b.*w*.) could inhibit ulcerative colitis in rats fed both a control diet and a cafeteria diet.

2.19. Histopathological study

Microscopic observation of obese rats' colons showed minimal histological alterations. However, AA administration to rats fed a standard diet resulted in histological deterioration characterized by extensive inflammation cell infiltration, congestion of the epithelial cells of the colonic fragments, and the developments of necrotic lesions and edematous regions (Fig. 2A), along with a higher histological score (Fig. 2B). Necrosis and inflammatory cell infiltration were more severe in CD-fed rats with colitis. Importantly, SP treatment significantly protected against obesity and/or colitis-provoked colon histological injuries (Fig. 2C–H).

Table 1
Amino acids composition of spirulina after HPLC-FLD analysis
The data are expressed as means−standard error of the mean (SEM) (n = 3).

Amino acid content in mg/kg	
Aspartic acid	90.78 ± NA
Glutamic acid	159.72 ± NA
Serine-Glutamine-Histidine	41.25 ± NA
Arginine-Glycine-Threonine	119.16 ± NA
Alanine	118.55 ± NA
Tyrosine	45.14 ± NA
Phenylalanine	29.47 ± NA
Isoleucine	46.52 ± NA
Leucine	99.41 ± NA
Valine-methionine	35.84 ± NA
Cystine	14.23 ± NA
Lysine	35.65 ± NA

Table 2
Effect of SP 500 mg/kg on body weight, total abdominal fat, weight gain and food intake of SD-fed and CD-fed rats administered, or not, with AA (3 % v/v, 5 ml/kg b.*w.*). The results are represented as mean S.E.M. (n = 7). One-Way ANOVA was employed for multi-group comparison, $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥ vs CD, #, ##, ### vs SD + AA, and £, ££, £££ vs CD + AA respectively.

Diet	Groups	Final Body mass (g)	weight gain (g)	Food intake (g)	Total abdominal adipose weight (g)
SD	**Control**	260.41 ± 12.32	26.37 ± 3.21	110.26 ± 9.62	4.21 ± 0.97
	SP	261.61 ± 10.14	14.71 ± 2.21**	123.16 ± 7.62	5.92 ± 0.78
	AA	269.35 ± 8.15*	27.33 ± 3.45	109.04 ± 5.84	4.5 ± 0.87
	AA + SP	260.44 ± 11.54#	15.01 ± 4.32*##	111.76 ± 11.01	5.23 ± 0.54
CD	**Control**	301.97 ± 7.32**	52.52 ± 6.65***	140.94 ± 10.21**	13.49 ± 1.04***
	SP	245.87 ± 11.54¥¥¥	23.51 ± 2.32¥¥	101.97 ± 8.65¥¥	8.15 ± 1.31¥
	AA	302.55 ± 9.65**	50.68 ± 9.65***	139.14 ± 8.95**	13.05 ± 2.01***
	AA + SP	270.35 ± 10.12£	23.88 ± 1.32£	117.85 ± 7.62£	8.16 ± 1.05£

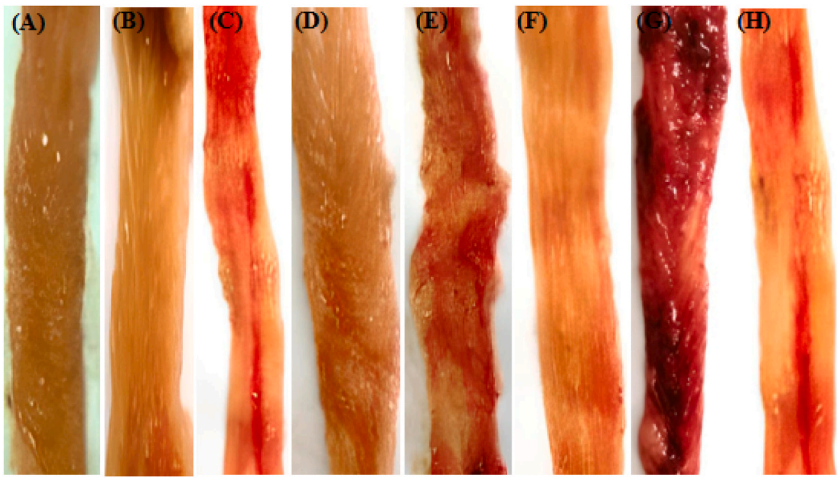

Fig. 1. Effect of spirulina (SP 500 mg/kg) on AA-induced alteration in colon morphology of SD-fed and CD-fed rat. Animals was pre-treated with SP (500 mg/kg, *b.w.*, p. o) or distilled water, and challenged with a single anal treatment of AA (3 % v/v, 5 mL/kg b.*w.*) or NaCl (0.9 %, 5 ml/kg 1, *b.w.*) for 24 h. (A) SD, (B) SD + SP, (C) CD, (D) CD + SP, (E) SD + AA, (F) SD + AA + SP, (G) CD + AA, (H) CD + AA + SP.

Table 3
Impact of SP (500 mg/kg) on colon length/weight and the gross lesion score of SD-fed and CD-fed rat treated, or not, with AA (3 % v/v, 5 ml/kg b.*w.*). The results are expressed as mean S.E.M. (n = 7). One-Way ANOVA was utilized for multi-group comparison, $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥vs CD, #, ##, ###vs SD + AA, and £, ££, £££ vs CD + AA respectively.

Diet	Group	Gross inflammatory score	Colon weight/length ratio (mg/cm)	protection percentage
SD	**Control**	0 ± 0	86.21 ± 7.52	–
	SP	0 ± 0	68.94 ± 6.42	51.85
	AA	3.28 ± 0.18***	155.41 ± 8.71***	–
	AA + SP	1.85 ± 0.14##	106 ± 9.33###	31.79
CD	**Contol**	1.5 ± 0.18**	141.24 ± 9.25***	–
	SP	0.87 ± 0.12¥	99.6 ± 5.89¥¥	29.43
	AA	3.85 ± 0.14***	187.09 ± 11.55***	–
	AA + SP	2.14 ± 0.21£	129.52 ± 8.9 £	30.77

2.20. Effect of SP, AA and CD on colonic oxidative stress

SP effect on colonic oxidative stress is depicted in Fig. 3. Obesity, colitis and their combination provoked a state of colonic oxidative stress, as evidenced by important lipoperoxidation presented by elevated levels of MDA (Fig. 3A) in obese and/or colitis rats in comparison with those fed a SD. Additionally, there was a notable depletion of the antioxidant enzymes activity such as SOD (Fig. 3B), CAT (Fig. 3C) and GPx (Fig. 3D), along with a significant reduction of thiol groups levels (Fig. 3E) and reduced glutathione (Fig. 3F). Notably, treatment with spirulina (500 mg/kg b.*w.*) significantly restored the prooxidant/antioxidant balance of the colonic fragments of all groups without exception.

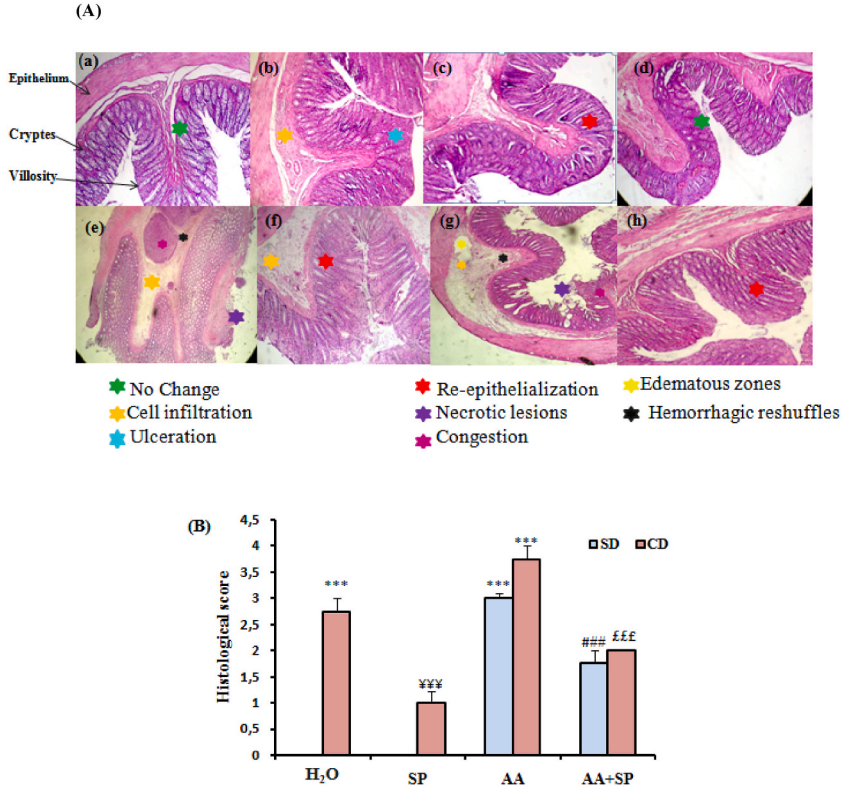

Fig. 2. Effect of spirulina (SP 500 mg/kg) on histological alteration in colonic mucosa associated with CD and AA treatments (A)and histological score (B). Animals was pre-treated with SP (500 mg/kg, *b.w.*, p.o) or distilled water, and challenged with a single anal treatment of AA (3 % v/v, 5 mL/kg b.w.) or NaCl (0.9 %, 5 ml/kg 1, *b.w.*) for 24 h. (a) SD, (b) CD, (c) CD + SP, (d) SD + SP, (e) SD + AA, (f) SD + AA + SP, (g) CD + AA, (h) CD + AA + SP, (Magnification ×10).For the histological score, multi-groups comparison by One-way ANOVA. $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥vs CD, #, ##, ###vs SD + AA, and £, ££, £££ vs CD + AA respectively.

2.21. Effect of SP, AA and CD on colonic reactive oxygen species (ROS)

Induction of colitis in normal or obese rats resulted in a significant increase inreactive oxygen species (ROS) levels in colon tissues (Fig. 4), leading to elevated amounts of hydrogen peroxide (Fig. 4A), hydroxyl radical (Fig. 4B) and superoxide anion (Fig. 4C). SP administration exhibited potent ROS scavenging activity, as evidenced by a substantial decrease in all colon reactive oxygen species in animals fed a standard alimentation as well as those received a cafeteria diet.

2.22. Impact of SP, AA and CD on plasma scavenging activity

Plasma scavenging activity of SD-fed rats or CD-fed rats decreased significantly *($p < 0.05$)* due to induction of colitis. It is noteworthy that the PSA levels were elevated in non-colitis obese groups in comparison with SD-fed group. However, these levels were significantly restored in all groups treated with spirulina (Fig. 5).

2.23. SP, AA and CD effect on metabolic parameters

As shown in Table 4, plasma concentrations of glycemia, TG, CHOL, and LDL elevated in the ulcerative colitis CD-fed rats compared with the non-colitis SD-fed rats, with more pronounced effects observed in the colitis obese rats. Administration of SP significantly ameliorated all these metabolic disorders.

2.24. SP, AA and CD effect on calcium levels and free iron

To evaluate the involvement of intracellular mediators in the protective impacts of SP, we measured the quantity of free iron and calcium in the colonic mucosa and plasma. We observed a significant elevation in the levels of free iron, and calcium following the induction of obesity and/or colitis, with higher levels in detected in colitis obese rats (Table 5). However, after SP treatment, there was significant inhibition observed in both calcium and free iron amounts.

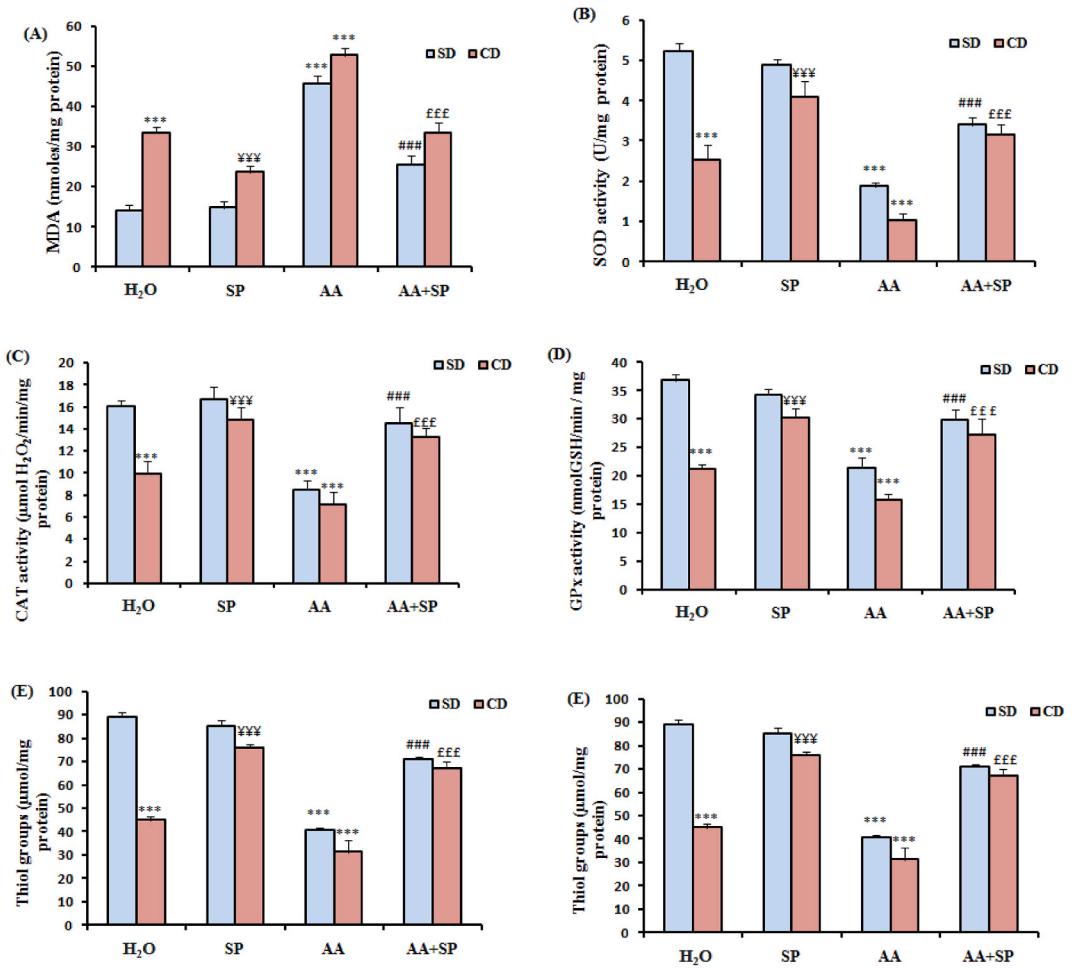

Fig. 3. Effect of SP (500 mg/kg,*b.w., p.o*) on colonic oxidative stress of SD-fed and CD-fed rat treated, or not, with AA (3 % v/v, 5 mL/kg b.*w.*) such as MDA (A), SOD (B), CAT (C) and GPx (D), thiol groups (E) and GSH (F). Animals was pre-treated with SP (500 mg/kg, *b.w., p.o*) or distilled water, and challenged with a single anal treatment of AA (3 % v/v, 5 mL/kg b.*w.*) or NaCl (0.9 %, 5 ml/kg, *b.w.*) for 24 h. The data are expressed as mean S.E.M. (n = 7),different groups comparison by One-way ANOVA. P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001 were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥vs CD, #, ##, ###vs SD + AA, and £, ££, £££ vs CD + AA respectively.

2.25. Impact of SP, AA and CD on inflammation

Effect of SP on colitis inflammation during obesity and/or colitis is shown in Fig. 6. Obesity, colitis, or their combination caused a significant elevation in plasma CRP levels (Fig. 6A) and ALP activity (Fig. 6B) as well as increased levels of colonic mucosa cytokines such as IL-1β (Fig. 6C), IL-4 (Fig. 6D), IL-6 (Fig. 6E) and TNFα (Fig. 6F). Notably, inflammation was more severe in colitis CD-fed rats compared to other groups. However, SP treatment effectively protected against obesity and/or colitis-induced inflammation, demonstrating potent anti-inflammatory activity.

3. Discussion

Obesity is associated with low-level inflammation [48,49], but it may exacerbate other inflammatory diseases [50–52]. In this study, we evaluated the amino acid composition as well as the protective impact of SP against colonic inflammation provoked in normal and obese groups.

The link between obesity and colonic inflammation induced by acetic acid has not been widely studied experimentally, which constituted a significant obstacle in the implementation of our experimental protocol. Furthermore, a significant lack of available or reliable data about the effect of spirulina on obesity-associated colitis prevented us to compare our results with previous researches.

Protein analysis revealed that SP is rich in amino acids. Using HPLC-FLD analysis, we identified 17 amino acids (AA), separated into 2 classes: essential amino acids such as phénylalanine, isoleusine, leusine, methionine, and lysine, as well as non-essential amino acids like aspartic acid, glutamic acid, alanine, glycine, and tyrosine. These AA are considered synergistic antioxidants [53]. Different forms

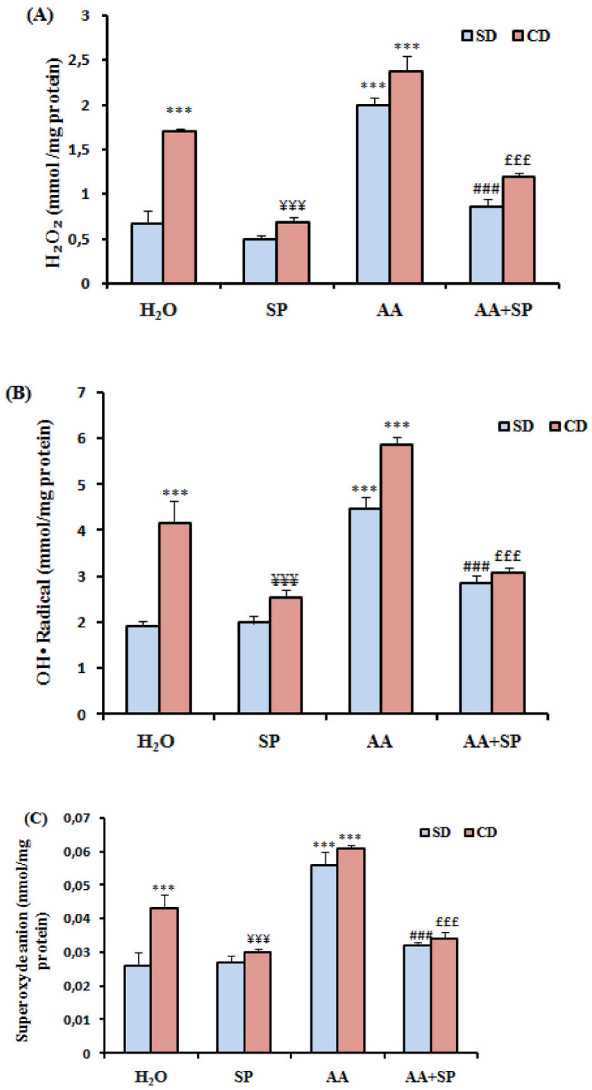

Fig. 4. Effect of SP (500 mg/kg, *b.w., p.o*) on colonic mucosa Hydrogen peroxide (A), hydroxyl radical (B) and superoxide anion (C) levels of SD-fed and CD-fed rat treated, or not, with AA (3 % v/v, 5 mL/kg b.w.). Animals was pre-treated with SP (500 mg/kg,*b.w., p.o*) or distilled water, and challenged with a single The data are expressed as mean S.E.M. (n = 7).All groups comparison by One-way ANOVA. $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥vs CD, #, ##, ###vs SD + AA, and £, ££, £££ vs CD + AA respectively.

of amino acids, including glycine, tyrosine, alanine, tryptophan, glutamine, and valine, exhibit antioxidant and anti-inflammatory properties [54–56]. They possess effective ROS scavenging activity due to their diverse side chains [53,57,58].

In vivo, we established that SP pre-treatment decreases hyperlipidemia provoked by CD, as accompanied with a significant weight loss, reduced food intake, and decreased weight gain, and abdominal fat accumulation, consistent with several previous studies [59, 60]. This weight gain induced by CD is attributed to its high caloric intake [31] and energy content, primarily in the form of saturated fatty acids, consequently leading to obesity development [61,62]. The anti-hyperlipidemic properties of SP, may be explained to its high levels of the amino acids, especially glutamic acid [63,64]. Additionally, several research have indicate the significant impact of glutamine treatment on ulcerative colitis [65]. and ischemia/reperfusion [66].

In the past decades, robust epidemiological studies have established a close link between IBD and dietary factors, particularly high fat intake [67–69]. In our study, we first observed that rectal treatment of acetic acid induced colonic mucosal ulceration with standard diet groups. More importantly, these colonic ulcerations were significantly more intense and hemorrhagic in the rats fed the CD. This morphological damage was evidenced by an elevate wet weight/length compared to SD group. Indeed, our findings indicate that obesity, as well as AA, altered intestinal barrier integrity and led to severe injuries of the adherent mucosal layer, including goblet cell loss, villus destruction, and crypt damage [1,70,71].

Especially in obese rats with colitis [72–74], our histological analysis has demonstrated necrotic destruction of colonic epithelial cells and inflammatory cellular infiltration in the colitis SD groups, but to a greater extent in colitis-CD groups. This alteration was

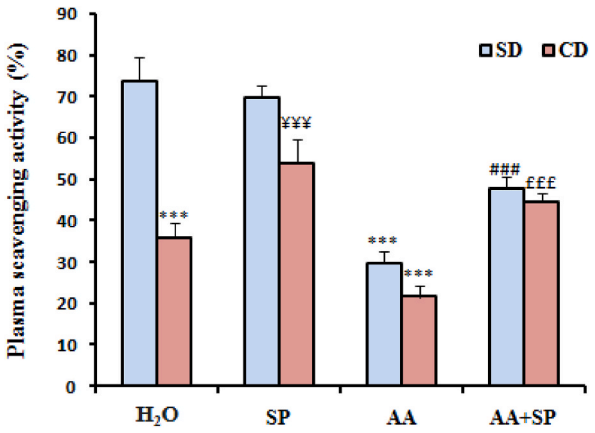

Fig. 5. Effect of SP (500 mg/kg,*b.w., p.o*) on plasma scavenging activity changes of SD-fed and CD-fed rat treated, or not, with AA (3 % v/v, 5 mL/kg b.w.). Animals was pre-treated with SP (500 mg/kg,b.w., p.
o) or distilled water, and challenged with a single anal treatment of AA (3 % v/v, 5 mL/kg b.w.) or NaCl (0.9 %, 5 ml/kg 1, *b.w.*) for 24 h. The data are expressed as mean S.E.M. (n = 7),multi-group comparison by One-way ANOVA. P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001 were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥ vs CD, #, ##, ### vs
SD + AA, and £, ££, £££ vs CD + AA respectively.

Table 4
Effect of spirulina (500 mg/kg) on glycemia disruption with lipid metabolisms of SD-fed and CD-fed rat treated, or not, with acetic acid (3 % v/v, 5 ml/kg b.*w.*). Animals was received SP (500 mg/kg) or water, and administered with anal treatment of acetic acid (3 % v/v, 5 ml/kg b.*w.*) or saline solution (0.9 %, 5 ml/kg) for 24 h. The results are defined as mean S.E.M. (n = 7). One-Way ANOVA using for multi-group comparison, P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001 were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥ vs CD, #, ##, ### vs SD + AA, and £, ££, £££ vs CD + AA respectively.

Diet	Group	GLY (Mm/l)	CHOL (mM/l)	TRIG (mM/l)	HDL CHOL (mM/l)	LDL CHOL (mM/l)
SD	**Control**	5.03 ± 0.20	1.18 ± 0.06	0.52 ± 0.06	0,55 ± 0,03	0.52 ± 0.05
	SP	5.04 ± 0.19	1.16 ± 0.09	0.55 ± 0.05	0.49 ± 0.02	0.49 ± 0.03
	AA	8.58 ± 0.29**	2.56 ± 0.06*	0.99 ± 0.03**	0.28 ± 0.03**	0.92 ± 0.02**
	AA + SP	5.23 ± 0.21##	1.29 ± 0.02##	0.64 ± 0.02##	0.51 ± 0.05##	0.56 ± 0.02##
CD	**Control**	8.89 ± 0.12**	2.99 ± 0.05***	1.55 ± 0.05***	0.23 ± 0.02***	0.95 ± 0.04**
	SP	5.12 ± 0.21¥	1.21 ± 0.05¥	0.65 ± 0.05¥¥	0.51 ± 0.03¥¥	0.67 ± 0.02¥
	AA	9.12 ± 0.20***	3.10 ± 0.05**	1.90 ± 0.06***	0.19 ± 0.02***	1.13 ± 0.03***
	AA + SP	6.71 ± 0.42£	1.61 ± 0.07£	0,71 ± 0,03£	0.45 ± 0.06£	0.71 ± 0.03£

Table 5
Effect of SP on calcium and free iron amounts in colonic mucosa and plasma of SD-fed and CD-fed rat treated, or not, with acetic acid (3 % v/v, 5 ml/kg b.*w.*). Animals was received SP (500 mg/kg, b.w., *p.o*) or water, and treated with a single anal administration of AA (3 % v/v, 5 ml/kg b.*w.*) or saline solution (0.9 %, 5 ml/kg) for 24 h. The data are represented as mean S.E.M. (n = 7). One-Way ANOVA was employed for multi-group comparison, P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001 were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥ vs CD, #, ##, ### vs SD + AA, and £, ££, £££ vs CD + AA respectively.

		Calcium(mM/l)		Free iron (μM/l)	
Diet	Group	Plasma	Colonic mucosa	Plasma	Colonic mucosa
SD	**Control**	1.46 ± 0.02	3.84 ± 0.26	5.08 ± 0.42	6.31 ± 0.37
	SP	1.54 ± 0.02	3.73 ± 0.36	5.21 ± 0.33	6.01 ± 0.24
	AA	3.37 ± 0.06**	8.25 ± 0.64***	10.57 ± 0.19***	12.39 ± 0.41***
	AA + SP	1.71 ± 0.09###	4.91 ± 0.31##	6.14 ± 0.31##	6.97 ± 0.27###
CD	**Control**	2.96 ± 0.21**	7.38 ± 0.36**	9.65 ± 0.39**	11.96 ± 0.45**
	SP	1.63 ± 0.07¥	4.13 ± 0.38¥¥	5.68 ± 0.21¥¥	6.71 ± 0.29¥¥
	AA	3.76 ± 0.08**	9.12 ± 0.28***	11.91 ± 0.34***	12.75 ± 0.43***
	AA + SP	1.87 ± 0.18£	5.46 ± 0.31 £	7.11 ± 0.27£	7.08 ± 0.31£

consistent with findings from several studies where acetic acid-caused ulcerative colitis manifested as massive erosion in the epithelial surface of the rat's colon, severe exfoliation and erosion of epithelium from the colonic surface and down into the crypts, as well as extensive destruction of the mucosal interstitial [14,33,75]. Moreover, obesity exacerbates these alterations.

The aggravation of histological alterations due to a high calorie diet following acetic acid administration is manifested by an epithelial lining that is almost completely sloughed off, irregular arrangement of cells, significantly reduced goblet cells, and

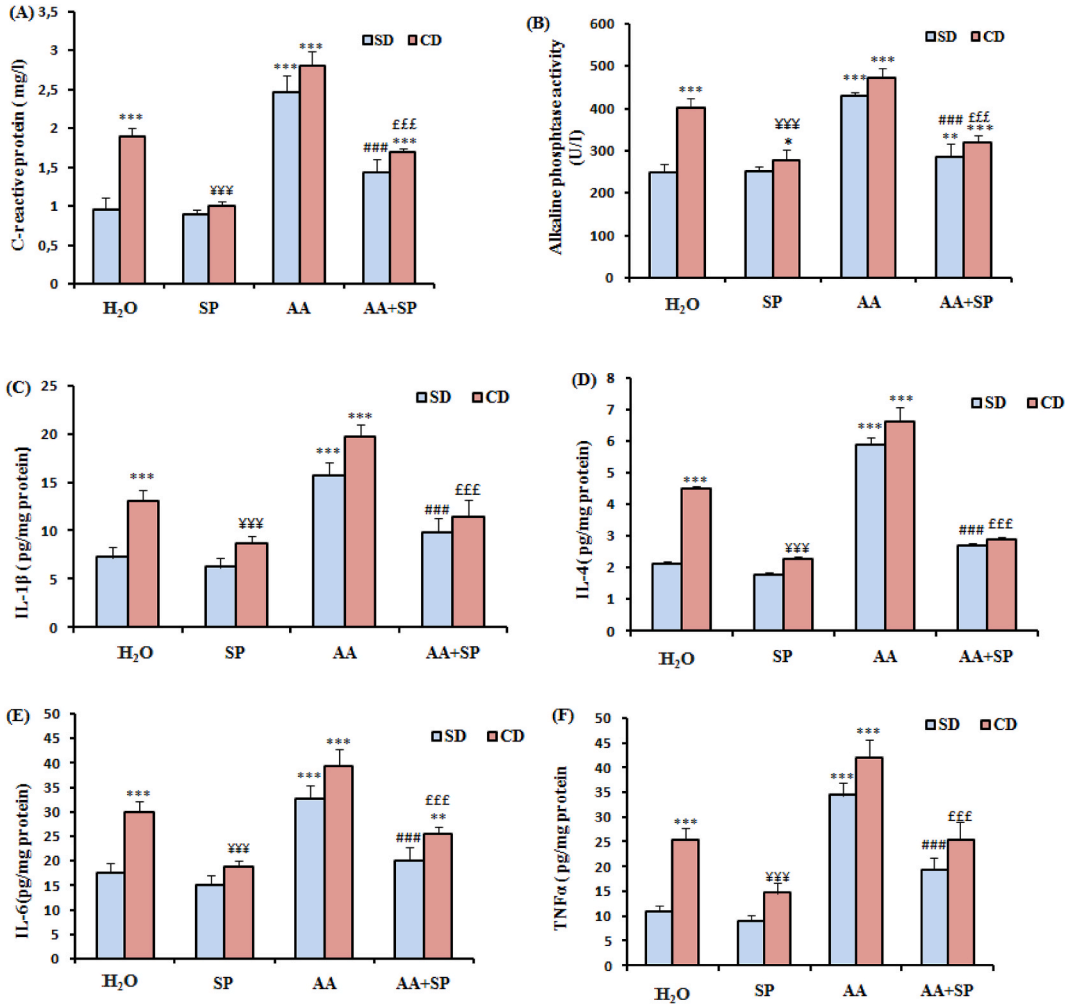

Fig. 6. Effect of SP (500 mg/kg, *b.w., p.o*) on colitis inflammation during obesity and/or colitis of SD-fed and CD-fed rat treated, or not, with AA (3 % v/v, 5 mL/kg b.w.) such as CRP (A), ALP (B) IL-1β (C), IL-4 (D), IL-6 (E) and TNFα (F). Animals was pre-treated with SP (500 mg/kg, *b.w., p. o*) or distilled water, and challenged with a single anal treatment of AA (3 % v/v, 5 mL/kg b.*w.*) or NaCl (0.9 %, 5 ml/kg 1, *b.w.*) for 24 h. The data are expressed as mean S.E.M. (n = 7), multi-group comparison by One-way ANOVA. $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ were denoted as *, **, *** vs SD, ¥, ¥¥, ¥¥¥vs CD, #, ##, ###vs SD + AA, and £, ££, £££ vs CD + AA respectively.

infiltration of inflammatory cells, particularly leucocytosis [72,74,76]. Importantly, the anti-obesity and anti-colitis impacts of spirulina are largely indicated to the abundance of amino acid compounds such as glutamine, glycine, arginine, and tryptophane, which effectively restore the morphological and histological changes [77] and maintain colonic mucosa permeability [78]. Glycine and glutamine also prevent gastric injury and decrease acid secretion induced by pylous-ligation. These amino acids possess effective cytoprotective and anti-ulcer activity [64,79,80]. In addition, hydrophobic amino acids such as proline and leucine demonstrated a powerful anti-hyperlipidemic effect [5]. In our research, we also observed the impacts of combining a hypercaloric diet-induced hyperlipidemia with experimental colitis on the colonic redox balance. The results exhibited that AA-induced colitis led to colonic oxidative stress state. Indeed, intoxication with AA resulted in lipid peroxidation, as manifested by a significant elevation of MDA amounts, and decrease of antioxidant enzymes activity including CAT, SOD and GPx, as well as in non-enzymatic antioxidants (GSH and –SH) levels. Moreover, there was a tissue accumulation of reactive oxygen species, including H_2O_2, $OH^{\bullet}$ and $O_2^{\bullet-}$ in the colons of all animals fed a standard diet.

This colonic oxidative stress was observed in CD-fed rats was particularly severe: lipoperoxidation was more pronounced, and alterations in enzymatic and non-enzymatic antioxidants, as well as plasma scavenging activity (PSA) and ROS overproduction, were more severe compared to non-obese colitis rats. These findings corroborate with several data, indicating that obesity exacerbates ulceration in the colonic mucosa by promoting oxidative stress in the colon [81–83]. Obesity can lead to mitochondrial dysfunction and increased ROS production due of electron transport occurring on the mitochondrial membranes [84,85].

In addition, intestinal inflammation serves as a significant source of ROS generated by activated phagocytic cells through the stimulation of the NADPH oxidase complex [86,87]. This enzyme uses molecular oxygen to produce superoxide anions, which

subsequently generate hydroxyl radicals [88,89] and are considered the most harmful ROS causing DNA damage [90]. Therefore, all the oxidative damages observed in our study were effectively mitigated by the administration of SP, whether in rats fed SD or CD. More importantly, SP is very rich in amino acids such as alanine, gulutamic acid, aspartic acid, arginin, and leucine [91,92] and the presence of these various amino acids confers synergistic antioxidant activities [53,93,94]. These amino acids act as effective ROS scavengers [62]. Prior studies has demonstrated that glycine reduces oxidative stress associated with various diseases and inhibits ROS production [95–97]. Additionally, cysteine plays a potent role in increasing cellular glutathione (GSH) levels [98–100].

Furthermore, mitogen-activated protein kinases (MAPKs) have garnered significant attention as potent mediators of crucial effect of SP [101]. It has been shown to modulate the ERK1/2, JNK, p38 and IkB signaling pathways to develop its antioxidants, immunomodulatory, and anti-inflammatory activities [102].

Furthermore, we found that CD and intra-rectal AA intoxication disrupted metabolic parameters like glycemia, total cholesterol, triglyceride, LDL, and HDL-cholesterol. Similar disturbances were observed when colitis was associated with diabetes [75,103].

Actually, the excess supply of fatty acids is primarily stored in adipose cells, and the low oxidative self-regulating potential of lipid substrates results in dysfunction and an increase in fatty mass [104,105]. Recent research has reported that numerous bioactive constituents in SP have hypocholesterolemic activities, thereby reducing dyslipidemia risk [106,107]. Similarly, it has been shown that SP-derived peptides exhibit anti-hyperlipidemic effects by decreasing body weight and reducing serum glycemia in mice [108]. Proline (NEAA) has shown significant hypoglycemic properties, reducing glucose production in the liver by inhibiting glucose-6-phosphate, gluconeogenesis and glycogenolysis [109,110]. Moreover, we have also shown that that experimental colitis was manifested by tissue overload of free iron and calcium in the colons of animals fed a SD but, more importantly, in animals fed a cafeteria diet. All these alterations have been effectively mitigated by taking spirulina. Indeed, free iron stimulates the secretion of the radical hydroxyl ($OH^{\bullet}$) via the Fenton reaction [111], thereby causing membrane lipoperoxidation [16,112,113]. While this observed hypercalcemia may result from the release of calcium from cellular organelles like the sarcoplasmic and endoplasmic reticulum, or its importation from extracellular spaces through the injured plasma membrane [30,114]. On the other side, glycine, an amino acid found in spirulina, blocks the stimulation of degradative enzymes like protease, calcium-dependent protease, and calpain, which are involved in cell damage [115]. Importantly, we have observed that SP protects against obesity and colitis induced injuries, as evidenced by a significant decrease in colon CRP and ALP levels, along with reductions in cytokines including TNFα, IL-1β, IL-4, and IL-6. The inflammatory state in colitis-induced obese rats was notably more severe than in non-obese colitis rats. Indeed, hyperlipidemia is associated with chronic damage to adipose tissue [116], induced by the accumulation of fatty acids and the transformation of monocytes into macrophages, which contribute to maintaining local injury [8].

In fact, M1 macrophages generate various cytokines like TNF-α, IL-1, and IL-6, which play a role in the secretion of T lymphocyte within adipose cells [117,118]. Several studies have confirmed that being overweight is intimately linked to abnormal expression of pro-inflammatory cytokines such as leptin, TNFα and IL-6, along with other markers of inflammation like serum amyloid, CRP and fibrinogen. Additionally, an increase in intestinal permeability was also observed. All of these biochemical disturbances can alter the intestinal flora and decrease immune tolerance, thereby facilitating the apparition of inflammatory bowel diseases [118].

In this research, spirulina showed powerful anti-inflammatory properties, which are related with the diversity of its amino acids, like glutamine, aspartic acid, alanine, tryptophan, and glycine [54,119]. These amino acids are involved in its anti-inflammatory role. Dietary glycine has been shown to blocks the systemic overexpression of cytokines gene including TNFα, IL-6, and IL-1β following hemorrhagic shock and clinical sepsis in rats [96,97]. Other studies have also demonstrated that arginine administration can regulate pro-inflammatory and anti-inflammatory cytokines as well as stimulate lymphocyte proliferation in Peyer's patches in an animal model with colitis [120,121]. Additionally, supplementation of tryptophan has been found to enhance the overexpression of pro-inflammatory cytokines in a porcine model of colitis [122].

However, almost 90 % of therapeutic molecules tested on animals in the preclinical phase and which indicate that the treatment is effective and safe, fail in humans during clinical trials. The reasons are generally related to differences in metabolism and symptomatology between species. This can prevent us from carrying out clinical studies on humans without being sure of the safety of spirulina.

4. Conclusion

Our study has for the first time reveals that spirulina shows promising potential in mitigating obesity and colon injuries caused by CD and/or acetic acid. We present the first experimentally validated study of reductions in weight gain, metabolic disturbances, inflammation, histopathological alteration and ROS overproduction, making it a safe and effective strategy for addressing obesity and promoting gut health. Clinical trials in human needed to validate these findings leading to the improvement and development of spirulina-based interventions targeting obesity and associated gastro-intestinal disorders.

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Ethical declaration

The ethical standards of this study were upheld through approval from the Biomedical Ethics Committee (CEBM) for the care and use of animals Tunisia (Reference: JORT472003, dated September 5, 2020).

Data availability statement

The data featured in this study are accessible upon request from the corresponding author.

CRediT authorship contribution statement

Fatma Arrari: Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Mohamed-Amine Jabri:** Supervision, Methodology, Funding acquisition, Formal analysis. **Ala Ayari:** Formal analysis, Data curation. **Nouha Dakhli:** Conceptualization. **Chayma Ben Fayala:** Formal analysis. **Samir Boubaker:** Supervision. **Hichem Sebai:** Supervision.

Declaration of competing interest

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