Clinical Epigenetics

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First evidence of epigenetic modulation of human gene methylation by microalga *Aphanizomenon flos-aquae* (AFA) in inflammation-related pathways in intestinal cells

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Abstract

The microalga *Aphanizomenon flos-aquae* (AFA) has garnered attention for its potential therapeutic benefits in various health conditions, primarily through its use in nutraceutical formulations. While biological effects of AFA have been extensively studied in preclinical models, including murine systems, its nutrigenomic and epigenetic impacts remain underexplored. This study investigates the potential epigenetic mechanisms of AFA, focusing on its ability to modulate DNA methylation, a key regulatory process in gene expression. Specifically, we examined the influence of AFA on the methylation status of genes encoding pro-inflammatory interleukins, as these cytokines play a crucial role in immune response modulation and inflammation. Given the known impact of AFA on inflammatory markers, we aimed to determine whether the effects of AFA involve direct or indirect modulation of DNA methylation patterns in genes associated with inflammation. Our findings, presented here for the first time, reveal the capacity of AFA to influence DNA methylation, with implications for its role in cellular regulatory processes. These results warrant further investigation into precise mechanisms of action of AFA and its potential in clinical applications targeting inflammation-related pathways.

Keywords DNA methylation, Nutrigenomics, Microalgae, Epigenetics, Gene regulation, Inflammation

Introduction

Chronic inflammation is pivotal in the pathogenesis of numerous chronic diseases, facilitating their onset and progression [1]. Persistent stimuli such as infection, tissue injury or metabolic imbalance trigger a prolonged inflammatory response. Unlike acute inflammation, which is a physiological process that acts against harmful agents and promotes repair, chronic inflammation causes tissue damage, immune dysregulation, and abnormal tissue remodeling [2]. Accumulating evidence suggests that chronic inflammation is a common factor in the pathogenesis of chronic diseases, including cardiovascular and

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neurodegenerative disorders, metabolic syndrome, and certain cancers [2, 3, 3, 4].

The dynamic interaction between inflammatory mediators, immune cells, and the tissue microenvironment perpetuates a cycle of inflammation and damage, underlining inflammation as a therapeutic target for chronic disease management. Modulating inflammation through epigenetic processes may be a promising strategy to reduce basal inflammation and prevent various pathologies.

DNA methylation, an essential epigenetic modification involving the addition of a methyl group to cytosine residues of CpG dinucleotides, typically results in gene silencing and plays a critical role in the development, differentiation, and pathogenesis of the disease [5]. Inflammation has emerged as a significant modulator of DNA methylation patterns, with growing evidence suggesting a bidirectional relationship between the two [6, 7]. In inflamed tissues, alterations in DNA methylation patterns have been observed, often characterized by global hypomethylation and site-specific hypermethylation [8]. These changes can influence the expression of genes involved in inflammatory pathways, including cytokines, chemokines, and immune receptors. Moreover, aberrant DNA methylation patterns have been implicated in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, obesity and metabolic syndrome [9]. Conversely, DNA methylation alterations can also contribute to the regulation of inflammatory responses by modulating the expression of key inflammatory genes [10].

Understanding the intricate interplay between DNA methylation and inflammation is crucial for unraveling the molecular mechanisms underlying inflammatory diseases and identifying potential targets for therapeutic intervention.

In recent years, the spotlight has intensified on complementary medicine approaches harnessing the power of natural ingredients. Among these, *Aphanizomenon flos-aquae* (AFA), also known as "Klamath algae," has emerged as a notable candidate with promising therapeutic potential, particularly in chronic diseases [11, 12] and as a functional powder for foods [13].

AFA, a cyanobacterial species thriving in the Upper Klamath Lake located in Oregon, USA, is revered as a nutritional powerhouse and is often consumed as a "superfood" [14]. Renowned for its nutritional richness, AFA has emerged as a prominent component in various nutraceutical formulations utilized across different pathologies [15, 16].

Studies have underscored the neuroprotective effects of AFA, along with their capacity to ameliorate psychological stress and improve menopausal well-being [11]. Moreover, previous studies have highlighted AFA's therapeutic potential in murine models, demonstrating its efficacy in mitigating inflammatory responses and improving overall health outcomes, as well as in mitigating components of intestinal inflammation during experimental colitis [12].

While the clinical benefits of AFA and its derivatives are becoming increasingly evident, further investigations are warranted to elucidate its mechanisms of action.

Since IL1β-induced inflammation alters the methylation of promoters of pro-inflammatory genes, [10], and considering the beneficial effects on DNA methylation observed in inflammatory diseases with dietary nutraceuticals [17], studying AFA from a nutrigenomic standpoint could offer significant benefits.

This study explores the nutrigenomic effects of AFA, specifically focusing on its influence on DNA methylation patterns of inflammation-related genes. Using DNA derived from colon tissue of rats and utilizing Caco-2 cells as a model system, we employed different treatment paradigms to simulate AFA administration either subsequent to an inflammatory stimulus or concurrently with an inflammatory stimulus. This approach aims to elucidate whether AFA's mechanism of action involves direct or indirect modulation of DNA methylation. Our investigation seeks to uncover novel insights into the molecular pathways through which AFA exerts its therapeutic effects, shedding light on its potential as a nutraceutical agent targeting epigenetic regulation in inflammatory processes.

Methods

Solubilization of AFA

The AFA extract was kindly provided by Nutrigea Research s.r.l. (Republic of San Marino). 5 mg of AFA powder was solubilized in 5 mL of PBS (pH = 7.4; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4) to obtain a concentration of 1 mg/mL. The solution was placed at 37 °C for 20 min, with a vortex step after 10 min. The insoluble fraction was removed by centrifugation at 14,000 rpm for 30 min at 4 °C. The supernatant was collected, filtered by using a 0.45 µm filter (Sartorius), aliquoted (1 mL/vial), and stored at − 20 °C. We will call this fraction "AFA extract."

Animals

Colonic tissues were obtained by adult Wistar male rats (8–9 weeks old, 250–300 g) randomly assigned into six groups ($n = 5$ animals/each): (1) control group (sham group); (2) group with colitis (DNBS); (3) group with colitis + AphaMax® (50 mg/Kg/day); (4) group with colitis + AphaMax® (100 mg/Kg/day); (5) group + AphaMax® (50 mg/Kg/day); (6) group + AphaMax® (100 mg/Kg/day).

To induce the colitis intracolonic (i.c) instillation of 2, 4-dinitrobenzensulfonic acid (DNBS; Sigma-Aldrich Inc., St. Louis, MO, USA), was performed as already described [18–20]. The experimental protocol, followed throughout the study, was conducted in the conformity of the Italian D.Lgs 26/2014, following the criteria outlined by the European Community Council Directive 2010/63/UE, recognized and adopted by the Italian Government, and approved by the Ethical Committee for Animal Experimentation of the University of Palermo and by the Italian Ministry of Health (Authorization n 921/2018–released Rome, Italy).

AFA extract (Nutrigea Research s.r.l., Borgo Maggiore, Republic of San Marino) was dissolved in 0.5 mL water, and then sonicated (twice for 60 s) immediately prior to the gavage. 50 or 100 mg/kg AFA solution, which we will refer to as AFA, was administered by oral gavage once a day for 14 days starting 7 days before the induction of colitis (day-7).

Briefly, rats were fasted overnight and then, under light anesthesia with 1% isoflurane (Merial Italia Spa, Assago, MI, Italy), DNBS (15 mg) dissolved in a solution of 50% ethanol, was deposited in the colon through an 8 cm plastic catheter (PE90).

To avoid reflux actions, the rats were kept for 5 min in a Trendelenburg position and then allowed to recover with food and water supplied. The control group (sham group) received i.c instillation of vehicle alone (50% ethanol).

None of the rats died during the study or was excluded from the study for any reason. Assessment of colitis-induced damage was performed minimizing the suffering of animals, in a blinded fashion as previously described [18–21].

At the end of the experimentation period rats were killed and colon collected for analyses of DNA methylation.

Cell culture and treatments

In our experiments, working concentrations of AFA were fixed at 10 μg/mL (AFA10) and 200 μg/mL (AFA200). The reason for this choice is that pilot studies showed that AFA was not cytotoxic for Caco-2 cells, whatever the experimental treatment (data not shown). Therefore, we wanted to test both low concentrations and the highest concentration that does not exhibit toxic effects.

The Caco-2 cell line, derived from a human colon adenocarcinoma (American Type Culture Collection, ATCC), was used between passages 20 and 30 and cultured at 37 °C in a humidified atmosphere of CO_2/air (5/95, v/v) in Dulbecco's MEM supplemented with 25 mM HEPES, 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) (Hyclone Perbio-Sciences), 1% penicillin (10 9 103 U/mL)-streptomycin (10 mg/mL), and 1% (v/ v) non-essential amino acids (NEAA) (Invitrogen) as described by [17]. Differentiated cells, grown to confluence for 18–21 days in 12-well plates replacing the media every 3 days, were used for the experiments.

For the treatments, cells were washed with phosphate buffer saline, pH 7.4 (PBS), and each treatment was applied to cells in culture medium containing 1% (v/v) FBS.

Co-treatment Cells were incubated with or without 25 ng/mL of IL-1β (SignalChem) and with different concentration of AFA for total time of 24 h.

Pre-treatment Following incubation, cells were preincubated with or without 25 ng/mL of IL-1β (SignalChem) for 24 h. Subsequently, the culture medium was replaced with fresh medium with or without AFA at different concentrations for 24 h. Control cells were incubated with medium alone.

After incubation, the supernatants were collected, and DNA or RNA were extracted.

Cytotoxicity of AFA extract on Caco-2 cells was checked in pilot studies using the Trypan Blue (Sigma Chemical Co.) exclusion method and the MTT (Sigma Chemical Co.) assay.

Genomic DNA isolation

Isolation of genomic DNA from differentiated Caco-2 cells and colon tissue was carried out with the PureLink Genomic DNA Kit (Invitrogen, UK) as previously described. The obtained DNA was quantified by NanoDrop® ND-1000 [22].

Epigenomic assessment of DNA methylation

To assess the possible genomewide changes in DNA methylation, Methylation-Sensitive Arbitrarily-Primed PCR (MeSAP-PCR) was performed as previously described by [17]. This is a technique that involves methylation-sensitive restriction of genomic DNA, coupled with two consecutive PCR reactions to identify altered methylation patterns at different sites with a preference for those rich in GC. This technique, which provides a qualitative and semi-quantitative estimate of DNA methylation levels at the genomic level, allows us to highlight differences in methylation between genomes.

Gene promoter methylation status assessment by methylation-sensitive restriction endonuclease-PCR (MSRE-PCR)

For the analysis of gene promoter methylation status, MSRE-PCR was performed as described by Bellavia et al. [23]. Genomic DNA from differentiated cells was separately digested with HpaII/MspI and HhaI methylation-sensitive restriction endonucleases (MSREs) and amplified by PCR in the presence of specific primers for

the cg sites of *IL6, IL8* and *IL10* genes, important for their expression [24–26]. Were studied seven sites for *IL6* gene (four for HhaI MSRE: IL6/1, IL6/2, IL6/4, IL6/6 and three for HpaII/MspI MSRE couple: IL6/3, IL6/5, IL6/7), two sites for *IL8* gene (IL8/1 and IL8/2 for HpaII/MspI MSRE couple) and three sites for *IL10* gene (two for HpaII/MspI MSRE couple: IL10/1, IL10/3 and one for HhaI MSRE: IL10/2).

Primers were designed using the bioinformatics software BLAST (http://blast.ncbi.nlm.nih.gov/Blast.igi).

Primers sequences and PCR settings are listed in supplementary files Tables 1–8.

RNA extraction and real-time PCR

Caco-2 cells were grown in 6-well plates and treated with or without 25 ng/mL of IL-1β following co-treatment or pre-treatment model as mentioned above. RNA was extracted using the commercially available (Qiagen GmbH Hilden, Germany), according to manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using QuantiTect Reversion Trascription Kit (Qiagen GmbH Hilden, Germany). RT-QPCR was performed in 96-well plates using the Step-One Plus Real-Time PCR system (Applied Biosystem). For quantitative SYBR®Green real-time PCR, using Quantinova SYBR®Green PCR Kit, Quantitect® Primer Assay was purchased from Qiagen.

Relative changes in gene expression between control and treated samples were determined with the $\Delta\Delta C_t$ method. Final values were expressed as fold change.

ELISA Test

Pro-inflammatory cytokines levels (IL-6 and IL-8) and the anti-inflammatory IL-10 cytokine level were quantified using specific ELISA kits (SEA079Hu, Cloud-Clone Corp., Wuhan, China; orb437375, Biorbyt Biotechnology Co., Wuhan, China) (SEA056Hu, Cloud-Clone Corp., Wuhan, China). The assays were performed using culture supernatants of differentiated Caco-2 cells treated with IL1β and AFA10/200 μg/mL for 24 h, following the manufacturer's instructions. The absorbance was finally measured in a microplate reader (Glomax, Promega Milan, Italy) at 450 nm.

Statistical analysis

Statistical analysis was conducted using Prism for Windows, (GraphPad Software Inc., San Diego, CA, USA). We employed one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. A significance level of $p < 0.05$ was used to determine statistically significant differences. Data are presented as means ± standard deviation (SD).

Results

DNA methylation of rat colonic tissue

As previous demonstrated AFA treatment is able to reduce inflammatory condition during experimental colitis in rats [12]. We want to evaluate if AFA anti-inflammatory effects should be related to a possible epigenetic mechanism such as DNA methylation.

Our data showed (Fig. 1A–C) in colon tissue from DNBS-group a global DNA demethylation compared to the sham group; the treatment of colitis groups with AFA50 or AFA100 leads to a DNA hypermethylation compared to the control group, in particular the group treated with AFA50 and AFA100 showed a DNA slight hypermethylation compared to the sham group (Fig. 1C).

Epigenetic effects of AFA on DNA methylation in differentiated Caco-2 cells

To investigate whether DNA methylation undergoes alterations following treatments with IL1β and AFA, we performed the MeSAP-PCR (Fig. 2A, C, E), using the experimental models previously described. Our findings reveal significant changes in DNA methylation patterns in response to both IL1β and AFA treatments. As shown in Fig. 2B treatment with IL1β for 24 h induced a slight hypomethylation compared to the control. Simultaneous co-treatment with IL1β and AFA10 led to hypermethylation, whereas co-treatment with IL1β and AFA200 led to hypomethylation. In contrast, after 24 h of IL1β pre-treatment followed by fresh DMEM for another 24 h (Fig. 2D), DNA hypomethylation was induced in Caco-2 cells. However, when IL1β pre-treatment was followed by AFA10 and AFA200 treatment foe 24 h, an increase in methylation was observed. The treatment with AFA10 or AFA200 alone for 24 h in differentiated Caco-2 cells induced a slight hypermethylation (Fig. 2F).

(See figure on next page.)

Fig. 1 Representative MeSAP-DNA fingerprinting and relative scanning densitometry **A**, **B** indicating genomic DNA methylation of rat colon tissue treated with: **A** (from left) Sham group (CTR); 2,4-dinitrobenzene sulfonic acid (DNBS); 2,4-dinitrobenzene sulfonic acid + AFA 50 mg/Kg/day (DNBS + AFA50); **B** (from left) 2,4-dinitrobenzene sulfonic acid + AFA 100 mg/Kg/day (DNBS + AFA100); AFA 50 mg/Kg/day (AFA50); AFA 100 mg/Kg/day (AFA100); and relative graphic representations of densitometry analysis **C** band pattern variation, in terms of intensification/weakening and appearance/disappearance was evaluated by densitometer scanning of mono- digested DNA (MD) in comparison with double-digested DNA (DD)

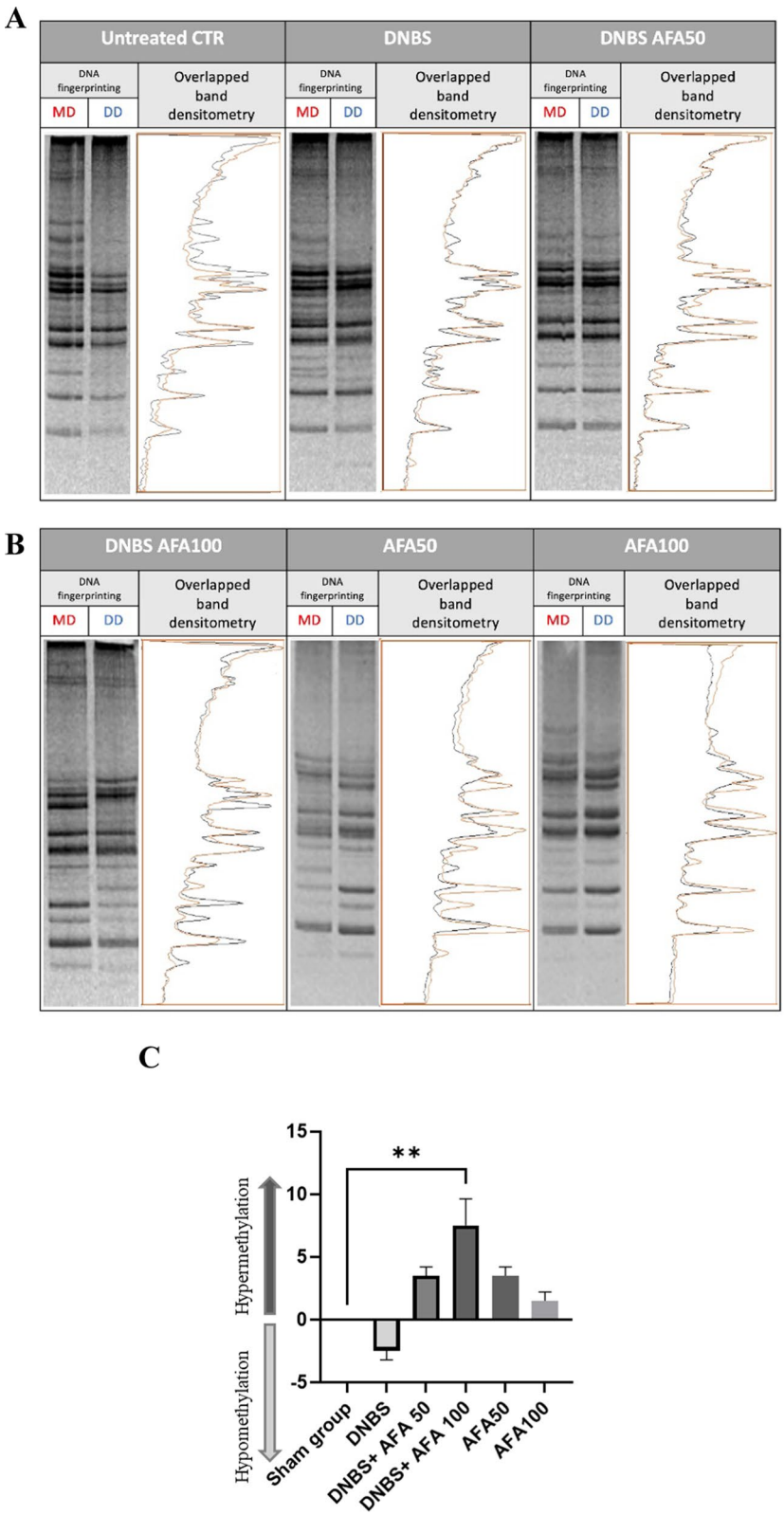


Fig. 1 (See legend on previous page.)

DNA methylation in the promoter region of inflammation-related genes

To assess whether AFA had an effect on the methylation of the promoter region of certain genes associated with the inflammatory process, we investigated the upstream region of the *IL6*, *IL8* and *IL10* pro-inflammatory and anti-inflammatory genes in Caco-2 cells co-treated with IL1β and AFA for 24 h and in cells pre-treated with IL1β for 24 h and subsequent AFA treatment at different concentrations in fresh media, for another 24 h.

IL6 gene

In the *IL6* gene promoter (Fig. 3) we examined the methylation status of seven restriction sites containing methylable CpGs. For the co-treatments experimental scheme, this study showed that when we administered IL1β for 24 h, it induced significant demethylation at the IL6/1, IL6/3, and IL6/6 sites. Only at the IL6/5 site IL1β induced hypermethylation compared to the untreated control. Subsequent treatment with AFA restored the methylation of the IL6/1 and IL6/6 sites in a dose-dependent manner to levels comparable to the untreated control. For the IL6/3 site, AFA10 did not induce a change in methylation compared to IL1β treatment, whereas AFA200 induced hypermethylation of this site compared to IL1β treatment. Conversely, AFA200 induced hypomethylation at the IL6/4 and IL6/7 sites.

For the pre-treatments, the study of CpG site methylation in the promoter of the *IL6* gene (Fig. 4) showed that treatment with IL1β for 24 h followed by fresh DMEM induced demethylation at sites IL6/2 and IL6/5 but did not affect the methylation status of sites IL6/3, IL6/4 and IL6/7, confirming what was also observed by Caradonna et al. [10]. When cells were treated with AFA10 following by IL1β insult, an increase in methylation was observed compared to treatment with IL1β alone at all analyzed sites. However, treatment with AFA200 induced hypomethylation compared to untreated control at all analyzed sites.

IL8 gene

The study of CpG site methylation in the promoter of the *IL8* gene (Fig. 5) in cells co-treated with IL1β and AFA for 24 h at different concentrations has shown that the IL8/1 site undergoes slight demethylation following treatment with AFA at both concentrations compared to the untreated control, which is already moderately methylated. Only AFA10 is able to induce demethylation of the IL8/2 site, which is highly hypermethylated in the untreated control.

For pre-treatments with or without IL-1β, as depicted in Fig. 6, IL1β induced a reduction in methylation at both analyzed CpG sites at the promoter of the *IL8* gene. Subsequent treatment with AFA at both tested concentrations did not induce further significant changes in the methylation of these two sites compared to treatment with IL1β alone.

IL10 gene

In the promoter region of the *IL10* gene, encoding an anti-inflammatory cytokine, we examined two CpG sites. The methylation of the analyzed CpG sites in the promoter of the *IL10* gene (Fig. 7) was not affected by treatments with either IL1β or AFA10. Only AFA200 induced a non-significant reduction in methylation in the two analyzed sites.

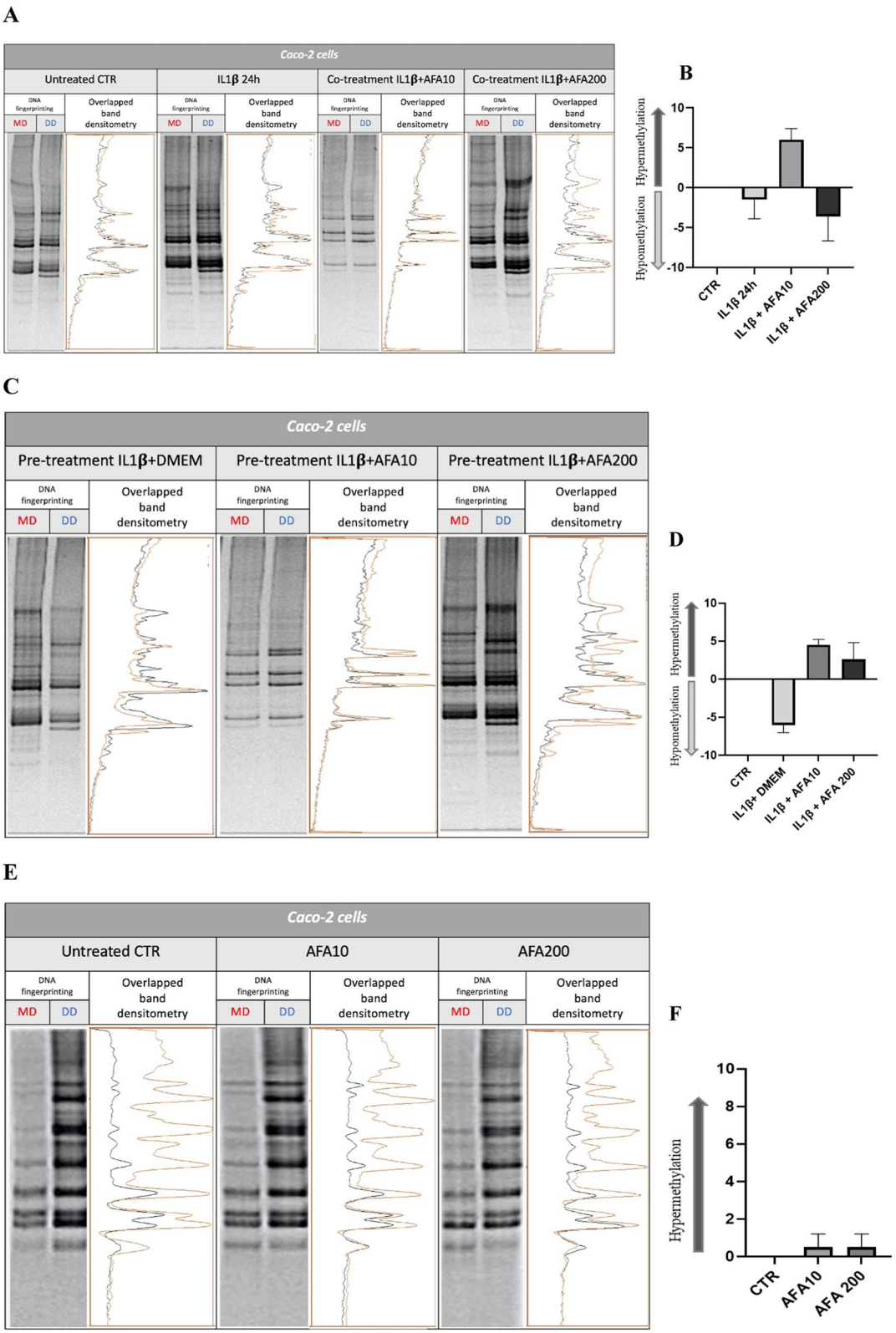
Instead, the results of IL1β pre-treatments demonstrate a slight demethylation at both CpG sites compared to untreated cells, as shown in Fig. 8. Interestingly, treatment with AFA10 following IL1β insult induces an increase in methylation compared to untreated control or IL1β-treated cells. AFA200 appears to reduce methylation of *IL10/2*.

Cytokines expression

The gene expression levels of the cytokines IL6 and IL8 were evaluated following various treatments and compared to untreated control Caco-2 cells. Figure 9A shows that the co-treatment of IL1β and AFA increases the expression of IL6 in a dose-dependent manner. Furthermore, while pre-treatment with IL1β for 24 h replaced with fresh medium increased the expression of IL6, subsequent treatment with AFA10 further significantly increases the expression of IL6 compared to the untreated control (Fig. 9B). In Fig. 9C and D, the levels of IL-6 released into the medium, as assessed by

(See figure on next page.)

Fig. 2 Representative MeSAP-DNA fingerprinting and relative scanning densitometry (**A**, **C**, **E**), indicating genomic DNA methylation of Caco-2 differentiated cells: **A** (from left) untreated (CTR), 25 ng/mL IL-1β 24 h (IL-1β 24 h), co-treatment 25 ng/mL IL-1β 24 h + 10 ng/μL 24 h (co-treatment IL-1β + AFA10), co-treatment 25 ng/mL IL-1β 24 h + 200 ng/μL 24 h (co-treatment IL-1β + AFA200); **C** (from left) 25 ng/mL IL-1β 24 h + only DMEM 24 h (IL-1β + DMEM), 25 ng/mL IL-1β 24 h + 10 ng/μL 24 h (IL-1β + AFA10), 25 ng/mL IL-1β 24 h + 200 ng/μL 24 h (IL-1β + AFA200); **E** (from left) untreated (CTR), AFA10 ng/μL treatment 10 ng/μL 24 h (AFA10), AFA 200 ng/μL 24 h (AFA200), and relative graphic representations of densitometry analysis for co-treatments **B** or subsequent pre-treatments **D** or treatment of differentiated Caco-2 cells with AFA10 and AFA200 **F**. Band pattern variation, in terms of intensification/weakening and appearance/disappearance was evaluated by densitometer scanning of mono-digested DNA (MD) in comparison with double-digested DNA (DD)



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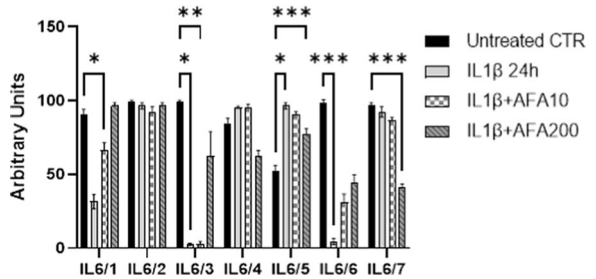

Fig. 3 MSRE-PCR revealing methylation status of CG sites in the promoter region of IL6 gene, from Caco-2 cells treated with IL-1β, co-treated with IL-1β + AFA10 and IL-1β + AFA200. Densitometry of the bands is shown by histograms, which represent the mean of three experiments. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.005$

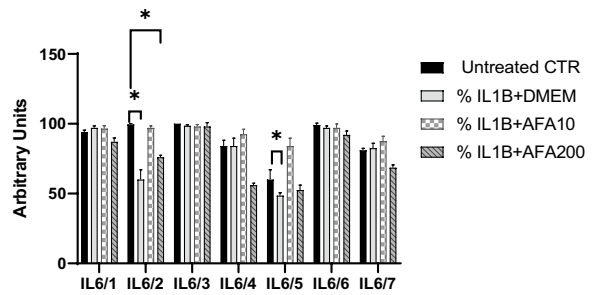

Fig. 4 MSRE-PCR reveals methylation status of CG sites in the promoter region of IL6 gene, from Caco-2 cells treated with IL-1β, subsequent treated with AFA10 and AFA200. Densitometry of the bands is shown by histograms, which represent the mean of triplicate experiments with similar results. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$

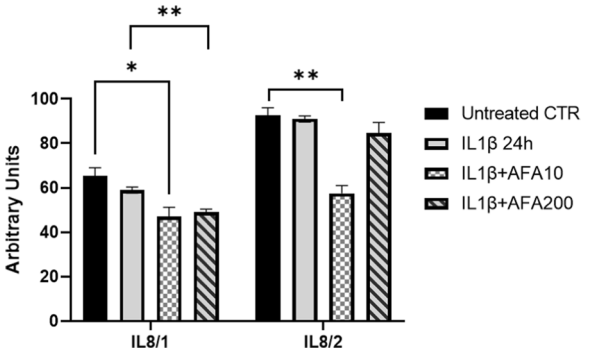

Fig. 5 MSRE-PCR revealing methylation status of CG sites in the promoter region of IL8 gene, from Caco-2 cells treated with IL-1β, co-treated with IL-1β + AFA10 and IL-1β + AFA200. Densitometry of the bands is shown by histograms, which represent the mean of triplicate experiments with similar results. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$

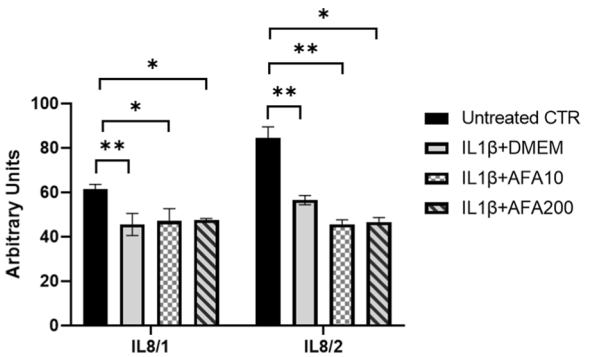

Fig. 6 MSRE-PCR revealing methylation status of CG sites in the promoter region of IL8 gene, from Caco-2 cells treated with IL-1β, subsequent treated with AFA10 and AFA200. Densitometry of the bands is shown by histograms, which represent the mean of triplicate experiments with similar results. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$

ELISA assay, are shown. As observed, AFA significantly increases IL-6 release following the inflammatory insult. In contrast, the co-treatment with IL-1β and AFA does not significantly alter IL-6 release.

Similarly, the expression of IL8 was analyzed. As seen in Fig. 10A, treatment with IL1β for 24 h significantly increases the expression of IL8. However, the co-treatment with AFA10 and AFA200 significantly reduces the expression of IL8. The release of cytokines into the medium was also investigated, and as shown in Fig. 10C. Treatment with IL-1β alone for 24 h increased the release of IL8 into the medium. However, when the cells were co-treated with AFA along with the inflammatory insult, a reduction in the release of the pro-inflammatory cytokine IL8 was observed. Likewise, pre-treatment for 24 h with IL1β followed by replacement with fresh medium for the subsequent 24 h increases the expression of IL8 compared to the untreated control. Treatment with AFA10 following IL1β pre-treatment reduces the expression of IL8 to the levels of the untreated control. No effect is induced by treatment with AFA200 (Fig. 10B). The same trend (although not statistically significant) can also be observed in the release of the cytokine IL8 into the medium following treatment with the IL1β and subsequent treatment with AFA (Fig. 10D).

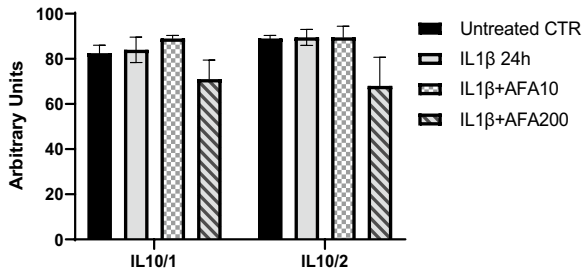

Fig. 7 MSRE-PCR revealing methylation status of CG sites in the promoter region of IL10 gene, from Caco-2 cells treated with IL-1β, co-treated with IL-1β + AFA10 and IL-1β + AFA200. Densitometry of the bands is shown by histograms, which represent the mean of triplicate experiments with similar results. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$

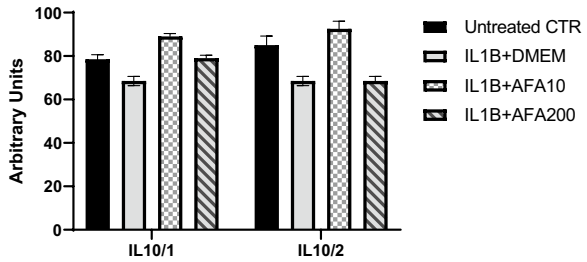

Fig. 8 MSRE-PCR revealing methylation status of CG sites in the promoter region of IL10 gene, from Caco-2 cells treated with IL-1β, subsequently treated with AFA10 and AFA200. Densitometry of the bands is shown by histograms, which represent the mean of triplicate experiments with similar results

Regarding the expression levels of IL10, the basal levels of this cytokine were so low that it was not possible to detect this cytokine with any of the techniques used.

DNMTs expression

One of the mechanisms for establishing variations in methylation patterns that may be associated with the establishment of a phenotypic condition such as inflammation is the increase in the activity or quantity of DNA methyltransferases (DNMTs). DNMTs are enzymes responsible for DNA methylation. For this reason, we studied the expression of DNMTs in Caco-2 cells after co-treatment with IL1β and AFA at different concentrations (Fig. 11A) or treated with the inflammatory stimulus and with AFA (Fig. 11B).

The treatment with IL1β alone for 24 h induced an increase in the expression of DNMT3A, which was restored to control levels by the co-treatment with IL1β and AFA at any concentration (Fig. 11A). Instead, the treatment of Caco-2 cells with IL1β for 24 h followed by replacement with fresh medium induced a significant reduction in the expression of DNMT3A compared to the untreated control (Fig. 11A). Treatment with IL1β for 24 h followed by subsequent treatment with AFA10 further reduced the expression of DNMT3A. However, subsequent treatment with AFA200 did not appear to have an additional effect compared to treatment with IL1β alone (Fig. 11B).

Figure 12 shows that, regardless of the treatment conditions, the expression levels of DNMT3B are increased following treatment with IL1β alone or IL1β and AFA at any concentration. Specifically, in Fig. 12A, a significant increase in the levels of DNMT3B expression is shown following treatment with IL1β for 24 h and in a dose-dependent manner following co-treatment with IL1β and AFA.

In the co-treatment of IL1β and AFA, it was observed that treatment of Caco-2 cells with IL1β alone induced an increase in DNMT1 levels. However, co-treatment of IL1β and AFA at different concentrations restored the expression levels of DNMT1 to those of the untreated control (Fig. 13A). The treatment with the inflammatory stimulus (IL1β) for 24 h followed by fresh medium induced a slight increase in the expression of DNMT1. This is reversed to control levels when cells are treated with AFA10. Treatment with AFA200, on the other hand, did not reduce the expression of DNMT1 induced by IL1β (Fig. 13B).

Discussion

Chronic inflammation promotes disease development and progression by causing prolonged tissue damage, immune dysregulation, and abnormal tissue remodeling [1]. Persistent stimuli like infection or injury trigger sustained inflammation, leading to cardiovascular and neurodegenerative diseases, metabolic syndrome, and cancers [2]. In this context, inflammation is a key therapeutic target for many diseases [3, 4].

DNA methylation, is key regulators of gene expression and genomic stability, often leading to gene silencing [5]. Inflammation may influence DNA methylation patterns [6, 7]. Inflamed tissues show global hypomethylation and site-specific hypermethylation, affecting genes involved in inflammatory pathways, which contributes to the development of diseases [8]. Understanding this interplay is crucial for identifying therapeutic targets.

Recently, natural compounds have been explored for their therapeutic potential. AFA, a cyanobacterial species from Upper Klamath Lake, Oregon, is recognized as a "superfood" due to its nutritional value [14, 27]. Studies highlight AFA's neuroprotective effects, stress reduction, and menopausal well-being improvements [11]. AFA also mitigate inflammatory condition during experimental colitis in murine model [12]. Given AFA's documented

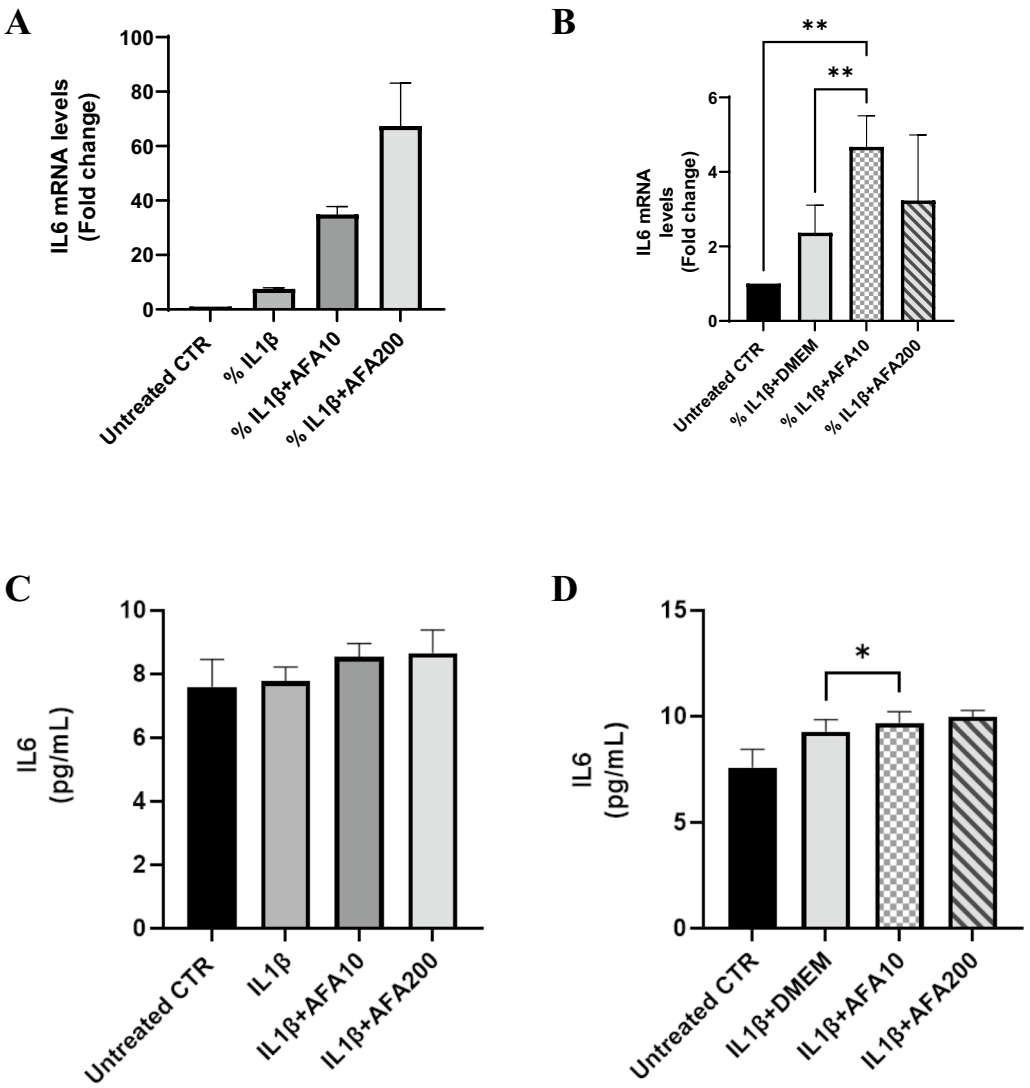

Fig. 9 Expression of IL6 gene in Caco-2 differentiated cells treated with IL-1β and AFA. **A** Histogram showing the IL6 mRNA levels after co-treatments with IL-1β and AFA10/AFA200; **B** Histogram showing the IL6 mRNA levels after treatments with IL-1β for 24 h after incubation, the culture medium was replaced with fresh medium with or without AFA10/AFA200. **C** Histogram showing the IL6 released levels after co-treatments with IL-1β and AFA10/AFA200; **D** Histogram showing the IL6 released levels after pre-treatments with IL-1β for 24 h and then treatment with or without AFA10/AFA200. Data in the histograms are presented as the mean of triplicate experiments. Statistical analysis performed with one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$

impact on inflammation, we hypothesize that AFA may exert its effects through the modulation of DNA methylation patterns. We aim to uncover new insights into AFA's therapeutic mechanisms, emphasizing its potential as a nutraceutical agent for epigenetic regulation in inflammatory processes.

This study investigates the nutrigenomic and epigenetic effects of AFA, focusing on its influence on DNA methylation patterns of inflammation-related genes. Previous data obtained from colon tissue of rats treated with AFA prompted us to further investigate the mechanism of action of AFA and determine if it could be considered a modulator of DNA methylation. Indeed, analyzing DNA methylation of colon tissue from rats (Fig. 1A, B), we observed that DNBS-induced colitis led to demethylation compared to the sham group. However, treatment with AFA resulted in hypermethylation compared with the sham group (Fig. 1C). Specifically, treatment with AFA alone leads to an increase in global DNA methylation, furthermore, when administered in the presence of an inflammatory insult, AFA100 is able to determine a greater hypermethylating effect. This is likely because its

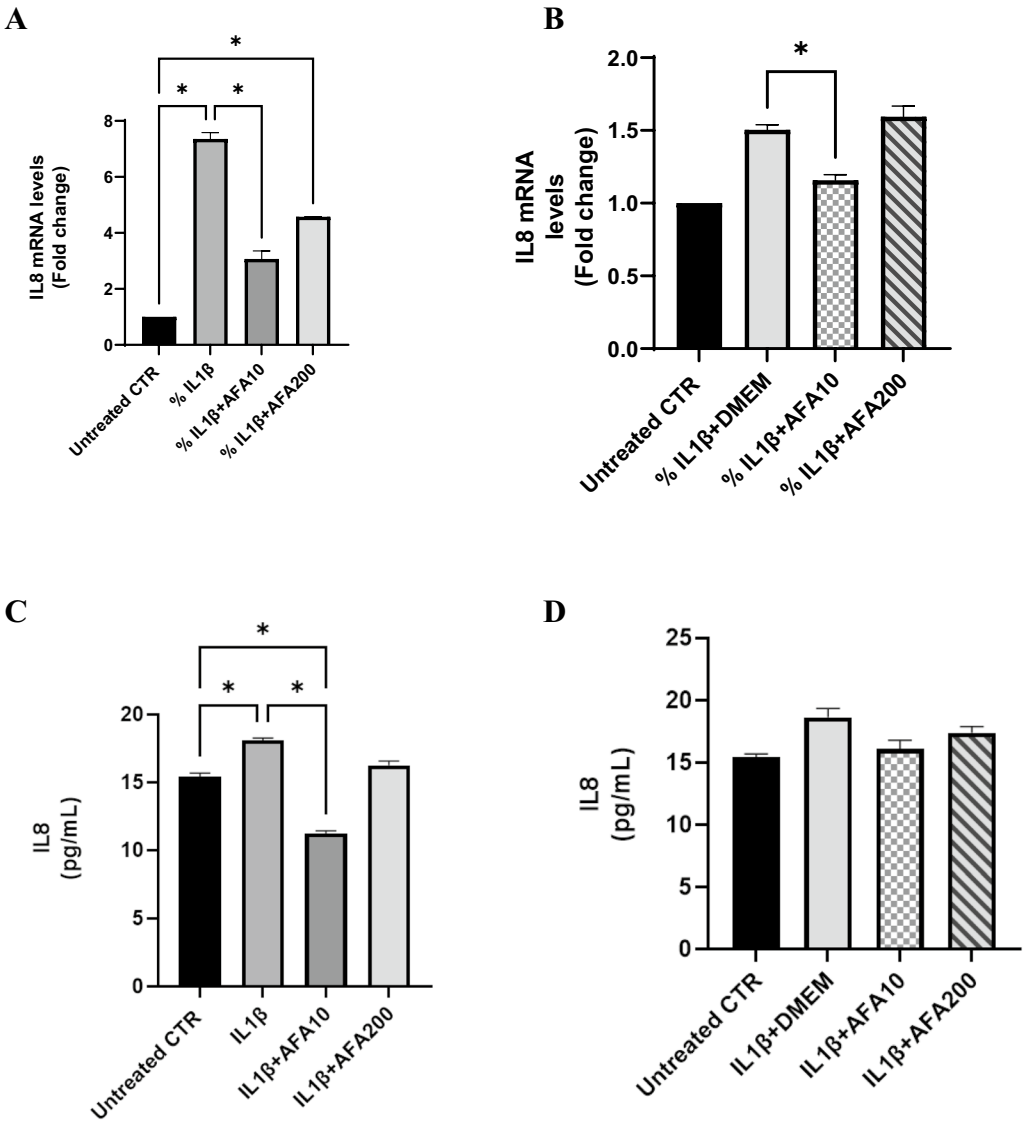

Fig. 10 Expression of IL8 gene in Caco-2 differentiated cells treated with IL-1β and AFA. **A** Histogram showing the IL8 mRNA levels after co-treatments with IL-1β and AFA 10/AFA200; **B** Histogram showing the IL8 mRNA levels after treatments with IL-1β for 24 h after incubation, the culture medium was replaced with fresh medium with or without AFA10/AFA200. **C** Histogram showing the IL8 released levels after co-treatments with IL-1β and AFA10/AFA200; **D** Histogram showing the IL8 released levels after treatments with IL-1β for 24 h after incubation, the culture medium was replaced with fresh medium with or without AFA10/AFA200. Data in the histograms are presented as the mean of triplicate experiments. Statistical analysis performed with one-way ANOVA and Tukey's multiple comparisons test. Differences were considered significant at $^{*}p < 0.05$

action probably contributes to regulating other mechanisms that influence the global DNA methylation status, such as microRNA expression.

By utilizing Caco-2 cells as a model system, we employed different treatment models to represent either AFA administration occurring simultaneously with an inflammatory stimulus or AFA treatment following an inflammatory stimulus (model of chronic inflammation). We observed that different treatment models exhibited varying effects of AFA on the modulation of global DNA methylation. The difference in the timing of AFA administration may influence its ability to modulate the methylation landscape, leading to the observed variability in DNA methylation patterns. Specifically, treatment with IL1β for 24 h induced a slight hypomethylation compared to the control. Simultaneous co-treatment with IL1β and AFA10 led to hypermethylation. Contrarily, co-treatment with IL1β and AFA200 led to hypomethylation

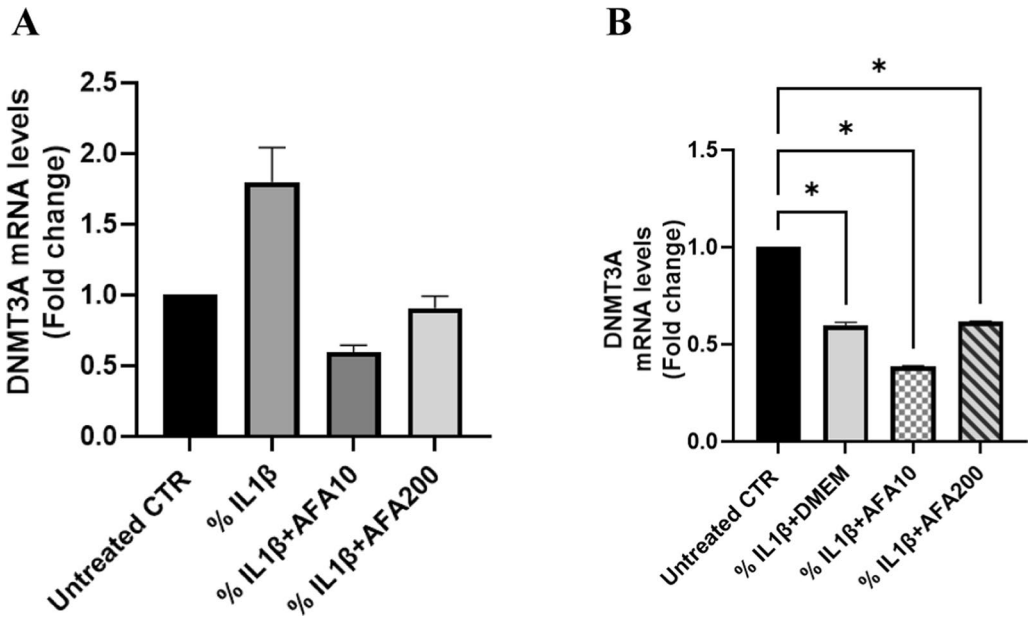

Fig. 11 Expression of DNMT3A gene in Caco-2 differentiated cells treated with IL-1β and AFA. **A** Histogram showing the DNMT3A mRNA levels after co-treatments with IL-1β and AFA 10/AFA200; **B** Histogram showing the DNMT3A mRNA levels after treatments with IL-1β and AFA10/AFA200. Data in the histograms are presented as the mean of three experiments with similar results. Statistical analysis performed with one-way ANOVA and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$

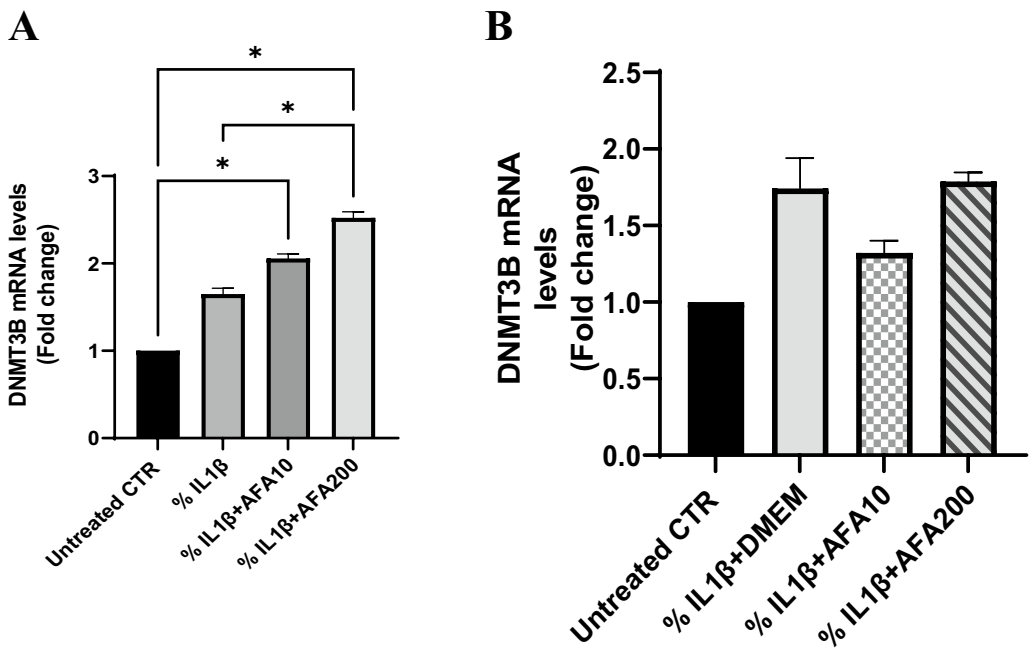

Fig. 12 Expression of DNMT3B gene in Caco-2 differentiated cells treated with IL-1β and AFA. **A** Histogram showing the DNMT3B mRNA levels after co-treatments with IL-1β and AFA 10/AFA200; **B** Histogram showing the DNMT3B mRNA levels after treatments with IL-1β and AFA10/AFA200. Data in the histograms are presented as the mean of triplicate experiments with similar results. Statistical analysis performed with one-way ANOVA and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$

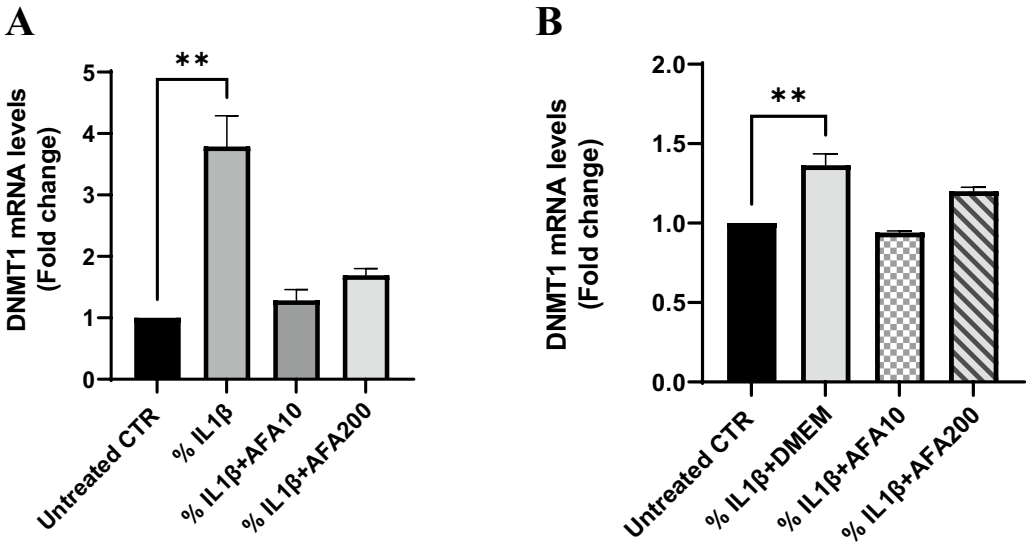

Fig. 13 Expression of DNMT1 gene in Caco-2 differentiated cells treated with IL-1β and AFA. **A** Histogram showing the DNMT1 mRNA levels after co-treatments with IL-1β and AFA10/AFA200; **B** Histogram showing the DNMT1 mRNA levels after treatments with IL-1β and AFA10/AFA200. Data in the histograms are presented as the mean of triplicate experiments with similar results. Statistical analysis performed with one-way ANOVA and Tukey's multiple comparisons test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$

(Fig. 2B). Similarly, treatment with IL1β for 24 h followed by fresh medium for another 24 h led to global DNA demethylation. However, when AFA10 and AFA200 were added after the inflammatory stimulus (IL1β for 24 h), global hypermethylation was induced compared to the untreated control (Fig. 2D). These results are consistent with existing literature and confirm the relationship between inflammatory status and changes in DNA methylation pattern [28–30].

In particular, inflammation induces global DNA demethylation, making it more unstable. Here, most treatments with AFA restore methylation status, counteracting the induced inflammatory effects and likely leading to a more stable DNA. Furthermore, it was observed that solely AFA200 co-treated with IL1β, induced hypomethylation. This phenomenon suggests an inability of AFA 200 to mitigate the impact of the simultaneous inflammatory stimulus on DNA methylation. Intriguingly, this combination exhibits an additive effect concerning methylation in contrast to the inflammatory stimulus alone. To the best of our knowledge, no studies to date have demonstrated the effect of microalgae on human DNA methylation, highlighting a crosstalk between the animal and plant kingdoms. With this work, we are the first to assert that microalgae can interact with and modulate human/animal DNA methylation, demonstrating their functional role in cross-kingdom interaction.

We also studied the methylation of promoters of certain genes encoding pro-inflammatory and anti-inflammatory interleukins, as it has been shown that these are subject to epigenetic regulation [10]. We examined single methylation sites, revealing the different effects of AFA depending on the experimental treatment method. In particular, an overview of the *IL6* gene (Fig. 3) suggests that in cases of co-treatments (the inflammatory stimulus and AFA at different concentrations), AFA significantly affects DNA methylation at specific sites compared to the inflammatory stimulus alone. We hypothesize that certain methylation sites in the *IL6* gene promoter exhibit varying sensitivities to external agents, which interact in some way with the epigenetic machinery. Indeed, following IL1β-AFA co-treatment, an increase in IL6 gene expression is observed (Fig. 9A).

It is worth noting that IL-6 has a dual role, acting as both a pro-inflammatory and an anti-inflammatory cytokine. The pro-inflammatory effects of IL-6 are primarily mediated through classical signaling via the membrane-bound IL-6 receptor (mIL-6R), which leads to the activation of inflammatory pathways. Conversely, IL-6 can also exhibit anti-inflammatory properties through trans-signaling via soluble IL-6 receptors (sIL-6R), promoting the production of anti-inflammatory molecules [31, 32]. Given the proven anti-inflammatory effects of AFA, the increase in IL6 expression following AFA treatment (Fig. 9A, B) regardless of whether it was co-treated with the inflammatory stimulus or added afterward, leads us to believe that it probably promotes the anti-inflammatory role already demonstrated for IL6.

For the *IL8* gene, co-treatment with AFA10 and IL1β significantly reduces methylation at only one methylation

site, whereas AFA200 does not induce any changes compared to the inflammatory stimulus alone (Fig. 5). However, the gene expression levels are significantly reduced by co-treatment with AFA at both concentrations compared to cells treated with IL1β alone (Fig. 10A). This suggests that mechanisms beyond DNA methylation are involved in the anti-inflammatory effect exerted by AFA.

By changing the experimental setup, the impact of AFA on DNA methylation varied. Specifically, replicating an acute inflammatory stimulus followed by resolution (IL1B + DMEM) or AFA treatment administered post-inflammatory stimulation. Regarding the study of methylated sites of *IL6* gene promoter (Fig. 4) following IL1β treatment for 24 h and subsequent, replacement with fresh medium, demethylation was observed only at certain sites (IL6/2 and IL6/5) (Fig. 4). Subsequent administration of AFA10 consistently induced hypermethylation across all methylated sites compared to the untreated control. However, treatment with AFA200 reduced methylation in many sites compared to the untreated control (Fig. 4). This nonuniform behavior of AFA prompted us to consider the potential implications. The overall results suggest that, when AFA is administered concurrently with the inflammatory stimulus, it fails to counteract the IL1β effects (Figs. 3, 5). However, when AFA10 was administered following the inflammatory stimulus, which was no longer present, it was able to contribute to the restoration of methylation (Figs. 4, 6). Furthermore, these latter results have demonstrated a dose-dependent effect of AFA, indicating that its outcome relies on the concentration at which it is administered.

Considering that one of the factors contributing to an altered pattern of DNA methylation is a change in the expression of DNMTs [22], we aimed to investigate the effect of AFA on the expression of DNMTs in both experimental model studied. We analyzed the expression levels of DNMTs responsible for de novo DNA methylation (DNMT3A and DNMT3B), whose substrates are symmetrically unmethylated on both DNA strands. These results, however, show a different behavior of AFA depending on the experimental model. On the one hand, AFA co-treatment with the inflammatory stimulus (Fig. 11A), reduced the expression of DNMT3A, compared to treatment with the inflammatory stimulus alone. Similarly, when AFA was added following the inflammatory stimulus (Fig. 11B), it significantly reduced the expression of DNMT3A. On the other hand, AFA was able to significantly increase the expression of DNMT3B when added in co-treatment with IL1β (Fig. 12A), or when AFA was administered following the inflammatory stimulus (Fig. 12B). Although AFA increases the expression (both gene and protein levels data are shown in the supplementary files) of DNMT3B, the activity of these enzymes could also be regulated by AFA. In this study, we observed (Figs. 1, 2) an increase in global DNA methylation along with an increase in DNMT3B expression. Therefore, we hypothesize that, at least for DNMT3B, AFA may enhance its expression thereby also increasing its overall enzymatic activity, enabling it to produce detectable changes in global DNA methylation. We then analyzed DNMT1 (Fig. 13), responsible for DNA methylation maintenance, and observed an increase in DNMT1 following treatment with the inflammatory stimulus alone (Fig. 13A). However, co-treatment with AFA reduced the expression of DNMT1, bringing it back to levels similar to the untreated control (Fig. 13A). AFA treatment following IL1β did not alter the levels of DNMT1 compared to the untreated control (Fig. 13B).

These findings lead us to two different considerations. The different actions of AFA toward the different DNMTs (maintenance or de novo) suggests that the mechanism by which AFA induces epigenetic changes may preferentially affect de novo methylation rather than altering pre-existing methylation. This likely explains the different behaviors of AFA even in the two experimental models. Additionally, it is known that the two de novo DNMTs (DNMT3A and DNMT3B) act on different DNA portions [33]. The decrease in one (DNMT3A) and the increase in the other (DNMT3B) could be the result of AFA's specificity in activating one pathway over another.

Based on these results, we can confirm that AFA's anti-inflammatory effect operates through an epigenetic mechanism. AFA can modulate DNA methylation in human cells; however, further research is essential to fully understand the mechanisms through which AFA influences DNA methylation and its potential therapeutic effects in inflammatory processes.

Conclusions

Our study reveals significant findings on the impact of *Aphanizomenon flos-aquae* (AFA) on DNA methylation in the context of inflammation. By examining the effects of AFA on DNA methylation patterns during inflammatory conditions, in colon tissue from rats and using Caco-2 cells, we discovered that AFA could modulate global DNA methylation and gene-specific methylation differently depending on the experimental model and gene of interest. In More specifically, AFA treatment following an inflammatory stimulus resulted in hypermethylation more often than not, potentially stabilizing DNA and counteracting inflammation-induced demethylation. We also explored the methylation of promoters for genes encoding interleukins, revealing that AFA's effects are site-specific and dependent on the treatment model. For instance, co-treatment with AFA and IL1β significantly influenced *IL6* gene methylation and expression,

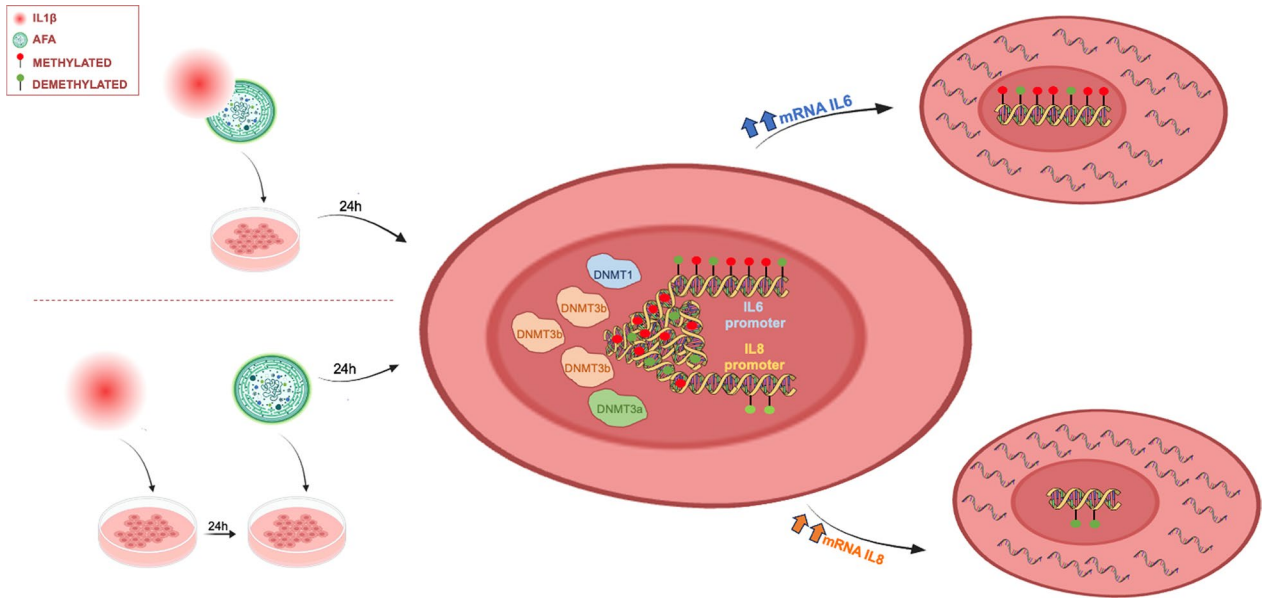

Fig. 14 A schematic diagram of the experimental plans involving differentiated Caco-2 cells co-treated for 24 h with the inflammatory stimulus (IL1β) and AFA and pre-treated for 24 h with the inflammatory stimulus (IL1β) and subsequently with AFA at different concentration for an additional 24 h. The image shows that AFA induces an alteration in the expression levels of DNA methyltransferases (DNMTs), consequently leading to global DNA hypermethylation. At a specific gene level, AFA induces hypomethylation of IL8 and IL6 genes, resulting in increased gene expression

suggesting differential sensitivity of methylation sites to external agents. Furthermore, AFA demonstrated distinct effects on DNMT expression, indicating that its epigenetic modulation may preferentially affect de novo methylation pathways (Fig. 14).

These findings for the first time suggest the microalgae's ability to modulate DNA methylation in human or animal models, highlighting a new aspect of cross-kingdom interaction. Our results suggest that AFA has the potential to serve as a nutraceutical agent targeting epigenetic regulation in inflammatory processes. Future research is essential to further elucidate the underlying mechanisms and therapeutic potential of AFA.

Abbreviations

AFA	Microalga *Aphanizomenon flos-aquae*
DNBS	2, 4-dinitrobenzensulfonic
MeSAP-PCR	Methylation-sensitive arbitrarily-primed PCR
MSREs	Methylation-sensitive restriction endonucleases
DNMTs	DNA methyltransferases

Glossary

Aphanizomenon flos-aquae (AFA)	A species of cyanobacteria, also known as "Klamath algae," known for its nutritional richness and potential therapeutic benefits, particularly in chronic diseases.
Epigenetics	The study of changes in gene function that do not involve changes in the DNA sequence, such as DNA methylation.
DNA Methylation	An epigenetic mechanism involving the addition of a methyl group to cytosine residues in CpG dinucleotides, often resulting in gene silencing.
CpG Dinucleotides	Regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide; common sites for DNA methylation.
Inflammation	A biological response to harmful stimuli, such as pathogens or damaged cells, which can be acute or chronic. Chronic inflammation is implicated in various diseases, including cardiovascular and neurodegenerative disorders, metabolic syndrome, and cancers.
Pro-inflammatory Interleukins	Cytokines that promote inflammation, such as IL1β, IL6, and IL8.
Anti-inflammatory Cytokines	Cytokines that reduce inflammation, such as IL10.
Nutrigenomics	The study of how foods and their constituents affect gene expression.
Colitis	Inflammation of the colon, which can be experimentally induced in rats for research purposes.
Caco-2 Cells	A human epithelial colorectal adenocarcinoma cell line used as a model for the intestinal barrier in research.
MSRE-PCR (Methylation-Sensitive Restriction Enzyme PCR)	A technique used to study DNA methylation patterns by using enzymes that cut DNA at specific sites depending on the methylation status.
DNBS (2,4-Dinitrobenzenesulfonic acid)	A chemical used to induce colitis in experimental models.
Cytokines	Small proteins important in cell signaling, with roles in the immune system.
Nutraceutical	A food or food product that provides health and medical benefits, including the prevention and treatment of disease.
Chronic Diseases	Long-term medical conditions that are generally progressive, such as heart disease, diabetes, and arthritis.
Hyper- and Hypomethylation	Hyper-refers to an increase in methylation of DNA, while hypo- refers to a decrease in methylation.

DMEM (Dulbecco's Modified Eagle Medium)	A type of cell culture medium used to grow cells.
CpG Sites	Specific regions of DNA with a cytosine followed by a guanine; often sites of methylation.
MTT Assay	A colorimetric assay for assessing cell metabolic activity. It is based on the reduction of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial enzymes of viable cells.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-01849-5.

Supplementary file 1.

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This article is dedicated to the memory of Dr. Stefano Scoglio (1957-2024), who made significant contributions to the promotion of research on Klamath algae as a source of biomolecules with potential applications in human health.

Author contributions
F.N., F.C., D.N., conceptualization and design of the study; G.D.S. provided the microalgae; S.V., P.S.C., S.M, A.C., acquisition of data; F.N., S.V., F.C., analysis and interpretation of data, F.N. and S.V. drafting the article; F.N. F.C., R.C., D.N., M.G.Z. revision of the article critically for important intellectual content; F.N., F.C., D.N., M.G.Z., P.P. final approval of the version to be submitted. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analyzed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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