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Dietary freeze-dried beer prevents inflammation in DSS-induced chronic ulcerative colitis in mice†

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Inflammatory bowel disease (IBD) is a complex condition that is influenced by numerous factors, including genetic, immune and environmental factors. In the search for new therapies, nutritional interventions including dietary polyphenols are becoming increasingly important in the management of IBD. The present study aimed to investigate the antioxidant and anti-inflammatory effects of commercial freeze-dried beer, an interesting product resulting from the fermentation of cereals, rich in polyphenols, in (I) an *in vitro* model of inflammation using lipopolysaccharide (LPS)-stimulated THP-1 human derived macrophages and (II) a murine dextran sodium sulfate (DSS)-induced chronic colitis model, for elucidating the action mechanism involved. According to the results, commercial freeze-dried beer exhibits antioxidant, anti-inflammatory and immunomodulatory properties in LPS-stimulated THP-1 human macrophages by reducing reactive oxygen species (ROS), tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels. Moreover, *in vivo* results showed that preventive treatment with dietary freeze-dried beer improved murine DSS-induced chronic colitis by attenuation of the clinical and histological signs of colonic damage. Colonic cytokine levels in animals fed with commercial freeze-dried beer reached values near basal levels. In addition, pro-inflammatory cyclooxygenase (COX)-2 and microsomal prostaglandin E synthase (mPGES)-1 protein expressions were significantly downregulated *via* inhibition of nuclear transcription factor kappa B (NF-κB) translocation. This inhibition may be mediated by an induction of the antioxidant nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway and a reduction of signal transducer and activator of transcription (STAT)-3 phosphorylation orchestrating its potent anti-inflammatory and immunomodulatory effects. Overall, our results suggest that dealcoholized beer may be effective in the management of immune-mediated inflammatory diseases in which macrophages are crucial, including IBD, providing clues for developing useful dietary interventions against inflammation-associated pathologies.

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1. Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) characterized by chronic inflammation of the rectum and colon. Abdominal pain, diarrhoea, unintentional weight loss, bloody and/or pus-like stools are some of the clinical symptoms UC patients exhibit.[1,2] Accumulating evidence shows that this inflammatory disorder is multifactorial, comprising interactions between genetic, immune, and environmental factors. Although the etiology of IBD is largely unknown, it involves an aberrant innate immune response in genetically susceptible individuals.[3] An increase in inflammatory mediators, such as pro-inflammatory cytokines or reactive oxygen species (ROS), and upregulation of certain enzymes, such as cyclooxygenase (COX)-2, play an important role in this immune dysregulation.[4] Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) is an important transcription factor against inflammation and oxidative stress that acts by regulating the expression of endogenous antioxidant enzymes, such as heme oxygenase-1 (HO-1).[5] The Nrf-2/HO-1 axis has been reported to be a modulator of the inflammatory response in experimental colitis. Since Nrf-2 is an important initiator of the antioxidant

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response in UC, the upregulation of this pathway may be an alternative strategy for the treatment of UC.[3,6] Another nuclear factor involved is nuclear transcription factor kappa B (NF-κB), which controls the activation of some pro-inflammatory cytokine genes supporting a key role in the pathogenesis of UC.[1] In addition, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is involved in cell function mediating the inflammatory immune response.[7] Particularly, STAT3 is a transcription factor implicated in innate and acquired immunity, making it a relevant target in the pathogenesis of UC. This factor is activated by phosphorylation of various growth factors and cytokines, which induce the expression of genes for pro-inflammatory enzymes and mediators.[8,9]

The murine dextran sodium sulfate (DSS)-induced model of colitis is one of the most widely used experimental models for studying the pathogenesis and innovative treatments for UC. DSS is a chemical colitogen that disrupts the colonic epithelium inducing intestinal inflammation. Symptoms and epithelial damage are very similar to those found in human patients with UC.[2,3]

In the search for new therapeutic strategies, nutritional intervention has attracted remarkable interest in the management of IBD. Scientific evidence suggests that individual dietary factors and patterns may be involved in the risk of developing UC. A balanced diet with the right nutrients can provide an alternative way to improve health, particularly immune function, to help manage various diseases.[10,11] Since ancient times, many of the medicines have been derived from natural products leading to important advances in the treatment of diseases.[12] These natural products include those rich in polyphenols, which have been called "essential elements for life" due to their significant impact on health.[13] Among the foods rich in phenolic compounds are extra virgin olive oil, wine, and beer. In fact, Redondo *et al.* have previously suggested several health benefits of moderate beer consumption for bone health, cardiovascular health or the immune system.[14] The phenolic compounds of beer come mainly from hops (30%) and malts (70–80%), but not all beers contain the same amounts or types of polyphenols.[15] Variations are mainly due to the choice of starting raw materials, the variety of malts and hops and the brewing process. The yeast strain used in the brewing process also affects the content of phenolic compounds, and there are even special yeast strains designed to improve the phenolic profile.[16] This results in different types of beers with different concentrations of polyphenols.[17] Currently, many studies support the beneficial role of dietary polyphenols in IBD.[18] Indeed, the protective effects of phenolic compounds against UC have been demonstrated in DSS-induced C57BL/6 mice, presumably through suppression of inflammation and apoptosis.[19] In addition, Maia *et al.* have investigated the effects of a phenolic extract from Pale Ale craft beer powder on the attenuation of DSS-induced acute colitis in BALB/c mice.[20]

There is no doubt that dietary phenolic compounds play a remarkable role in IBD, ameliorating the inflammatory response; however, evidence for the preventive effects of dietary beer treatment on chronic colitis is still very limited. Therefore, the aim of this study was to elucidate the anti-inflammatory and antioxidant effects of different types of commercial beers on the lipopolysaccharide (LPS)-stimulated THP-1 human monocytic cell line and the beneficial preventive effects of dietary commercial Guinness beer treatment in a murine model of DSS-induced chronic colitis.

2. Materials and methods

2.1. Beer selection

Five types of beers were selected, 3 lager type: "5 estrellas Mahou" (Mah1) (Pale Lager), "Cruzcampo Pilsen" (Cr1) (Pilsen), and "Reserva Roja Alhambra" (Alh1) (Bock), and 2 ale type: "Cruzcampo IPA" (CrIpa1) (IPA) and "Guinness" (Gui1) (Stout). These beers were lyophilized, dissolved in dimethylsulfoxide (DMSO) and added to the cultures at indicated concentrations.

2.2. Lyophilisation of beers

Lyophilisation of beers was carried out on a VirTis lyophilizer (The VirTis Company®, Inc., Gardiner, NY, USA). For this purpose, 100 mL of each beer sample were frozen at −20 °C for 24 hours (h), and at −80 °C for 12 h. Then, water and alcohol were removed by freeze-drying until a non-humid solid residue of constant weight was obtained. Yields of 2.3–5.1% w/w were obtained in the lyophilisation.

2.3. Extraction and analysis of phenolic compounds

The lyophilized beer residue, corresponding to 10 mL of beer, was dissolved in 10 mL of ethanol : water (1 : 1 v/v). Prior to qualitative and quantitative analysis, NaCl was added until a saturated solution was obtained (approximately 1.5 g). Ethyl acetate (10 mL) was added, and the resulting mixture was shaken on an agitator (Vortex Stirrer "Vortex Vib" 7001725, J.P. Selecta® S.A., Barcelona, Spain) for 1 minute (min). To assist the phase separation, the mixture was centrifuged (6000 rpm for 15 min) using a Z 206 A centrifuge (HERMLE Labrotechnik GmbH®, Wehingen, Germany) and the supernatant extracted was carefully transferred to a 50 mL evaporating flask. This extraction step with ethyl acetate was repeated three times. The pooled ethyl acetate extracts were then evaporated to dryness under reduced pressure at 35 °C. The residue was redissolved in 2 mL of methanol : water (1 : 1 v/v) and then filtered through a 0.45 μm membrane (Syringe filters PTFE 13 mm, 0.45 μm, Análisis Vínico®, Ciudad Real, Spain).

Qualitative analysis by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry/mass spectrometry (UHPLC-HRMS/MS). Phenolic compounds were analysed using a Thermo Scientific liquid chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS, connected to a quadrupole-orbitrap QExactive hybrid mass spectrometer (ThermoFisher Scientific®, NY, USA) with a HESI ionization probe. Xcalibur software was used for instru-

ment control and data acquisition. Separation was carried out using an Acquity BEH C18 column (1.7 μm particle size, 100 × 2.1 mm, Waters®) at 40 °C at a flow rate of 0.5 mL min^{-1}. A binary gradient consisting of (A) water and (B) methanol both containing 0.1% formic acid was used with the following elution profile: 5% B (1 min), linear gradient to 100% B (9 min), 100% B (2 min) and finally 5% B (3 min). The injection volume was 5 μL.

A data dependent acquisition method (Top5) was used in negative mode at resolution of 70 000 and 17 500 at *m*/*z* 200 FWHM for full scan and product ion scan, respectively. HESI source parameters were as follows: spray voltage, 3.0 kV; S lens level, 50; capillary temperature, 320 °C; sheath and auxiliary gas flow, 60 and 25, respectively (arbitrary units); and probe heater temperature, 400 °C. Trace Finder 5.1 software was used for data analysis. The identification was made by comparing the retention time, the exact masses of the pseudomolecular ion and their fragment ions (maximum deviation of 5 ppm) with data contained in a phenolic compound database with 87 compounds. Isotopic pattern scores higher than 80% were also required.

Quantitative analysis by high-performance liquid chromatography with diode-array detection (HPLC-DAD). The residue obtained after the extraction of the phenolic fraction was redissolved in 2 mL of methanol : water (1 : 1 v/v) and then filtered through a 0.45 μm membrane (Syringe filters PTFE 13 mm, 0.45 μm, Análisis Vínico®, Ciudad Real, Spain). Once filtered, the extracted phenolic fraction was injected into an HPLC system (Agilent Technologies® 1200, Waghaeusel–Wiesental, Germany), equipped with a diode array detector. The column was a Lichrospher 100RP-18 (4.0 mm i.d. × 250 mm; 5 μm, particle size, Darmstadt®, Germany) kept at 30 °C. The flow rate was 1.0 mL min^{-1}, and the gradient elution was performed using a mixture of water/*ortho*-phosphoric acid (99.5 : 0.5 v/v) (solvent A) and methanol/acetonitrile (50 : 50 v/v) (solvent B). The change in solvent gradient was programed as follows: from 95% (A) and 5% (B) to 70% (A) and 30% (B) in 25 min; 65% (A) and 35% (B) in 10 min; 60% (A) and 40% (B) in 5 min; 30% (A) and 70% (B) in 10 min and 100% (B) in 5 min, followed by 5 min of maintenance.

The chromatographic signals were obtained at 220 nm, 256 nm, 280 nm and 335 nm. The identification of the compounds was confirmed by the analysis of the corresponding standards, using the retention times, the absorbance spectra and HPLC-MS spectrometry. Quantitative results were expressed as milligrams of gallic acid per kilogram (mg kg^{-1}).

2.4. Cell culture

The THP-1 human monocytic leukemia cell line from the American Type Culture Collection (ATCC®, Manassas, VA, USA) was cultured in RPMI 1640 medium (GIBCO®, Grand Island, NY, USA) supplemented with 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and 10% heat-inactivated fetal bovine serum (FBS). The THP-1 cell line was grown in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.5. Cell viability assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich®, St. Louis, MO, USA) colorimetric assay was used to evaluate cell viability. The formation of purple formazan in viable cells allows the determination of cell viability. For differentiation into macrophages, THP-1 cells (10^4 cells per well) were seeded in the presence of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich®, St. Louis, MO, USA) at a final concentration of 8 nM for 72 h in 96-well plates (100 μL per well). Cells were incubated in the presence or absence of the different freeze-dried beers (1.56–100 μg mL^{-1}) for 24 h. After the treatment period, cells were exposed to MTT at a final concentration of 0.5 mg mL^{-1} at 37 °C for 3 h in the dark. Formazan products were solubilised in DMSO (PanReac®, Barcelona, Spain) and the absorbance was measured at 570 nm in a microplate spectrophotometer (Synergy HT, Biotek®, Bad Friedrichshall, Germany). The results were expressed as the percentage of formazan absorbance under the different treatment conditions with respect to that of untreated THP-1 cells.

2.6. Intracellular ROS scavenging

A 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) assay kit (Abcam®, Cambridge, UK) was used to quantify intracellular ROS in THP-1 cells according to the manufacturer's instructions. THP-1 cells were seeded in 96-well black plates (10^4 cells per well) and differentiated into macrophages for 72 h, as described above. Cells were incubated in the presence or absence of non-cytotoxic concentrations (25, 50 and 100 μg mL^{-1}) of freeze-dried beers for pre-treatment for 1 h and then stimulated with LPS (1 μg mL^{-1}). Dexamethasone (Dex, 1 μM) (Sigma-Aldrich®, St. Louis, MO, USA) was used as a positive reference compound. After 24 h, the fluorescence of the DCFDA product was determined with a fluorescence plate reader (Synergy HT, Biotek®, Bad Friedrichshall, Germany) at 485 nm for excitation and 535 nm for emission.

2.7. Determination of pro-inflammatory cytokines: TNF-α and IL-6

Pro-inflammatory cytokine levels were determined in human THP-1 macrophages by quantitative enzyme-linked immunoassay (ELISA). Cells were pre-treated with different concentrations of freeze-dried beer (25, 50 and 100 μg mL^{-1}) and Dex (1 μM) as a positive reference compound for 1 h. They were then stimulated with LPS (1 μg mL^{-1}) for 24 h to induce the inflammatory response. After incubation, supernatants were collected and stored at −80 °C. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured using TNF-α and IL-6 human ELISA kits (Diaclone®, Besancon Cedex, France), according to manufacturer's instructions.

2.8. Animals and diets

52 female C57BL/6 mice (4 week old) were provided by the Animal Production Centre of the University of Seville (Seville, Spain). They were maintained in our Animal Laboratory Centre

under standard conditions of temperature (24–25 °C), humidity (70–75%) and with a 12 h light/dark cycle. Animals were acclimated and maintained on a standard diet (SD) (Altromin®, Lage, Germany) and water *ad libitum* until they reached 6 weeks of age. Then, mice were randomized into five experimental groups: (1) the naïve group received the SD (naïve group) (n = 6), (2) the beer naïve group received the SD supplemented with 0.2% freeze-dried Guinness beer (naïve 0.2% group) (n = 6), (3) the DSS group received the SD (DSS group) (n = 12), (4) the DSS + 0.05% freeze-dried Guinness beer group received the SD supplemented with 0.05% freeze-dried Guinness beer (0.05% group) (n = 14) and (5) the DSS + 0.2% freeze-dried Guinness beer group received the SD supplemented with 0.2% beer (0.2% group) (n = 14) (dietary enrichment percentage selection was based on previous reports[21]). Diets were formulated according to the American Institute of Nutrition (AIN) standard reference diet, with or without freeze-dried Guinness beer and stored at −80 °C. Fresh diets were provided to the experimental groups for four weeks before colitis induction and until the day of sacrifice.

All experiments were performed in the Faculty of Pharmacy Animal Laboratory Center (University of Seville, Spain) in accordance with the Guidelines of the European Union regarding animal experimentation (Directive of the European Council 2012/707/EU) and according to a protocol approved by the Animal Ethics Committee of the University of Seville (CEEA-US2022-17) and by the Consejeria de Agricultura, Pesca y Desarrollo (Junta de Andalucia, 14/04/2023/0012), according to RD 53/1 February 2013.

2.9. Induction of colitis

Colitis was induced as described by Melgar *et al.*[22] After mice were fed for four weeks with the experimental diets, DSS + SD, DSS + 0.05% and DSS + 0.2% groups received 3% DSS (DSS group; MW: 40000, ICN Pharmaceuticals®, Costa Mesa, CA) in drinking water for five days followed by a regime of 21 days of water, simulating chronic colitis. Control healthy animals (SD and SD + 0.2%) were allowed to drink only water. Mice were euthanized with an overdose of ketamine/xylazine.

2.10. Evaluation of the severity of clinical colitis

During the experimental phase, mice were inspected for visible signs of pathology. The clinical activity of colitis was evaluated to determine the disease activity index (DAI) as described by Gommeaux *et al.* with slight modifications.[23] The presence of diarrhoea, rectal bleeding and weight loss were graded on a scale of 0 to 3 and the DAI was the average of the three values. All parameters were registered during the five days of DSS treatment, as well as during the 3 week follow-up period when mice were given pure water.

2.11. Macroscopic and histopathological evaluation

After the mice were sacrificed, colons were removed and cleaned in physiological saline, measured, and weighed to evaluate changes in the weight/length as an index of inflammation.

Table 1 Score assigned for histopathological evaluation

Score	Description
0	No inflammatory signs
1	A few inflammatory signs
2	Generalized inflammation in mucosa
3	Inflammatory nodules in mucosa
4	Loss of the glandular architecture in the mucosa and inflammation of the entire colon wall

Samples from different regions (proximal, transverse, and distal colon) were prepared for subsequent paraffin embedding. After removal, they were fixed in 10% neutral buffered formalin, embedded in paraffin by the usual procedure, cut into 4–5 μm thick sections and mounted on silane-coated slides. For general morphological and histological assessment, consecutive sections were stained with haematoxylin–eosin (H–E) and periodic acid-Schiff (PAS) staining, following standard procedures.

Colon sections were scored by blinded investigators on a scale of 0 to 4 as the degree of total inflammation and crypt damage (Table 1). The scores assigned to each group were evaluated as described by Maia *et al.* and Gong *et al.* with some modifications.[20,24]

2.12. Determination of pro-inflammatory cytokines: TNF-α, IL-1β, IL-6 and IL-17

Colon tissues were weighed and homogenized in a phosphate buffered saline solution (PBS; pH 7.2) containing a protease cocktail at 4 °C and centrifuged twice at 10 000g for 10 min. Supernatants collected from the colon tissue were stored at −80 °C and used for determining cytokine production with ELISA murine kits: TNF-α (Invitrogen®, Carlsbad, California, USA), IL-1β, IL-17 (R&D Systems®, Abingdon, UK) and IL-6 (Diaclone®, Besancon Cedex, France), according to the manufacturer's instructions. Values are expressed as picograms per milligram (pg mg^{-1}) of tissue.

2.13. Western blot analysis

Frozen colonic tissues were weighed and homogenized in ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 5 mM EGTA, 0.5 mM EDTA, 0.01 mg mL^{-1} leupeptin, 0.01 mg mL^{-1} pepstatin, 0.01 mg mL^{-1} aprotinin, 1 mM phenylmethylsulfonyl fluoride and 250 mM NaCl]. Homogenates were centrifuged twice at 4 °C, 10 000g for 10 min. Supernatants were collected and stored at −80 °C. The Bradford colorimetric method[25] was used to determine the protein concentration of the homogenate. Aliquots of supernatants containing equal amounts of protein (50 μg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-COX-2, rabbit anti-Nrf-2, rabbit anti-pSTAT-3, rabbit anti-IκB-α, rabbit anti-phospho-NF-κB p65 (Cell Signaling Technology®, Danvers, MA, USA, 1 : 1000), rabbit anti-HO-1 (Henzo®, Madrid, Spain, 1 : 1000) and rabbit anti-mPGES-1 (Abcam®,

Cambridge, MA, USA, 1 : 1000). Membranes were also incubated with anti-GAPDH antibodies (Cell Signaling Technology®, Danvers, MA, USA, 1 : 1000) to prove equal loading. After rinsing, the membranes were incubated at room temperature with horseradish peroxidase-labelled (HRP) secondary antibodies, anti-rabbit or anti-mouse (Cell Signaling Technology®, Danvers, MA, USA, 1 : 1500), in blocking solution for 2 h at room temperature. Immunosignals were captured with an Amersham Image 600 (GE Healthcare®, Chicago, IL, USA) and signals were analysed and quantified using Image Processing and Analysis in Java (ImageJ, Softonic).

2.14. Statistical analysis

Data were expressed as arithmetic mean ± standard error of the mean (SEM). GraphPad Prism Version 5.01 software (San Diego, CA, USA) was used to evaluate data. To assess the normality of the data, the Kolmogorov–Smirnov test was used. The statistical significance of any difference in each parameter between groups was assessed by one-way or two-way analysis of variance (ANOVA), using Bonferroni's multiple comparison test for parametric values. *P*-Values of <0.05 were considered statistically significant. Protein expression was normalized to the control to show the relative changes. For experiments involving histology or western blot, the figures shown are representative of at least 4–5 experiments performed on different experimental days.

3. Results

3.1. Phenolic compound analysis

Prior to the quantitative analysis, the beer samples were analysed by UHPLC-HRMS/MS to obtain qualitative information of the compounds present in the samples. Table 2 shows the phenolic compounds that were identified in the beer samples. Gallic acid, syringic acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid, isovanillic acid, ferulic acid, salicylic acid, and catechin were found in all beer samples while 3-*O*-methylgallic acid was only identified in Mah1. Furthermore, abscisic acid, a plant hormone, was also identified in all the samples. The presence of these compounds was also verified with bibliography. The diversity of materials and processes used in the beer production explains that some compounds were identified only in some samples. Thus, the frequency of identification of the compounds in the samples was expressed as the percentage of occurrence and as the number of samples in which they were identified.

Table 3 shows the concentration of the compounds whose identification was confirmed with standards, retention time and HPLC-MS, and they were identified by HPLC-DAD. The data showed that the most concentrated phenolic compound was gallic acid (0.69–3.23 mg kg^{-1}) and catechin (2.25–8.42 mg kg^{-1}) in all the samples. Gallic acid was more concentrated

Table 2 Phenolic compounds identified and frequency of identification in the 5 samples and the associated percentage. A percentage of 100% points out that the compound was identified in all samples: "Cruzcampo Pilsen" (Cr1), "5 estrellas Mahou" (Mah1), "Reserva Roja Alhambra" (Alh1), "Cruzcampo IPA" (Crlpa1) and "Guinness" (Gui1)

Phenolic compound	Cr1	Mah1	Alh1	Crlpa1	Gui1	% Presence	Frequency
Gallic acid	✗	✗	✗	✗	✗	100	5/5
Syringic acid	✗	✗	✗	✗	✗	100	5/5
Protocatechuic acid (3,4-dihydroxybenzoic acid)	✗	✗	✗	✗	✗	100	5/5
4-Hydroxybenzoic acid	✗	✗	✗	✗	✗	100	5/5
Caffeic acid	✗	✗	✗	✗	✗	100	5/5
Isovanillic acid	✗	✗	✗	✗	✗	100	5/5
Ferulic acid	✗	✗	✗	✗	✗	100	5/5
Salicylic acid	✗	✗	✗	✗	✗	100	5/5
Catechin	✗	✗	✗	✗	✗	100	5/5
p-Coumaric acid	✗	✗	✗	✗		80	4/5
3-Hydroxytyrosol	✗	✗		✗	✗	80	4/5
Homovanillic acid	✗	✗	✗		✗	80	4/5
Taxifolin	✗	✗	✗	✗		80	4/5
Isovitexin	✗	✗	✗	✗		80	4/5
Hyperoside (quercetin-3-*O*-galactoside)	✗	✗	✗	✗		80	4/5
Quercetin-3-*O*-glucoside	✗	✗	✗	✗		80	4/5
Kaempferol-3-*O*-glucoside	✗	✗	✗	✗		80	4/5
Luteolin-4′-*O*-glucoside	✗	✗	✗	✗		80	4/5
Gentisic acid	✗	✗			✗	60	3/5
Catechol (2-hydroxyphenol)		✗	✗		✗	60	3/5
Chlorogenic acid (3-*O*-caffeoylquinic acid)	✗		✗	✗		60	3/5
Vanillic acid		✗	✗	✗		60	3/5
4-*O*-Caffeoylquinic acid	✗		✗	✗		60	3/5
Epicatechin		✗	✗	✗		60	3/5
Sinapic acid	✗	✗	✗			60	3/5
Dihydrocaffeic acid		✗			✗	40	2/5
Vanillin			✗	✗		40	2/5
Phloretic acid		✗			✗	40	2/5
Rutin (quercetin-3-*O*-rutinoside)	✗			✗		40	2/5
Quercitrin (quercetin-3-*O*-rhamnoside)			✗	✗		40	2/5
3-*O*-Methylgallic acid		✗				20	1/5

Table 3 Concentration of the phenolic compounds identified in the beer samples expressed as milligrams of gallic acid per kilogram (mg kg^{-1}). "Cruzcampo Pilsen" (Cr1), "5 estrellas Mahou" (Mah1), "Reserva Roja Alhambra" (Alh1), "Cruzcampo IPA" (CrIpa1) and "Guinness" (Gui1)

Phenolic compound	Cr1	Mah1	Alh1	Crlpa1	Gui1
Gallic acid	0.707 ± 0.022	0.833 ± 0.040	2.474 ± 0.131	1.318 ± 0.258	2.474 ± 0.131
3,4-Dihydroxybenzoic acid	0.162 ± 0.008	0.075 ± 0.007	0.114 ± 0.001	0.041 ± 0.018	0.074 ± 0.017
Catechin	3.205 ± 0.049	2.990 ± 0.056	8.120 ± 0.423	3.439 ± 1.169	2.371 ± 0.170
Vanillic acid	0.030 ± 0.002	0.116 ± 0.003	0.043 ± 0.013	0.031 ± 0.004	0.058 ± 0.006
Syringic acid	0.067 ± 0.005	0.199 ± 0.003	0.245 ± 0.041	0.268 ± 0.069	0.021 ± 0.006
4-Hydroxybenzoic acid	0.092 ± 0.003	0.151 ± 0.004	0.246 ± 0.005	0.173 ± 0.035	0.527 ± 0.063
Hydroxytyrosol	0.327 ± 0.015	0.613 ± 0.019	0.318 ± 0.005	0.090 ± 0.002	0.118 ± 0.041
m-Coumaric acid	0.020 ± 0.002	0.023 ± 0.009	0.131 ± 0.029	0.022 ± 0.010	0.040 ± 0.022
Caffeic acid	0.060 ± 0.007	0.080 ± 0.004	0.157 ± 0.001	0.077 ± 0.051	0.066 ± 0.006
p-Coumaric acid	0.231 ± 0.003	0.380 ± 0.002	0.515 ± 0.009	0.131 ± 0.004	0.127 ± 0.033
Ferulic acid	0.421 ± 0.012	0.433 ± 0.001	0.887 ± 0.006	0.428 ± 0.105	0.270 ± 0.005
Luteolin	0.00 ± 0.000	0.019 ± 0.002	0.041 ± 0.005	0.037 ± 0.015	0.012 ± 0.004

than catechin in the black beer (Gui1). Among the remaining compounds, ferulic acid was the one that showed the most significant concentration (0.89 mg kg^{-1}). Luteolin was the phenolic compound that showed the lowest concentration, and it was not identified in Cr1 beer.

A principal component analysis (PCA) was carried out to study the differences in the phenolic compositions of the different beer types with a multivariate perspective. The objective was to examine the differences between the beer samples considering the whole set of identified phenolic compounds. Likewise, a PCA was also carried out to identify the phenols contributing to the variations observed in the PCA score plot. Fig. 1A and B show the PCA scores and loadings, respectively, plotted in the plane defined by the two principal components (PC1 and PC2). The two principal components explained 99.44% of the total variance, most of the variance being explained by PC1 (91.93%).

The score plot (Fig. 1A) shows the distribution of the beer samples in two groups along PC2, allowing to separate the black beers (Gui1, Gui2) from the rest of the samples. All the samples were in the negative side of PC1. According to the loading plot results (Fig. 1B), the compounds catechin and gallic acid show a clear influence in the model.

3.2. Effects of freeze-dried beers on THP-1 cell viability

MTT assay was performed to evaluate the effects of freeze-dried beers on THP-1 cell viability. After 24 h of treatment, the results showed that the viability of THP-1 cells treated with the different freeze-dried beers at concentrations of 1.56–100 μg mL^{-1} was not reduced. All of them showed a cell viability >80% (Fig. 2).

3.3. Effects of freeze-dried beers on intracellular ROS production

Intracellular ROS levels were measured to determine the effect of the different freeze-dried beer products on LPS-stimulated THP-1 macrophages. Treatment of cells with LPS (1 μg mL^{-1}) increased ROS levels compared to cells without stimulation (+++p < 0.001 *vs.* unstimulated control cells), whereas Dex (1 μM) reduced ROS production in these LPS-stimulated cells. The pre-treatment with freeze-dried beers was able to reduce ROS production compared to that in LPS-stimulated cells. Mah1, CrIpa1 and Gui1 showed the most significant reduction in response to the treatment, with the 50 and 100 μg mL^{-1} concentrations being the most effective (*p < 0.05, **p < 0.01 *vs.* LPS-stimulated cells) (Fig. 3). These results suggest that freeze-dried beer may have antioxidant activity.

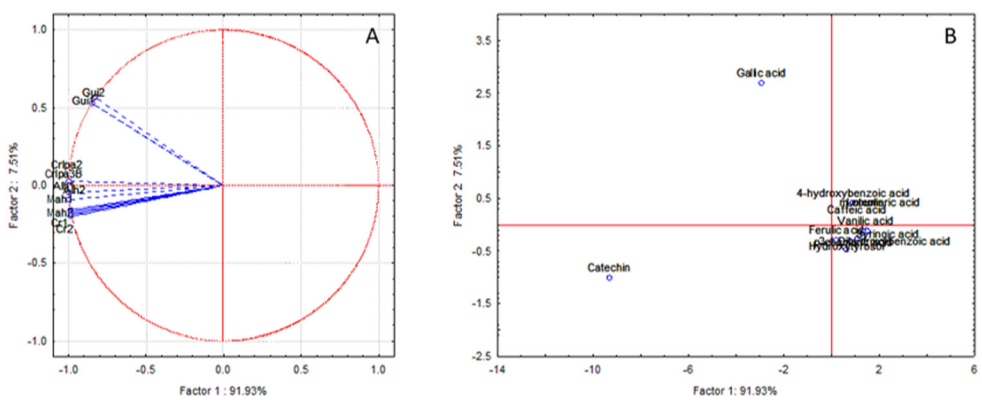

Fig. 1 Principal component analysis (PCA) results (score and loading plots) obtained with the phenolic compound concentration quantified in the beer samples. "Cruzcampo Pilsen" (Cr1), "5 estrellas Mahou" (Mah1), "Reserva Roja Alhambra" (Alh1), "Cruzcampo IPA" (CrIpa1) and "Guinness" (Gui1).

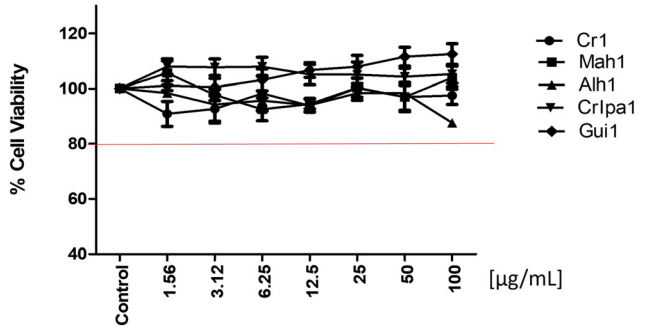

Fig. 2 Effect of freeze-dried beers on cell viability determined by MTT assay. Cells were treated with freeze-dried beers (1.56–100 μg mL^{-1}) for 24 h. Data are expressed as percentage of viability with respect to untreated control cells (100%) and represented as means ± SEM (n = 4). "Cruzcampo Pilsen" (Cr1), "5 estrellas Mahou" (Mah1), "Reserva Roja Alhambra" (Alh1), "Cruzcampo IPA" (CrIpa1) and "Guinness" (Gui1).

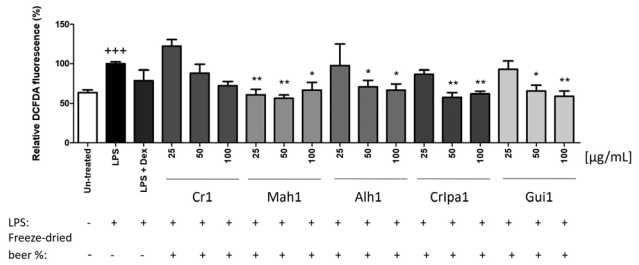

Fig. 3 Freeze-dried beers downregulate ROS levels in THP-1 stimulated cells. Cells were pre-treated with different concentrations (25, 50 and 100 μg mL^{-1}) and then stimulated with LPS for 24 h. (+++) $p < 0.001$ *vs.* unstimulated control cells; (*) $p < 0.05$, (**) $p < 0.01$ *vs.* LPS-stimulated cells. Data are represented as mean values ± SEM ($n \geq 5$). "Cruzcampo Pilsen" (Cr1), "5 estrellas Mahou" (Mah1), "Reserva Roja Alhambra" (Alh1), "Cruzcampo IPA" (CrIpa1) and "Guinness" (Gui1).

3.4. Effects of freeze-dried beers on pro-inflammatory cytokine production in culture supernatant

ELISA assays were used to determine the production of pro-inflammatory cytokines in the culture supernatant of LPS-stimulated THP-1 macrophages. Non-cytotoxic concentrations (25, 50 and 100 μg mL^{-1}) of freeze-dried beers were selected to study their effects on TNF-α and IL-6 production. Treatment of cells with LPS (1 μg mL^{-1}) increased the levels of TNF-α and IL-6 (Fig. 4A and B) compared with cells without the stimulus (+++$p < 0.001$ *vs.* unstimulated control cells). Dex (1 μM) reduced cytokine production to similar levels to those of the control unstimulated group. As shown in Fig. 4A, all freeze-dried beers significantly reduced TNF-α production, with Gui1 achieving the greatest reduction at all concentrations (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$ *vs.* LPS-stimulated cells). On the other hand, IL-6 levels were only significantly decreased by CrIpa1 and Gui1, the latter being the one that achieved the greatest reduction from 25 to 100 μg mL^{-1} (**$p < 0.01$, ***$p <$ 0.001 *vs.* LPS-stimulated cells). It should be noted that these two beers (CrIpa1 and Gui1) are ale beers, which have a higher

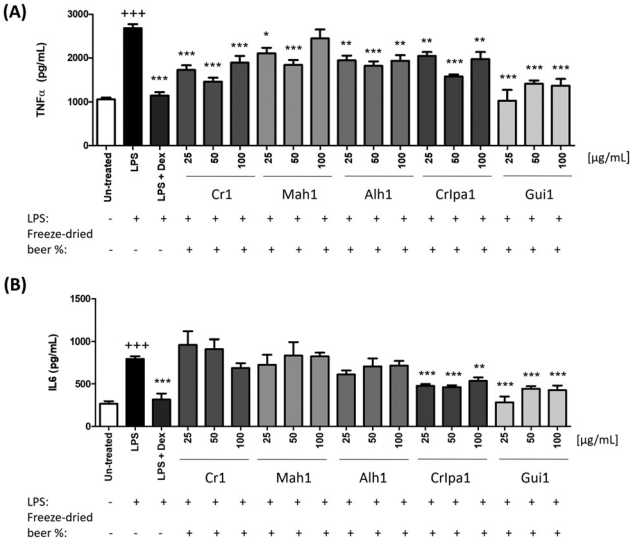

Fig. 4 Effects of freeze-dried beers on (A) TNF-α and (B) IL-6 production in LPS-stimulated THP-1 cells. Cells were pre-treated with different concentrations (25, 50 and 100 μg mL^{-1}) and then stimulated with LPS for 24 h. (+++) $p < 0.001$ *vs.* unstimulated control cells; (*) $p <$ 0.05, (**) $p < 0.01$, (***) $p < 0.001$ *vs.* LPS-stimulated cells. Data are represented as mean values ± SEM ($n \geq 5$). "Cruzcampo Pilsen" (Cr1), "5 estrellas Mahou" (Mah1), "Reserva Roja Alhambra" (Alh1), "Cruzcampo IPA" (CrIpa1) and "Guinness" (Gui1).

content of phenolic compounds.[15,26] Therefore, it is interesting to note that the highest anti-inflammatory activity was detected with the freeze-dried Guinness beer.

3.5. Effects of freeze-dried Guinness beer supplementation on DSS-induced chronic colitis

After *in vitro* experiments, freeze-dried Guinness beer was selected for its anti-inflammatory and antioxidant properties to further evaluate its effects on DSS-induced chronic colitis in mice. Body weight is an important parameter to evaluate the development of colitis. DSS-treated animals presented a marked loss of body weight on the ninth day compared to the naïve group (+++$p < 0.001$ *vs.* the naïve control group). However, DSS-treated mice supplemented with freeze-dried Guinness beer (0.05% or 0.2%) showed a significantly lower weight loss than DSS-treated mice on the 12th day (**$p < 0.01$ and ***$p < 0.001$ *vs.* DSS-treated mice, respectively) (Fig. 5A). In addition to weight loss, stool consistency and bleeding were observed to evaluate the DAI score. As expected, naïve animals showed no signs of symptoms, whereas the DSS-treated group exhibited a significant increase in the DAI score from day 4, peaking on day 9 (+++$p <$ 0.001 *vs.* the naïve control group). In contrast, administration of both doses of freeze-dried Guinness beer (0.05% and 0.2%) showed a significant decrease in the DAI score from day 6 compared to DSS-treated mice (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ *vs.* the DSS-treated group). Moreover, the reduction in the DAI was greater in the group supplemented with the higher dose of freeze-dried Guinness beer (Fig. 5B). It should also be noted that as this is a model of chronic colitis, recovery was observed in all

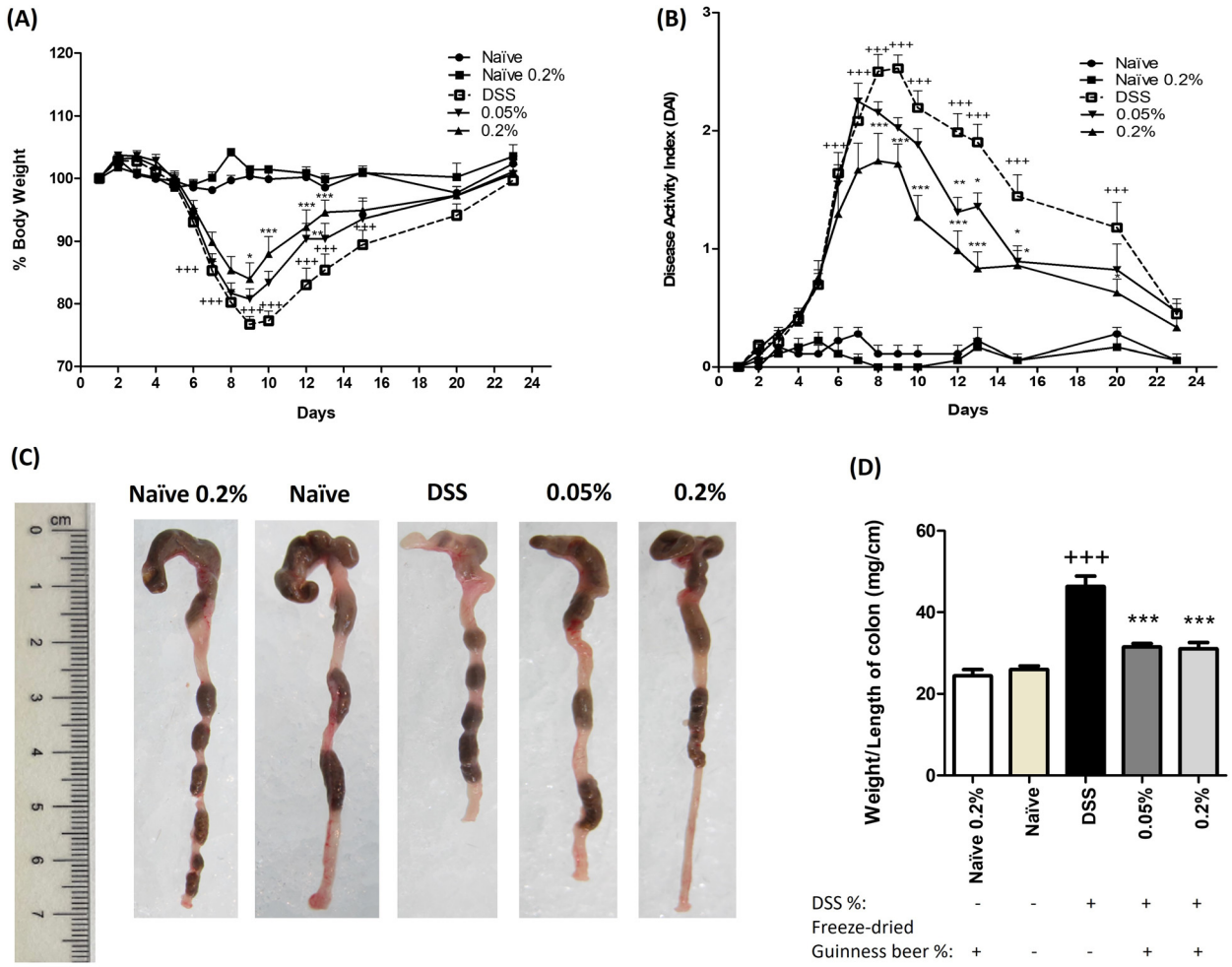

Fig. 5 Effects of Gui1 beer supplementation on the clinical and anatomical parameters of DSS-induced chronic colitis. (A) Body weight loss. Body weights were normalised to day 1 body weight. (B) Disease activity index (DAI) was determined as the average of weight loss, stool consistency and bleeding scores. (C) Macroscopic representative images of the colon from all groups. (D) Colon weight/length ratio. (+++) $p < 0.001$ *vs.* the naïve group; (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ *vs.* the DSS-treated group. Data are represented as mean values ± SEM. Naïve group ($n = 6$), naïve 0.2% group ($n = 6$), DSS group ($n = 12$), 0.05% group ($n = 14$) and 0.2% group ($n = 14$).

experimental groups after day 8–10, although it was slower in the DSS-treated group. As shown in Fig. 5C, macroscopic analysis revealed a shortening in colon length in DSS-treated mice compared to those on the experimental diets (0.05% and 0.2% groups). In line with these findings, the colon weight/length ratio (an indicator of inflammation) increased significantly in DSS animals compared to that in naïve animals (+++$p < 0.001$ *vs.* the naïve control group). However, the administration of diets supplemented with both doses of freeze-dried Guinness beer showed a marked anti-inflammatory effect by decreasing this indicator of colonic inflammation (***$p < 0.001$ *vs.* the DSS-treated group) (Fig. 5D).

3.6. Freeze-dried Guinness beer supplementation ameliorated microscopic colonic damage in DSS-induced chronic colitis

The different layers that constitute the wall of the large intestine are mucosa, submucosa, muscularis propria and serosa. The mucosa is composed of straight tubular glands with abundant mucous cells in the epithelium and includes a muscular layer, called the muscularis mucosae, which borders the submucosa. The connective tissue of the lamina propria in the mucosa is described as the cellular lax connective tissue, due to the presence of diffuse lymphoid tissue (MALT). Some nodular aggregates may appear in this part of the colon, but it is not an area where lymphoid follicles are abundant, either in the mucosa or in the submucosa. The muscularis propria is described as an inner circular layer and an outer longitudinal layer in which there is no significant presence of cells of the immune system.[27]

Microscopic analysis of the H–E-stained samples from the naïve control groups revealed the histological organisation described above, with a thinned and poorly cellular submucosa as the only relevant finding (Fig. 6A–D). However, samples from the DSS-treated group showed such an intense inflammatory response that cells penetrated the muscularis

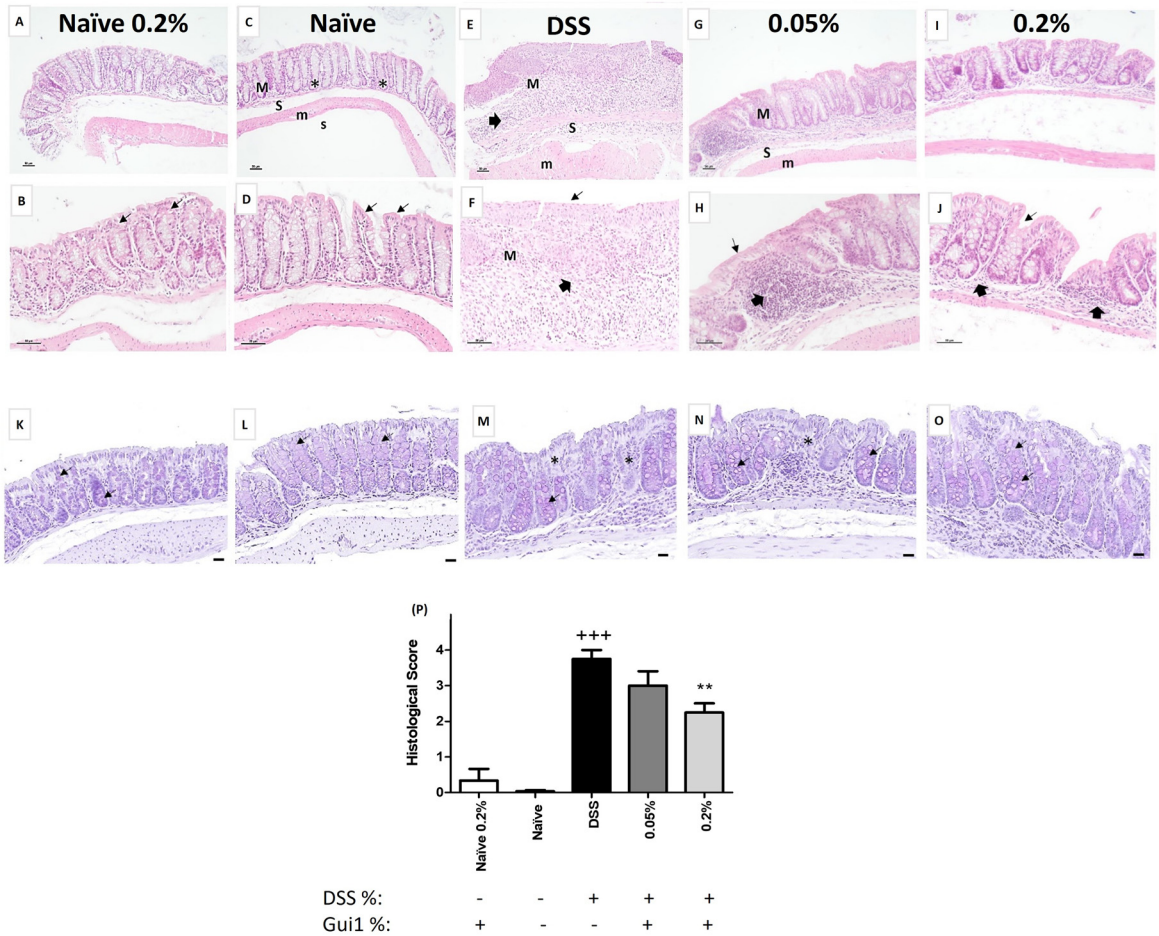

Fig. 6 Histopathological damage in the colon samples of the different groups. (A–J) H–E staining (20× and 10×). Micrograph C shows the histological layers organised in a normal colon. M, mucosa; S, submucosa; m, muscularis propria; s, serosa. The mucosa is composed of straight tubular glands (asterisks) with a simple cylindrical lining epithelium (B and D, arrows). In the DSS group samples, generalised inflammation (E and F, arrowheads) invades all layers, causing the loss of the glandular architecture, resulting in ulcers in some areas (F, arrow) with the loss of epithelium. At 0.05% dose of Gui1 beer an attenuation of the signs, with each of the histological layers being differentiated, was detected (G). However, some inflammatory nodules were observed (H, arrowheads) with alteration of the glandular structure and epithelium (H, arrow). When the dose was increased to 0.2%, the signs decreased, although some pathological signs continued to appear, these were mild and only caused an increase in cells at the level of the mucosa (J, arrowheads) without the loss of the glandular pattern (J, arrow). (K–O) PAS staining (20×). Comparative distribution of goblet cells between the different groups. Goblet cells were abundant among colonic epithelial cells at the level of the colon and were evenly distributed throughout the gland (K, L and O, arrows). However, in the inflamed mucosa they were localised in the glandular crypt (M and N, arrows) and were difficult to identify in the mucosal tracts invaded by lymphoid follicles (M and N, asterisks). (P) Histological score. (+++) $p < 0.001$ *vs.* the naïve group; (**) $p < 0.01$ *vs.* the DSS-treated group. Data are represented as mean values ± SEM. Naïve group ($n = 3$), naïve 0.2% group ($n = 3$), DSS group ($n = 4$), 0.05% group ($n = 4$) and 0.2% group ($n = 4$).

mucosae and invaded the rest of the colonic layers (Fig. 6E). The generalised inflammation would explain the increased wall thickness. In addition, inflammatory nodules of various sizes were identified in both the mucosa and submucosa. Even in some localised areas, a loss of the glandular architecture was detected due to the invasion of inflammatory cells (lymphocytes, polymorphonuclear and plasma cells) (Fig. 6F). Colon samples from the DSS group supplemented with 0.05% dose of freeze-dried Guinness beer showed some tissue damage. In these mice, inflammatory cells invaded the mucosa, although they did not penetrate the mucosal muscle and did not invade the submucosa. However, the normal architecture of this layer was lost in some areas, with the absence of intestinal glands (Fig. 6G and H). When the dose was increased to 0.2% of freeze-dried Guinness beer, mice showed some signs of inflammation, but they were much milder than in the DSS group and 0.05% group. The mucosal connective tissue of some of the samples showed a diffuse increase in inflammatory cells and mucosal thickening, but in none of the cases the glandular architecture was lost (Fig. 6I and J). These findings provide evidence for a protective effect of freeze-dried Guinness beer on colonic mucosal injury.

In addition, the histopathological score was calculated to evaluate microscopic damage (Fig. 6P) following that shown in

Table 1. Quantification of these results showed a significant increase in samples from the DSS group compared to those from naïve animals (+++$p < 0.001$ *vs.* the naïve control group). However, the mean score was significantly lower in the DSS group supplemented with the highest dose of freeze-dried Guinness (0.2%) (**$p < 0.01$ *vs.* the DSS-treated group). An abundant cell type in the glands of the large intestine is goblet cells. These cells are themselves carbohydrate-rich, unicellular intraepithelial mucus glands that can be clearly identified by the PAS technique. In all groups studied, active goblet cells were identified among the epithelial cells of the intestinal glands with no differences in cell staining intensity between the different groups. An important finding was that the number and distribution of goblet cells changed from normal with glandular disruption. Thus, in groups with inflamed mucosa, goblet cells occupied basal positions in the glands and were even unidentifiable at the surface level (Fig. 6K–O).

3.7. Effects of freeze-dried Guinness beer supplementation on pro-inflammatory cytokine production in DSS-induced chronic colitis

To assess whether freeze-dried Guinness beer could inhibit the production of pro-inflammatory cytokines in DSS-induced mice, specific ELISA assays were used to determine the levels of TNF-α, IL-1β, IL-6 and IL-17. DSS administration significantly increased TNF-α, IL-1β, IL-6 and IL-17 levels compared to those in naïve animals (+++$p < 0.001$ *vs.* the naïve control

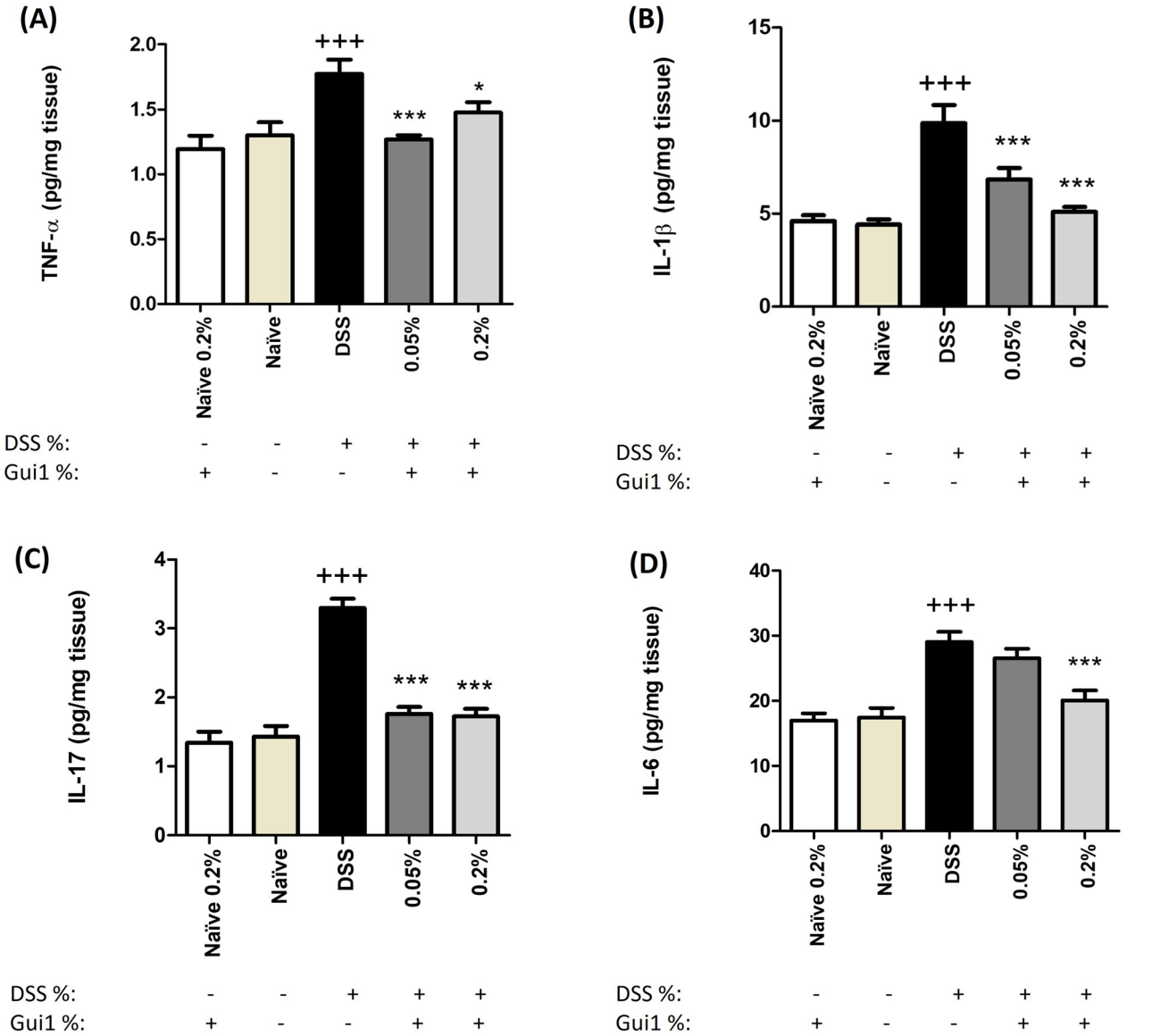

Fig. 7 Effects of Gui1 beer supplementation on the production of (A) TNF-α, (B) IL-1β, (C) IL-17 and (D) IL-6 in a DSS-induced chronic colitis model. (+++) $p < 0.001$ *vs.* the naïve group; (*) $p < 0.05$, (***) $p < 0.001$ *vs.* the DSS-treated group. Data are represented as mean values ± SEM. Naïve group ($n \geq 5$), naïve 0.2% group ($n \geq 5$), DSS group ($n \geq 9$), 0.05% group ($n \geq 9$) and 0.2% group ($n \geq 9$).

group). However, administration of freeze-dried Guinness beer at both doses (0.05% and 0.2%) significantly reduced the secretion of TNF-α, IL-1β and IL-17 (*$p < 0.05$, ***$p < 0.001$ *vs.* the DSS-treated group) (Fig. 7A–C). Regarding IL-6 production (Fig. 7D), only the highest dose was able to significantly reduce its production (***$p < 0.001$ *vs.* the DSS-treated group).

3.8. Inhibition of COX-2 and mPGES-1 protein expression after feeding a freeze-dried Guinness beer supplemented diet to mice with DSS-induced chronic colitis

COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) have been shown to be involved in inflammatory processes. COX-2 is an enzyme involved in the synthesis of various prostaglandins and specifically in the prostaglandin E_2 (PGE_2) biosynthesis pathway. PGE_2 is an important mediator of inflammatory responses and inflammatory bowel disease, and its production is also dependent on the induction of mPGES-1.[28,29] To demonstrate the anti-inflammatory properties of freeze-dried Guinness beer, COX-2 and mPGES-1 protein levels were analysed. The DSS-treated group showed a significant increase in the production of both proteins (+++$p <$ 0.001 *vs.* the naïve control group). In contrast, after supplementation with freeze-dried Guinness beer at both doses (0.05% and 0.2%), the expressions of COX-2 and mPGES-1 proteins were significantly reduced compared to those in the DSS-treated group (***$p < 0.001$ *vs.* the DSS-treated group) (Fig. 8A).

3.9. Freeze-dried Guinness beer supplementation increased the Nrf-2/HO-1 antioxidant signaling pathway in DSS chronic colitis

The transcription factor Nrf-2 plays a key role in the response to oxidative stress and can regulate the expression of antioxidant enzymes such as HO-1. We therefore determined the expression of Nrf-2 and HO-1 proteins by western blotting to assess whether freeze-dried Guinness beer could regulate the Nrf-2/HO-1 antioxidant signaling pathway. Supplementation with freeze-dried Guinness beer at a dose of 0.05% showed an upward trend in Nrf-2 and HO-1 expression, the latter being significant compared to that in the DSS-treated group (*$p <$ 0.05 *vs.* the DSS-treated group). Increasing the dose to 0.2% significantly upregulated Nrf-2 and HO-1 expression (***$p <$ 0.00 1 *vs.* the DSS-treated group) (Fig. 8B). These findings, along with the reduction of ROS production, support a potential role of freeze-dried Guinness beer in the antioxidant response.

3.10. Effects of freeze-dried Guinness beer dietary supplementation on NF-κB in DSS-induced chronic colitis

The NF-κB complex is a family of transcription factors that play a key role in cellular process and in immunoinflammatory

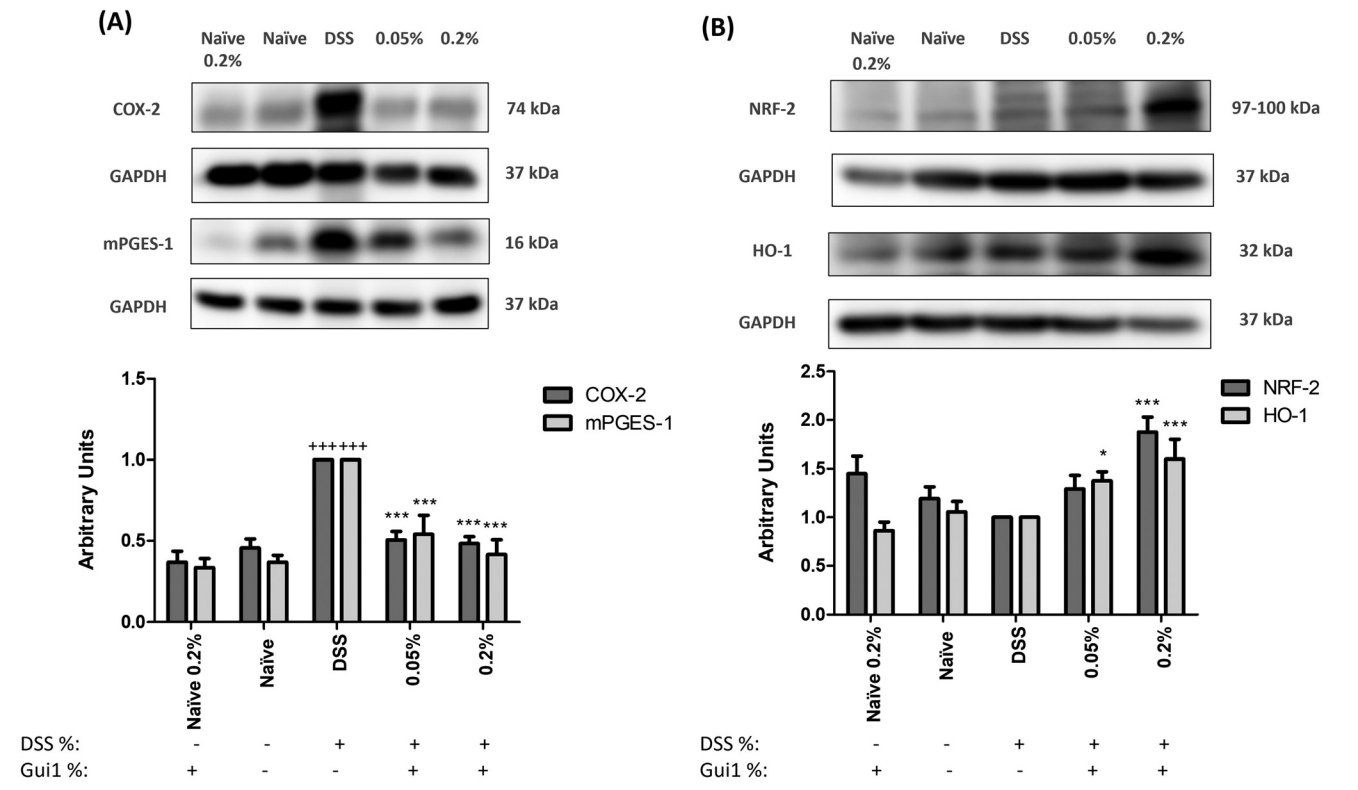

Fig. 8 Effects of Gui1 beer supplementation on (A) COX-2 and mPGES-1 expression and (B) Nrf-2 and HO-1 protein expression in a DSS-induced chronic colitis model. Densitometry was performed following normalization to the control (GAPDH housekeeping gene). (+++) $p < 0.001$ *vs.* the naïve group; (*) $p < 0.05$, (***) $p < 0.001$ *vs.* the DSS-treated group. Data are represented as mean values ± SEM ($n = 6$).

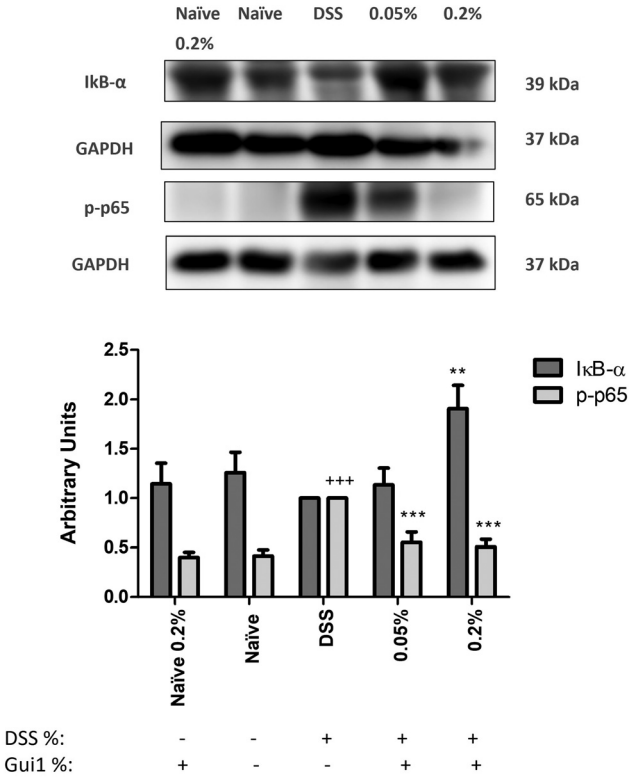

Fig. 9 Effects of Gui1 beer supplementation on IκB-α degradation and NF-κB p-p65 in colonic mucosa. Densitometry was performed following normalization to the control (GAPDH housekeeping gene). (+++) $p < 0.001$ *vs.* the naïve group; (**) $p < 0.01$, (***) $p < 0.001$ *vs.* the DSS-treated group. Data are represented as mean values ± SEM ($n = 7$).

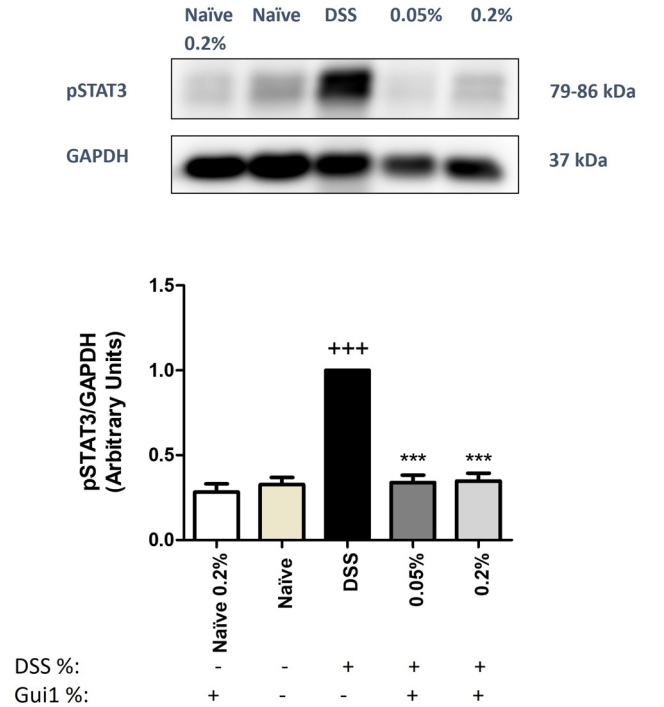

Fig. 10 Effects of Gui1 beer supplementation on STAT3 phosphorylation in colonic mucosa. Densitometry was performed following normalization to the control (GAPDH housekeeping gene). (+++) $p < 0.001$ *vs.* the naïve group; (***) $p < 0.001$ *vs.* the DSS-treated group. Data are represented as mean values ± SEM ($n = 7$).

response. Activation of NF-κB is controlled by IκB-α, which binds to NF-κB dimers. After degradation of IκB-α, the NF-κB p-p65 subunit is free to translocate to the cell nucleus and regulate the expression of pro-inflammatory genes. Levels of both proteins were measured, and as shown in Fig. 9, IκB-α expression exhibited a downward trend in the DSS-treated group, consistent with the up-regulation of the NF-κB complex (+++$p < 0.001$ *vs.* the naïve control group). Dietary supplementation with 0.05% freeze-dried Guinness beer tended to reduce IκB-α degradation, whereas the 0.2% dose group was able to significantly reduce the degradation of IκB-α in the DSS-treated mice (**$p < 0.01$ *vs.* DSS-treated mice). Consequently, both doses significantly reduced the expression of the NF-κB p-p65 subunit (***$p < 0.001$ *vs.* the DSS-treated group). This suggests that freeze-dried Guinness beer could have an inhibitory effect on the NF-κB complex in UC mice.

3.11. Effects of dietary supplementation with freeze-dried Guinness beer on STAT3 phosphorylation

As mentioned above, the JAK/STAT signaling pathway plays an important role in the function of cells that mediate the inflammatory immune response. Specifically, STAT3 is a transcription factor involved in the pathogenesis of UC. Phosphorylation of STAT3 leads to its activation, which induces the expression of pro-inflammatory genes. Our results showed a significant increase in phosphorylated STAT3 in the DSS-treated group (+++$p < 0.001$ *vs.* the naïve control group). In contrast, mice given freeze-dried Guinness beer at both doses showed a significant decrease in the phosphorylation of this transcription factor (***$p < 0.001$ *vs.* the DSS-treated group) (Fig. 10).

4. Discussion

IBD is a complex condition that is influenced by several factors, including diet, genetics, microbial, environmental and immunological factors. The main forms of IBD are Crohn's disease (CD) and UC, chronic and relapsing conditions that affect the gastrointestinal tract. Despite the existence of various strategies to control IBD, such as drug treatment or lifestyle changes, there is a need to look for new therapies as the use of drugs can lead to various side effects. Therefore, nutritional intervention and the use of bioactive compounds are becoming increasingly important in the treatment of IBD.[30,31] In general, natural polyphenols present health-protective properties, which are likely attributed to their effect on oxidative stress and inflammation. In fact, previous research has demonstrated the therapeutic potential of dietary polyphenols in many immunoinflammatory diseases, including IBD. These natural products produced from the secondary metabolism of plants are found in a wide range of foods and beverages, such

as wine and beer.[32–35] Beer, in particular, is an interesting product resulting from the fermentation of cereals. It is rich in a variety of important nutrients such as polyphenols. The amount of phenolic compounds present in beer depends on factors such as the types of hops, malts and yeast strains used, and the brewing process.[16] Data from several studies show a beneficial effect of polyphenol intake on inflammatory biomarkers, thus preventing inflammatory diseases.[36,37] Apart from being rich in polyphenols, beer is an attractive product in terms of flavour, appearance and is also very common in society. This makes beer an excellent product for studying the potential benefits associated with its moderate intake. Moderate alcohol consumption is defined as up to 1 drink (a 330 mL can of beer containing about 4% w/v alcohol) per day for women and up to 2 drinks per day for men.[17] Experimental studies attribute many health-promoting properties to beer. Moderate beer consumption has been shown to be associated with increased bone density in older women compared to those who abstain or abuse beer consumption.[14] In addition, moderate consumption of beer has also been attributed to a reduction in the risk of atherosclerosis,[21] as well as a decrease in the incidence of cardiovascular diseases, hypertension, diabetes, and certain types of cancer.[26,38] The preventive role of beer intake in IBD has not been studied in-depth, and the molecular mechanisms mediating the effects of beer intake are poorly understood. In this context, the aim of this research was to determine the anti-inflammatory effects of freeze-dried commercial beer treatment on LPS-induced inflammatory response in human macrophages and to elucidate its preventive effects in a murine chronic experimental UC model.

Initially, after the freeze-drying process, different commercial beers were analysed quantitatively and qualitatively to determine the phenolic compounds as described by Zhao *et al.*[39] For instance, they analysed thirty-four beers by HPLC and identified nine phenolic compounds including gallic acid, protocatechuic acid, catechin, vanillic acid, caffeic acid, syringic acid, epicatechin, *p*-coumaric acid and ferulic acid, the main ones being gallic and ferulic acid. All the phenolic compounds they identified were found in some of our beers, and specifically, gallic acid, protocatechuic acid, catechin, caffeic acid, syringic acid, and ferulic acid were identified in all beer samples. In addition to these nine compounds, approximately twenty other phenolic compounds were identified in our different samples. We also quantified the predominant phenolic compounds present in our samples, with gallic acid and catechin being the most abundant in all samples. Although the phenolic compound profile is coincidental in many varieties of beers, the phenolic content of each beer varies. These variations are mainly due to the choice of raw materials, the variety of malts, hops, yeast strains and the brewing process. These result in different types of beers with different concentrations of polyphenols.[17]

The suppressive effects of dietary polyphenols on multiple cell signaling pathways reveal their potential use in prevention and treatment of chronic inflammatory disorders, such as IBD.[40] In this sense, macrophages play a remarkable role in the immune response to inflammatory processes. Immune cells release reactive oxygen metabolites and large amounts of pro-inflammatory cytokines, such as IL-6 and TNF-α.[41–43] The production of ROS is also a critical factor in the development and progression of inflammatory diseases. ROS-mediated damage leads to oxidative stress, which alters the intracellular redox balance.[5] Therefore, the THP-1 cell line was selected because it closely approximates human macrophages and its robust *in vitro* capabilities for studying the relevant parameters in this work.[44] LPS-stimulated THP-1 macrophages have previously been reported to induce a significant increase in ROS production compared to the control group.[45] Our results revealed an overproduction of ROS in LPS-stimulated THP-1 human macrophages, whereas treatment with Mah1, CrIpa1 and Gui1 freeze-dried beers significantly reduced ROS levels. Additionally, the release of pro-inflammatory cytokines such as TNF-α and IL-6 by macrophages leads to the progression of inflammation in a large number of inflammatory diseases, including rheumatoid arthritis, psoriasis and IBD, among others.[45] Previous studies determined that several components present in beer, such as xanthohumol or melatonin, show anti-inflammatory activity by reducing the production of several pro-inflammatory cytokines.[35,46] In line with these findings, our results showed that commercial freeze-dried beers were able to reduce TNF-α and IL-6 levels in human LPS-stimulated macrophages. All beers assayed significantly reduced TNF-α production whereas IL-6 levels were significantly ameliorated by CrIpa1 and Gui1. In fact, Gui1 achieved the greatest reduction in both cytokines. These results revealed, for the first time, the antioxidant and anti-inflammatory properties of freeze-dried beers in human LPS-activated macrophages.

Once we tested the beneficial effects of freeze-dried beers in an *in vitro* screening THP-1 macrophage model, we attempted to elucidate the effects of dietary freeze-dried Guinness beer (Gui1) supplementation on a DSS-induced chronic colitis model. This model is widely used to study the pathogenesis and innovative treatments for UC. DSS induces intestinal inflammation by damaging the colonic epithelium, producing symptoms analogous to those found in UC patients.[2] Chronic colonic damage was induced in C57BL/6 mice by a short 5 day exposure to DSS followed by a 3 week rest period.[4] Supplementation with freeze-dried Guinness beer was successful in reducing the severity, extent and chronic colonic damage, corroborating previous *in vitro* studies. We observed that typical abnormalities (morphological, histological and clinical) in DSS-induced colitis were attenuated by pre-treatment with the freeze-dried beer. As expected, mice exposed to DSS developed an increase in inflammatory activity indicated by a significant body weight loss, increased DAI score, colon shortening and increased colon weight/length ratio. However, freeze-dried Guinness beer administration reduced body weight loss and colon weight/length ratio, as well as attenuated colon shortening and clinical symptoms of colitis. These results are consistent with those obtained in a study in which pale ale beer powder reduced the clinical and anatomical parameters in an acute murine colitis model.[20] In this context, it

has been reported that gallic acid, the major phenolic compound identified in freeze-dried Guinness beer, was able to reduce colonic damage.[47] In our study, dietary pre-treatment with commercial freeze-dried Guinness beer showed a reduction in histological damage by preventing inflammation in all layers and reducing the loss of the glandular architecture, which can lead to ulceration in some areas and the consequent loss of epithelium. In addition, the PAS technique revealed that the number and distribution of goblet cells changed from their normal pattern as the glandular disruption progressed. Hence, in the groups with inflamed mucosa, the goblet cells occupied a basal position in the glands and could not even be identified on the surface.

The inflammation that occurs in UC impairs intestinal epithelial function, leading to the recruitment of inflammatory cells to the site of injury. DSS induces an imbalance in the cytokine network, leading to high production of several pro-inflammatory Th1 and Th17 cytokines (including TNF-α, IL-1β, IL-6 and IL-17).[5,31] Consistent with our *in vitro* results, dietary supplementation with freeze-dried Guinness beer significantly reduced the levels of all these cytokines. Our results are along the same lines of a previous study described by Scherr *et al.*[48] showing that beer has anti-inflammatory potential by reducing these pro-inflammatory cytokines in respiratory disease.

COX-2 and mPGES-1 are enzymes that can be induced by pro-inflammatory cytokines and are expressed at sites of inflammation contributing to the development of intestinal damage. Both enzymes are upregulated in UC and are responsible for the overproduction of PGE_2, an important inflammatory mediator.[49,50] Cho *et al.* determined that synthesised xanthohumol, a compound found in certain types of beers, reduced COX-2 levels in a DSS-induced acute colitis model.[51] In contrast, the present study has shown that a dietary intervention with freeze-dried Guinness beer returned COX-2 and mPGES-1 production to basal levels. Thus, these results suggest that beer's beneficial effects may be mediated by inhibition of pro-inflammatory cytokines and an amelioration of DSS-induced COX-2 overexpression.

Overexpression of pro-inflammatory cytokines such as TNF-α and IL-1β in UC is associated with an activation of the NF-κB complex in colonic tissue. NF-κB can induce a strong immune response by regulating the transcription of many inflammatory factors. Activation of NF-κB involves degradation of the inhibitory protein IκB-α, which leads to the release and phosphorylation of NF-κB p65. Subsequently phosphorylated NF-κB p65 translocates to the nucleus where it regulates transcription of genes.[9] As expected, the inhibitory protein IκB-α was downregulated in the DSS group, whereas the expression of the NF-κB p-p65 subunit was significantly increased. In contrast, the freeze-dried Guinness beer pre-treatment inhibited the IκB-α degradation and consequently decreased NF-κB p-p65 levels, contributing significantly to the beneficial effect on colitis. These results are consistent with those obtained by Martinez *et al.* in which dealcoholized beer-rich diet showed a clear attenuation of the NF-κB complex, reducing the endothelial adhesion molecule expression in a model of atherosclerosis.[21] Additionally, JAK-STAT3 can act as an upstream regulator of NF-κB, promoting NF-κB signal transduction in innate and acquired immune cells. Phosphorylation of STAT3 leads to its activation, which regulates the expression of several genes such as pro-inflammatory enzymes and mediators. Consistent with previous studies, DSS promoted the phosphorylation of STAT-3 protein in a model of acute UC.[8] In contrast, freeze-dried Guinness beer pre-treatment was able to reduce this phosphorylation. Our results agree with those obtained by Takata *et al.*, who showed that STAT3 phosphorylation in lung epithelial-like A549 cells was significantly decreased after beer treatment.[52] Therefore, our findings suggest that dietary beer could inhibit the expression of pro-inflammatory cytokines by blocking the JAK/STAT pathway in chronic UC.

Finally, oxidative stress is also one of the key factors in the pathogenesis of UC, as high levels of ROS cause damage in the colon. Nrf2 is a transcription factor that mediates the cellular response to oxidative stress and regulates the expression of several antioxidant genes, such as HO-1. Xanthohumol, a chalcone polyphenol from hops, has previously been reported to activate the antioxidant Nrf2/HO-1 pathway.[53] Interestingly, we provide novel evidence that dietary commercial Guinness beer pre-treatment significantly up-regulates the expression of Nrf2/HO-1 proteins, thereby increasing the antioxidant response in UC. It has been previously shown that there is crosslink between Nrf2, STAT3 and NF-κB signaling pathways since the upregulation of Nrf2 inhibits STAT3 phosphorylation and, consequently, NF-κB inhibition. Nevertheless, Nrf2/HO-1 can also participate directly in the inactivation of NF-κB. The downregulation of NF-κB leads to a decrease in pro-inflammatory cytokines and the enzymes mPGES-1 and COX-2.[54,55] Our findings suggest that upregulation of Nrf2/HO-1 leads to downregulation of the JAK/STAT axis and inhibition of the NF-κB signaling pathway. For this reason, commercial Guinness beer may prevent the increase in pro-inflammatory cytokines and the enzymes mPGES-1 and COX-2 *via* the downregulation of NF-κB.

5. Conclusion

After an initial screening of the beer samples using HPLC-MS, subsequent analysis by HPLC-DAD enabled the quantification of 13 phenolic compounds typically found in this type of matrix. Among them, gallic acid and catechin were identified as the most abundant phenolic compounds. Our study has demonstrated, for the first time, that freeze-dried Guinness beer administration exerts antioxidant and anti-inflammatory effects in LPS-induced human macrophages and in a murine model of DSS-induced chronic colitis. The mechanisms underlying these protective effects appear to be related to ameliorated production of pro-inflammatory cytokines as well as downregulation of mPGES-1 and COX-2 protein expression *via* inhibition of NF-κB nuclear translocation. This inhibition may be mediated by the induction of the Nrf2/HO-1 antioxidant signaling pathway, which leads to reduction of STAT3 phosphoryl-

ation and, consequently, downregulation of NF-κB. Our results suggest that dealcoholized beer, a key bioactive food with multiple beneficial properties, may be effective in the management of immune-mediated inflammatory diseases where macrophages are crucial, including IBD, providing clues for developing useful nutritional interventions against inflammation-associated pathologies.

Author contributions

Conceptualization, M. S.-H. and E. T.; methodology, M. P.-S.; experimentation contribution, R. M.-G, J. A.-R. M. S.-H. and E. T.; chemical characterization, D. L. G.-G., A. L.-P. and M. M. O.-C.; histological assay, V. V.-R.; software, validation and formal analysis, M. P.-S.; investigation, M. P.-S.; writing – original draft preparation, M. P.-S., M. S.-H. and E. T.; writing – editing, M. P.-S., M. S.-H. and E. T.; reviewing – visualization and supervision, M. S.-H. and E. T.; funding acquisition, M. S.-H. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Submission declaration and verification

This manuscript has not and will not be published in whole or in part in any other journal.

Ethics approval

The study was approved by the Animals Ethics Committee of the University of Seville (ethical approval number: CEEA-US2022-17 and Junta de Andalucía, 14/04/2023/0012).

Data availability

All experimental data and detailed experimental procedures are available, and they will be shared when required.

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