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Freeze-dried jaboticaba (*Myrciaria jaboticaba*) peel powder, a rich source of anthocyanins and phenolic acids, mitigates inflammation-driven colorectal cancer in mice

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ABSTRACT

This study aimed to characterize the peel of jaboticaba (*Myrciaria jaboticaba* (Vell.) O. Berg) by spectrophotometry and chromatography, and investigate its effects on mice with inflammation-driven colorectal cancer (CRC). Freeze-dried jaboticaba peel (FDJP) showed a high antioxidant capacity *in vitro*, and to be mostly a source of cyanidin-3-*O*-glucoside, gallic acid, and ellagic acid. In the animal bioassay, FDJP added at 5% in the diet during 114 days completely abolished the formation of adenocarcinoma, which had an incidence of 75% in the non-treated group. Animals consuming FDJP showed low histology damage regarding inflammatory infiltrate, edema, and crypt distortion. FDJP significantly reduced the colonic levels of proinflammatory markers interleukin-1beta and cyclooxygenase-2, and demonstrated a probability to decrease the expressions of inducible nitric oxide synthase and nuclear factor kappa B. In summary, FDJP mitigated CRC, possibly by avoiding triggering inflammation. Such benefit may come from its rich content in anthocyanins and phenolic acids.

1. Introduction

Colorectal cancer (CRC) is the third most common and the second most deadly type of cancer worldwide (Sung et al., 2021). In the last years, a significant increase in incidence and/or mortality has been seen in developing countries, including the ones in Latin America and the Caribbean, such as Brazil, Chile, and Costa Rica (Piñeros et al., 2022; Sierra & Forman, 2016). By 2040, CRC is estimated to be the leading cause of cancer-related deaths in young individuals between 20 and 49 years of age in the United States of America (Rahib et al., 2021). The increasing adoption of a Western lifestyle is often referred to as one of the factors associated with such outcomes (Safiri et al., 2019). Additionally, as persisting inflammation can evolve into dysplasia,

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Abbreviations

AOM	azoxymethane
BSA	bovine serum albumin
C3G	cyanidin-3-*O*-glucoside
COX-2	cyclooxygenase-2
CRC	colorectal cancer
DAI	Disease Activity Index
D3G	delphinidin-3-*O*-glucoside
DSS	dextran sodium sulfate
ELISA	enzyme-linked immunosorbent assay
FDJP	freeze-dried jaboticaba peel
FRAP	ferric reducing antioxidant power
HPLC-DAD	high-performance liquid chromatography coupled with a diode array detector
iNOS	inducible nitric oxide synthase
IL-1β	interleukin-1beta
NO	nitric oxide
ORAC	oxygen radical absorbance capacity
P3G	pelargonidin-3-*O*-glucoside
p–NF–κB	phosphorylated nuclear factor kappa B
TNF-α	tumor necrosis factor-alpha
TPC	total phenolic compounds

individuals having other chronic conditions, such as diabetes, obesity, and inflammatory bowel diseases, may present an increased risk over time of developing CRC (Herrinton et al., 2012; Soltani et al., 2019).

CRC treatment is traditionally done through surgery in order to remove tumors and/or parts of the whole colon. These may be associated with radiotherapy or chemotherapy for patients in more advanced stages of the disease (Brenner et al., 2014). However, this process can culminate in an important decrease in the quality of life and well-being of individuals with CRC. Treatments do not always are effective and can cause adverse effects. When colectomy is needed, a high incidence of diversion colitis is observed (Son et al., 2013). Moreover, patients may experience psychosocial problems, which involve difficult family and social interactions, fear of relapse, and depression (Nicolussi & Sawada, 2009; Trnini et al., 2009). Alternative natural-based methods of handling CRC can potentially improve the present scenario but need further investigation.

By performing population and cohort studies, researchers have found that distinct types of diets or foods are implicated in CRC initiation and/or progression. While the consumption of red meat and total fat has been strongly associated with an increased CRC risk, healthy-linked foods such as fruits, legumes, low-fat dairy products, and whole cereals may exert a protective effect; although evidence for some of these are still weak (Ryan-Harshman & Aldoori, 2007a; Veettil et al., 2021). In that sense, experimental studies performed in the last decades have tested the impact of several plants or their bioactive compounds on CRC, indicating a positive correlation (Costea et al., 2018). Phenolic compounds, such as anthocyanins, mostly from functional fruits, have shown the capacity to reduce inflammation, oxidative stress, dysbiosis, and/or cell proliferation in a great variety of *in vitro* and *in vivo* CRC protocols (Medic et al., 2019). Anthocyanins are known to display their healing effects through colonic microbiota metabolization since they are not well absorbed in the large intestine. Circulating levels of anthocyanins have been mostly associated with microbial metabolism (Bars-Cortina et al., 2021).

Although still undervalued or little explored in comparison with other fruits, native Brazilian fruits represent promising anticancer crops according to preclinical studies (Machado et al., 2022; Nascimento et al., 2022; Reguengo et al., 2022). Jaboticaba (*Myrciaria jaboticaba*), a flavorful and popular berry native to the Atlantic Forest of Brazil, has shown a high antioxidant capacity *in vitro* and to be mostly a rich source of anthocyanins, but also dietary fibers, phenolic acids, and tannins (Inada et al., 2021). The anthocyanin composition and antioxidant capacity of jaboticaba equal or even surpass the ones of North American and European common berries (Rigolon et al., 2020), thus suggesting the pro-health potential of this Brazilian fruit. Especially, the peel of jaboticaba, a product usually discarded by both the industry and the customers, presents higher contents of bioactive compounds in comparison with the pulp, seed, and whole fruit, probably due to its dark purple-to-black color, an indication of anthocyanin richness (Inada et al., 2015). Recent studies have found that jaboticaba polyphenols or pectin have anti-CRC effects by reducing cell proliferation *in vitro* and/or restoring the healthy microbiota *in vivo* (Augusti et al., 2021; do Carmo et al., 2021; do Nascimento et al., 2020; Fidelis et al., 2021; Simas Frauches et al., 2021). However, some of the clinical aspects and molecular mechanisms behind that are still in an early stage of investigation or are poorly known.

No study to this date has tested the peel of jaboticaba in an animal model of CRC or identified if this fruit portion can reduce tumor incidence or regulate cancer-related inflammatory mediators. Ongoing investigations with jaboticaba peel may identify future alternative or complementary medicines for CRC. Therefore, our study aimed to understand 1. The proximate, spectrophotometric, and chromatographic compositions of freeze-dried jaboticaba peel (FDJP); and 2. The implications of dietary FDJP intake on mice with inflammation-driven CRC, considering the clinical, histopathological, and molecular aspects of the disease.

2. Material and methods

2.1. Chemicals

The main chemicals utilized in the experiment and analyses are described next. 2,4,6-tripyridyl-*S*-triazine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-mercaptoetanol, aprotinin, azoxymethane (AOM), bovine serum albumin (BSA), cyanidin-3-*O*-glucoside (C3G), delphinidin-3-*O*-glucoside (D3G), Folin-Ciocalteu reagent, Ponceau S, other flavonoids and phenolic acids standards, and Tween® 20 were all purchased from Sigma Aldrich (St. Louis, MO, USA). 2x laemmli sample buffer, 40% acrylamide/bis-acrylamide, ammonium persulfate, glycine, Protein Assay Dye Reagent Concentrate, sodium dodecyl sulfate, Triton X-100, tris, and tetrametiletilenodiamina were all purchased from BioRad Laboratories (Hercules, CA, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride was purchased from Cayman Chemical (Ann Arbor, MI, USA). Aprotinin, West Pico chemiluminescent substrate, and Western Blotting stripping buffer were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fluorescein was purchased from Synth (Diadema, SP, Brazil). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Peprotech (Rocky Hill, NJ, USA). Dextran sodium sulfate (DSS) was purchased from MP Biomedicals (Solon, OH, USA). Pelargonidin-3-*O*-glucoside (P3G) standard was purchased from PhytoLab (Vestenbergsgreuth, Germany). Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies were purchased either from Cell Signaling Technology or Promega (Madison, WI, USA).

2.2. Ethics, animals, and conditions

The study is in accordance with the ARRIVE guidelines and followed the guide for the care and use of laboratory animals of the National Institutes of Health (NIH Publications No. 8023, revised 1978). The animal experiment was approved by the Ethics Committee on the Use of Animals of the University of Campinas (UNICAMP), protocol number 5246–1/2019.

Male BALB/c mice (n = 18), three to four weeks of age, were obtained from the Multidisciplinary Center for Biological Investigation on

Laboratory Animal Science - CEMIB/UNICAMP. The animals were transported to an experimental room with controlled temperature (~19–21 °C) and humidity (~40%–60%), and 12/12 h dark/light cycles. Three per cage, the animals were acclimatized until six-to-seven weeks of age, receiving during this time water *ad libitum* and commercial mouse feed (Nuvilab CR-1, Nuvital).

2.3. Fruit, diets, and chemical analyses

The jaboticaba (*Myrciaria jaboticaba* (Vell.) O. Berg) fruit, cultivar Sabará, was obtained during May from a local producer in Casa Branca, São Paulo, Brazil, coordinates 21°53′42.1″S 47°02′10.9″W. The use of jaboticaba was registered on *SisGen/Brazil*, protocol number AD872CA. The peel was manually separated, freeze-dried in equipment and conditions similar to Leite-Legatti et al. (2012), and ground into powder.

Animals were divided into three groups of 6 animals each: *control* (*C*), *control cancer* (*CC*), and *jaboticaba cancer* (*JC*). Mice received during the whole experiment a standard AIN-93M diet (Reeves et al., 1993), with or without 5%-added FDJP powder (supplementary material 1). The chosen animal dose (5%) was based on successful CRC studies using anthocyanin-rich black raspberry (Chen et al., 2018; Huang et al., 2020), and would be equivalent to the consumption of 40–50 g of dried peel powder daily when translated to humans (supplementary material 2) (Reagan-Shaw et al., 2008). The experiment lasted 114 days, from which the initial 28 days were exclusively dedicated to the consumption of FDJP. The diet consumption, water intake, and body weight of mice were monitored during the whole experiment.

The FDJP and diets were extracted and analyzed following the studies and equipment cited in a recent study of our research group (Nascimento et al., 2021). Initially, proximate and spectrophotometric analyses were performed, including moisture, ashes, lipids, protein, total phenolic compounds (TPC) by Folin-Ciocalteu, total flavonoids, monomeric anthocyanins, and antioxidant capacity assays ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC).

The FDJP was also analyzed by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) for its specific content in anthocyanins (C3G, D3G, and P3G), other flavonoids and phenolic acids (catechin, epicatechin, quercetin, rutin, 4-hydroxybenzoic acid, ellagic acid, ferulic acid, gallic acid, *p*-coumaric acid, protocatechuic acid, syringic acid), and carotenoids (α-carotene, β-carotene, β-cryptoxanthin, and lutein), according to procedures by Santiago et al. (2010), Nascimento et al. (2017), and Pacheco (2009), respectively. Total carotenoids were determined by ultraviolet–visible spectroscopy (Pacheco, 2009). HPLC-DAD and spectroscopy analyses were performed by the Embrapa Food Technology (Rio de Janeiro, RJ, Brazil).

2.4. Colorectal cancer induction and clinical evaluation

After 28 days of FDJP consumption, animals received a single intraperitoneal injection of the carcinogenic AOM (10 mg/kg of body weight). On day 35, acute colonic inflammation was induced by replacing water bottles by new ones containing colitis-inducer DSS diluted in filtered water at 2% (w/v). The DSS solution was changed to a new one every two to 3 day as recommended by Chassaing et al. (2014). On day 44, bottles with DSS were changed to new ones containing only normal water until the end of the experiment (day 114). The long period after DSS withdrawal is necessary for tumors to develop and grow properly. The AOM/DSS protocol was based on Rosenberg et al. (2008) and Tanaka et al. (2003), and considers inflammation as an initial occurring factor leading to CRC.

During the period after the AOM injection, mice were analyzed for their body weight loss (%), anal bleeding, and stool consistency to compose the Disease Activity Index (DAI) (Gommeaux et al., 2007; Nascimento et al., 2020) Professional photographs were taken to represent the anal bleeding and stool consistency, which were later analyzed blindly, meaning without the knowledge of the experimental groups' identification, by two independent researchers (Nascimento et al., 2020). Mortality was also checked throughout the entire experiment. A scheme of the experimental protocol can be seen in supplementary material 3.

2.5. Euthanasia and tissue measurements

Euthanasia was performed on day 114 by an intraperitoneal injection of combined ketamine (300 mg/kg of body weight) and xylazine (30 mg/kg of body weight) solution, followed by cardiac puncture for blood collection. Serum was collected after centrifugation (10 min, 5000 rpm) for further antioxidant capacity analysis. Withdrawn tissues or organs were as follows: liver, spleen, and colon. All organs were weighted (g), the length of the colon was measured with a ruler (cm), and the colon weight/length (mg/cm) ratio was calculated.

At euthanasia, CRC tumors were counted macroscopically. The distal part of the colon plus rectum was kept in formaldehyde at 4% until histological processing and analysis. The remaining colonic tissue was stored at −80 °C for ELISA and Western Blotting analyses.

2.6. Histological processing and assessment

Histological processing of the colon followed by hematoxylin-eosin coloring and slide preparation was carried out routinely and standardized according to Nascimento et al. (2020). The incidence, multiplicity, and depth of tumors were assessed. The histological difference between adenoma and adenocarcinoma followed the guidelines of the World Health Organization (2000). Fibrosis and Necrosis incidence were also checked. Additionally, in order to assess the damage to the colon, a histopathological score was created, taking as reference some of the existing inflammation-related evaluations in the literature (Dieleman et al., 1998; Stucchi et al., 2000). Briefly, for this score, parameters such as inflammatory infiltrate, edema, vascularization, crypt distortion, and goblet cell depletion were considered (supplementary material 4). A researcher with experience in mouse models of colitis and CRC, blind to the identification of the groups, analyzed the slides. Histology photographs were taken at 10x/0.25 magnification using a Nikon Eclipse E−400 microscope (Tokyo, Japan) and software NIS-Elements.

2.7. Enzyme-linked immunosorbent assay and Western Blotting analyses

Colonic samples were homogenized in ice-cold radioimmunoprecipitation assay buffer composed of NaCl (150 mM), Tris HCl pH 8 (50 mM), Triton X-100 (1%), SDS (0.1%), and protease inhibitor aprotinin (2 μg/mL). Samples were centrifuged at 10,000 rpm, 4 °C, for 30 min, from which the supernatant was collected and later subjected to protein quantification by the Bradford colorimetric method.

Analyses of interleukin (IL) 1β and tumor necrosis factor-alpha (TNF-α) were performed by ELISA, according to the manufacturer's protocol.

For Western Blotting, samples were pre-treated with laemmli and β-mercaptoethanol, and heated at ~100 °C for 5 min for protein denaturation. Aliquots containing equal amounts of protein (40 μg) were then applied to polyacrylamide gels at 8% or 10% concentrations and separated at a constant voltage of 60 V (for 10 min) and 100 V (for the next 90–120 min). Proteins were electrophoretically transferred to nitrocellulose membranes through a wet system at 100 V for 90 min, and later on, checked for bands via Ponceau S staining. Membranes were incubated in a blocking buffer (5% BSA) for 120 min. Posteriorly, they were incubated overnight with primary antibodies cyclooxygenase-2 (COX-2) (rabbit, 1:1000, 5% BSA), inducible nitric oxide synthase (iNOS) (rabbit, 1:1000, 5% BSA), or phosphorylated nuclear factor kappa B (p–NF–κB) p65 (mouse, 1:1000, 5% non-fat dry milk). β-actin was used as a loading control antibody (rabbit or mouse, 1:1000, 5% BSA). Secondary antibodies were used at 1:1000 (rabbit, 5% BSA) or 1:2500 (mouse, 3% non-fat dry milk) concentrations for an incubation

Table 1
Proximate, spectrophotometric, and chromatographic characterization of the fruit and/or diets.

Analysis	FDJP powder*	Control diet**	5% FDJP diet**
PROXIMATE (%)			
Moisture	11.61 ± 0.77	10.81 ± 0.13**a**	7.99 ± 0.30**b**
Ashes	2.18 ± 0.13	2.35 ± 0.07**a**	2.42 ± 0.03**a**
Lipids	6.47 ± 0.51	11.50 ± 0.65**a**	11.28 ± 0.56**a**
Protein	1.55 ± 0.08	3.97 ± 0.47**a**	3.58 ± 0.25**a**
SPECTROPHOTOMETRIC			
TPC (mg GAE/g)	87.34 ± 6.64	0.46 ± 0.06**a**	1.44 ± 0.04**b**
Total flavonoids (mg QE/g)	6.92 ± 0.17	–	–
Monomeric anthocyanins (mg C3G/g)	5.64 ± 0.12	–	–
FRAP (μmol TE/g)	915.90 ± 20.19	0.50 ± 0.03**a**	11.19 ± 0.60**b**
ORAC (μmol TE/g)	821.8 ± 38.61	6.73 ± 0.43**a**	13.41 ± 0.44**b**
CHROMATOGRAPHIC (HPLC-DAD) (mg/100g)			
Anthocyanins			
Cyanidin-3-*O*-glucoside	980.7 ± 25.34	–	–
Delphinidin-3-*O*-glucoside	60.26 ± 0.77	–	–
Pelargonidin-3-*O*-glucoside	ND	–	–
Other flavonoids			
Catechin+	1.46 ± 0.07	–	–
Epicatechin#	ND	–	–
Quercetin+	0.93 ± 0.02	–	–
Rutin+	4.07 ± 0.11	–	–
Phenolic acids			
4-hydroxybenzoic acid#	0.51 ± 0.09	–	–
Ellagic acid+	235.30 ± 5.89	–	–
Ferulic acid#	0.83 ± 0.03	–	–
Gallic acid#	393.70 ± 7.84	–	–
p-Coumaric acid#	0.26 ± 0.02	–	–
Protocatechuic acid+	12.35 ± 0.07	–	–
Syringic acid&	5.53 ± 0.38	–	–
Carotenoids			
α-Carotene	0.15 ± 0.01	–	–
β-Carotene	0.85 ± 0.03	–	–
β-Cryptoxanthin	ND	–	–
Lutein	1.61 ± 0.07	–	–
Total carotenoids	3.21 ± 0.10	–	–

Results are expressed in mean ± standard deviation (SD). Student's t-test was performed between the diets; different letters indicate statistical difference ($p < 0.05$). *Results are in dried weight. **Results are in fresh weight. +Sum of free and hydrolyzed fractions. #Hydrolyzed fraction. **&**Free fraction**.** Abbreviations: FDJP: freeze-dried jaboticaba peel, FRAP: ferric reducing antioxidant power, GAE: gallic acid equivalent, HPLC-DAD: high-performance liquid chromatography coupled with a diode array detector, ND: not detectable, ORAC: oxygen radical absorbance capacity, QE: quercetin equivalent, TE: Trolox equivalent, TPC: total phenolic content.

time of 60 min. Immunodetection was performed using the West Pico chemiluminescent substrate kit. The signals were analyzed on chemiluminescence imaging Syngene's GeneGnome XRQ (Cambridge, EN, UK) and quantified on ImageJ software (Bethesda, MD, USA). When necessary, membranes were stripped to detect β-actin expression. Tris-buffered saline with Tween® 20 detergent was used between procedures to wash the membranes and as a basis for solutions.

2.8. Statistical analysis

Data from the composition parameters (difference between diets) were analyzed by Student's t-test. Data from the animal experiment were initially submitted to an outliers withdrawal by Grubs 5%,

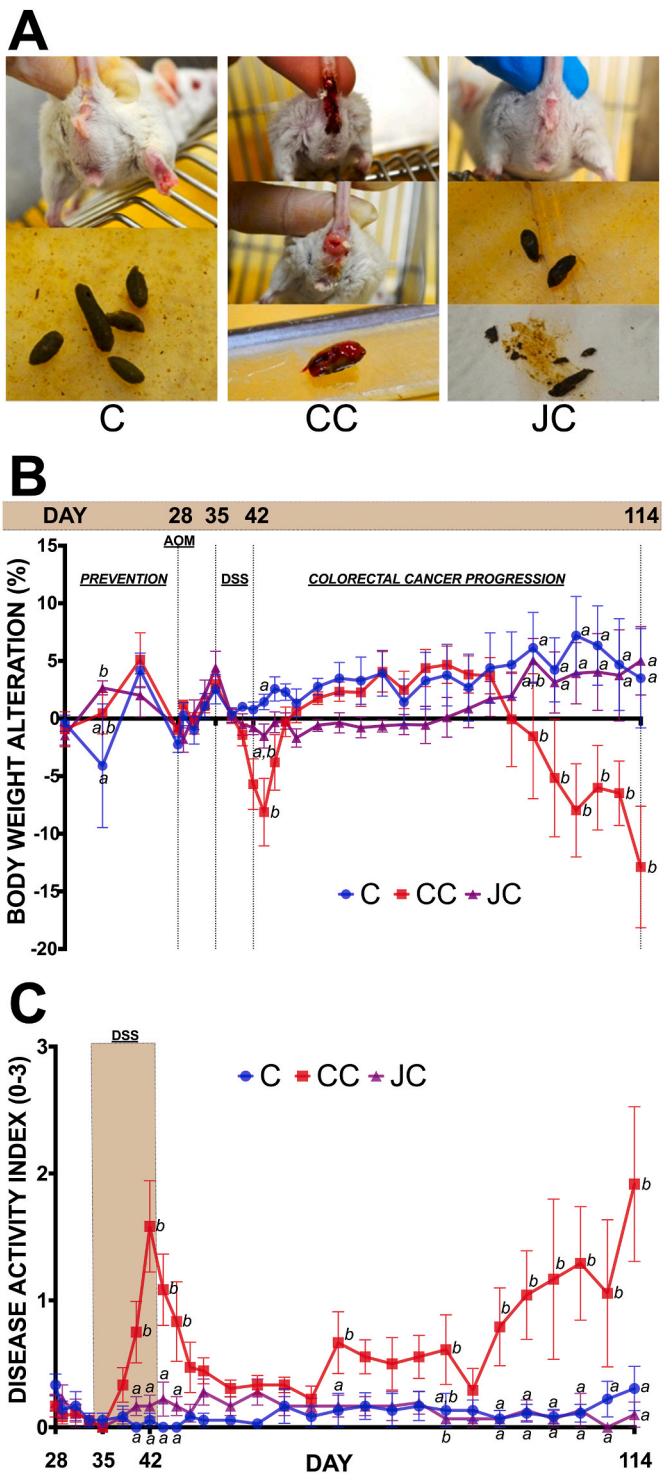

Fig. 1. Clinical results
Experimental groups: C – *control,* CC – *control cancer,* JC – *jaboticaba cancer.* Two-way ANOVA followed by Tukey ($p < 0.05$); different letters indicate statistical difference ($p < 0.05$). **A –** Photographic representations of bleeding, stool consistency, and prolapse (when applicable) during the colorectal cancer onset period. **B –** Body weight alteration (%) during the entire length of the experiment (days 0–114). Abbreviations: AOM: azoxymethane, DSS: dextran sodium sulfate. **C –** Disease Activity Index (combination of body weight loss, bleeding, and stool consistency scores) analysis starting from azoxymethane injection (days 28–114).

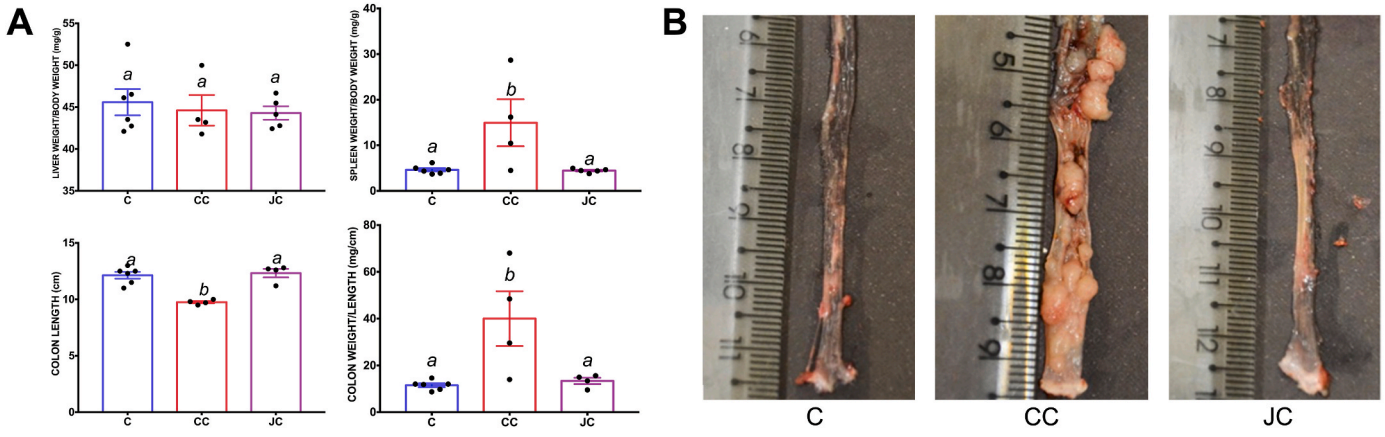

Fig. 2. Tissue measurements and photographic representation of the colon
Experimental groups: C – *control,* CC – *control cancer,* JC – *jaboticaba cancer.* One-way ANOVA followed by Tukey ($p < 0.05$); different letters indicate statistical difference ($p < 0.05$). **A** – Tissues (liver, spleen, colon) weight, length, and/or weight/length. **B –** Photographic representation of the colon at euthanasia.

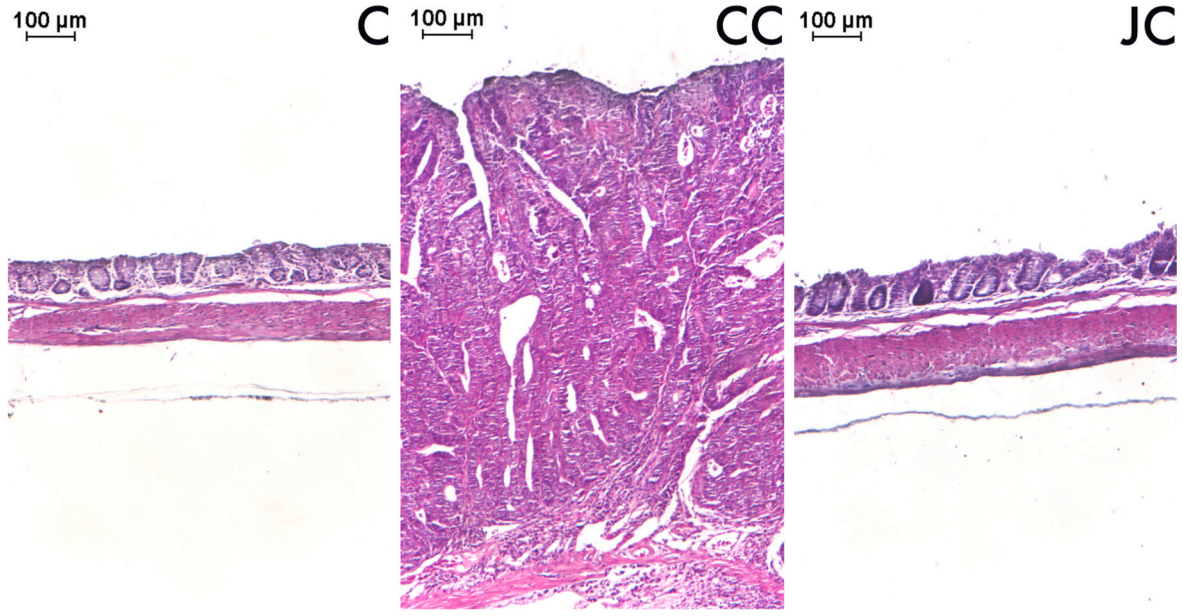

Fig. 3. Histology of the distal colon
Experimental groups: C – *control,* CC – *control cancer,* JC – *jaboticaba cancer.* Magnification 10×/0.25, scale 100 µm, Nikon Eclipse E−400 microscope (Tokyo, Japan), software NIS-Elements.

followed by a normality test considering values of kurtosis and skewness between −2 and 2 (George & Mallery, 2003). Later, data from the three experimental groups were compared by One-way or Two-way ANOVA followed by Tukey (if time was a variable involved). A $p < 0.05$ was considered to determine statistical difference.

3. Results and discussion

3.1. Chemical characterization of the fruit and diets

Spectrophotometric analyses indicated that FDJP is an interesting source of TPC and total monomeric anthocyanins, and has a high antioxidant capacity measured by both FRAP or ORAC methods. As expected, mouse diets enriched with 5% FDJP presented a significant increase in TPC and antioxidant capacity in comparison with the control diet ($p < 0.05$) (Table 1). A previous study of our research group showed similar results. A mouse diet enriched with 5% FDJP has a TPC that surpasses 1 mg of gallic acid equivalent (GAE)/g, and an antioxidant capacity (FRAP, ORAC) higher than 10 µmol of Trolox equivalent/g (R. de P. do Nascimento et al., 2021). According to do Socorro et al. (2010), the FDJP used in our study would be classified as a high source of TPC, as it has more than 50 mg GAE/g (dry matter). Well-known Western berries (blackberry, blueberry) have way less TPC and antioxidant capacity when compared to jaboticaba peel (Rigolon et al., 2020), making the latter a potential new functional food of the 21st century.

Regarding detailed anthocyanins content, the chromatographic analysis indicated two chromatogram peaks, which were relative to C3G and D3G. Quantitatively, C3G was present in a greater amount, contributing to almost 1% (dry weight) of FDJP's composition. A few contents in D3G (0.06%, dry weight) and none in P3G were detected. Supplementary material 5 displays the anthocyanins chromatogram. The anthocyanin profile found by our study corroborates those obtained by Inada et al. (2015), Leite-Legatti et al. (2012), Paludo et al. (2022), Plaza et al. (2016), and De Araujo Santiago et al. (2018), which also indicated C3G followed by D3G as the main anthocyanins of jaboticaba peel.

Table 2
Histopathology of the distal colon.

Parameter	Experimental group		
	C	CC	JC
INCIDENCE (%)			
No alteration or aberrant crypt foci	100 (6/6)	25 (1/4)	100 (5/5)
Adenoma	0 (0/6)	0 (0/4)	0 (0/5)
Adenocarcinoma	0 (0/6)	75 (3/4)	0 (0/5)
SCORE			
Inflammatory infiltrate (mucosa)	0.50 ± 0.22**a**	2.25 ± 0.47**b**	0.8 ± 0.20**a**
Inflammatory infiltrate (submucosa)	0.33 ± 0.21**a**	2.25 ± 0.47**b**	0**a**
Edema (mucosa)	0.83 ± 0.16**a**	1.75 ± 0.25**b**	0.80 ± 0.20**a**
Edema (submucosa)	0.33 ± 0.21**a**	2.25 ± 0.47**b**	0.20 ± 0.20**a**
Vascularization (mucosa)	1.00 ± 0.00**a**	1.75 ± 0.47**a**	0.80 ± 0.20**a**
Vascularization (submucosa)	0.33 ± 0.21**a**	1.50 ± 0.64**a**	0**b**
Crypt distortion	0.66 ± 0.21**a**	2.50 ± 0.50**b**	0.20 ± 0.20**a**
Goblet cell depletion	0.83 ± 0.16**a**	2.50 ± 0.50**b**	0.40 ± 0.24**a**
TOTAL (average)	0.60 ± 0.11**a**	2.09 ± 0.41**b**	0.40 ± 0.12**a**

Experimental groups: C – *control,* CC - *control cancer*, JC – *jaboticaba cancer.* Score results are expressed in mean ± standard error (SEM). One-way ANOVA followed by Tukey (n = 4–6) was applied to the score's parameters and total; different letters indicate statistical difference ($p < 0.05$).

Phenolic acids were also found in an expressive amount in FDJP's polyphenolic composition. Gallic acid and ellagic acid combined represented around 0.5% (dry weight) of FDJP's composition. Other phenolic acids, such as protocatechuic and syringic, were found in interesting amounts, but quantities were less meaningful (Table 1). Recently, Paludo et al. (2022) indicated that jaboticaba peel has also relevant contents of other phenolic compounds, including quercetin and myricetin derivatives, flavan-3-ol monomers, and proanthocyanidins. The quantities found, however, can significantly differ depending on the jaboticaba cultivar, with Sabará and Paulista being the most characterized so far by the literature (Paludo et al., 2022).

Small amounts of carotenoids were detected in FDJP. Lutein was found in higher quantities in comparison with α- and β-carotenes. β-cryptoxanthin was not detected in the FDJP sample (Table 1). The total carotenoids content found by our study (3.21 mg/100 g, dry weight) is almost double that of Quatrin et al. (2018), also using FDJP (1.78 mg/100 g), a difference which is probably associated with the fruits' distinct harvest and storage periods.

A previous publication of our research group has also shown that FDJP, treated very similarly to the present study, is also a rich source of total dietary fibers, around 30 g/100 g in dry basis, which may contribute to the health-related properties of the fruit (Lenquiste et al., 2012).

In general, among the analyses performed, FDJP showed to be mostly a rich source of C3G, gallic acid, and ellagic acid, which may have promoted increased TPC content and antioxidant capacity when added to a standard diet. Table 1 shows the complete results of the proximate, spectrophotometric, and chromatographic analyses performed for the FDJP and/or diets.

3.2. Diet and water intake, Disease Activity Index, and mortality

Weekly average diet consumption (g) did not differ significantly between the *control* (3.76 ± 0.06), *control cancer* (3.80 ± 0.07), and *jaboticaba cancer* (3.93 ± 0.07) groups, meaning that jaboticaba did not influence the amount of diet ingested. On the other hand, water consumption increased significantly in the *control cancer* and *jaboticaba cancer* groups in comparison with the *control* group ($p < 0.05$). This happened only during the period when DSS was added to the water bottles of the animals, suggesting that the colitis-inducing reagent is capable of increasing water intake.

During and after DSS intake, animals started to show signs of inflammation-driven CRC. At the peak of symptoms, mice in the *control cancer* group presented diarrhea, blood in the stools and anus, and even prolapse. Mice that consumed FDJP for 114 days did not show any of those signs (Fig. 1A). Especially, animals in the *control cancer* group showed significant body weight loss ($p < 0.05$), of up to 15%, due to colitis and tumor development (Fig. 1B). Accordingly, the DAI, which is a combination of body weight loss, bleeding, and stool consistency, also significantly increased only in the *control cancer* group ($p < 0.05$) (Fig. 1C). The consumption of FDJP prevented the clinical alterations analyzed in this CRC model, indicating its initial promising effects. Other animal studies, using anthocyanins extracted from black raspberry (*Rubus occidentalis*) and chokeberry (*Aronia melanocarpa*), have also reported the ability of these plant products in preventing body weight loss caused by CRC formation, therefore increasing the quality of life of mice (Li et al., 2021; Yu et al., 2021). In humans with CRC, a significant loss of body weight (5 kg) five years after the diagnostic is associated with worse survival, even in cases of obesity (Kocarnik et al., 2017). Studies with humans are necessary to understand if anthocyanins can also effectively improve the clinical symptoms and the quality of life of CRC patients.

Mice showed ~33% mortality (2/6) in the *control cancer* group and ~17% (1/6) in the *jaboticaba cancer* group. Deaths in the *control cancer* group occurred due to either AOM toxicity (likely) or symptoms from tumor formation, at days 90 and 98, respectively. Since the *jaboticaba cancer* group showed no signs of colitis or CRC progression, its single death was due to AOM toxicity and happened on day 86. According to our findings, AOM toxicity leading to death happens 8-to-9 weeks after AOM injection.

The consumption of natural plant products, including fruits, whole grains, and coffee has been inversely associated with all-cause mortality (Hoang et al., 2020). Particularly, daily fruit ingestion has been linked with reduced mortality for all causes of cancer (Sauvaget et al., 2003). Studies have not been able to identify a statistical correlation between mortality and fruit consumption, however, as reported by Luo et al. (2015), the intake of red/purple fruits can significantly reduce the risk for CRC. In addition, dietary fibers and anthocyanins, which are abundant in FDJP, have also been extensively associated with protection against CRC (Medic et al., 2019; Ryan-Harshman & Aldoori, 2007b).

3.3. Tissue measurements and macroscopic tumor counting

Liver weight was not changed by the CRC model or the intervention with FDJP. On the other hand, spleen weight and colon weight/length increased significantly in the *control cancer* group ($p < 0.05$). The colon length reduced considerably as a result of inflammation-driven CRC ($p < 0.05$). Animals that received FDJP showed no alterations in organ weight or length, meaning that their data were similar to the *control* group (Fig. 2A). Changes in body composition, including increased liver and spleen weights, have been seen in advanced cases of CRC. These are associated with cachexia-linked body weight loss and tissue's high metabolic rates in humans (Lieffers et al., 2009). In the AOM/DSS mouse model, an increase in spleen weight is commonly reported, being linked with enhanced inflammation and colonic damage (Chung et al., 2018; Leung et al., 2022; Ma et al., 2022).

On euthanasia day, tumor was evident in the *control cancer* group, which showed an average of 15.5 ± 6.27 points of tumor in the whole colon. Three out of four animals (75%) from the *control cancer* group had tumors. The macroscopic analysis also indicated that CRC tumors did not develop in the *jaboticaba cancer* group (Fig. 2B), therefore it seems that FDJP played a substantial role to avoid CRC initiation and progression. In similarity to our study, Guo et al. (2018) and Murakami

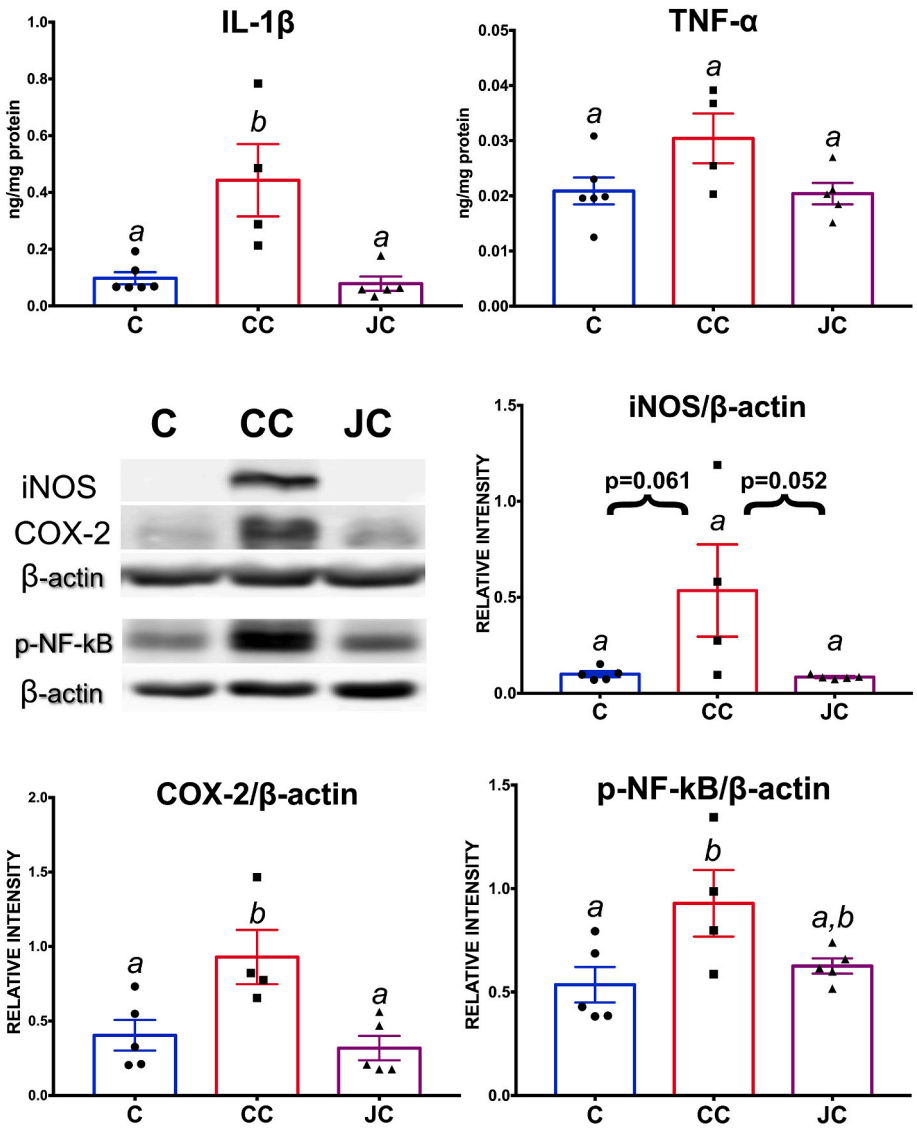

Fig. 4. Inflammation-related markers on the colon
Experimental groups: C – *control,* CC – *control cancer,* JC – *jaboticaba cancer.* One-way ANOVA followed by Tukey ($p < 0.05$); different letters indicate statistical difference ($p < 0.05$). The interleukins were analyzed by enzyme-linked immunosorbent assay kits, while the other proteins by Western Blotting. Abbreviations: IL-1β: interleukin-1beta, TNF-α: tumor necrosis factor-alpha, iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase-2, p–NF–κB: phosphorylated nuclear factor kappa B.

et al. (2013) also found a 100% inhibitory effect when using isolated curcumin from turmeric (*Curcuma longa*). The authors tested the chemopreventive effects of the isolated compound, added at 2% or 0.5% in the diet, respectively, on mice with colon carcinogenesis. In both studies, the interventions, which lasted for 16–18 weeks, completely abolished tumor incidence and multiplicity (Guo et al., 2018; Murakami et al., 2013).

Recently, Briata et al. (2021) performed a randomized, double-blind, placebo-controlled clinical trial in which they provided a supplement containing curcuminoids (Meriva®) plus anthocyanins (Mirtoselect®) to preoperative patients with colorectal adenomatous polyps. The mixture was highly promising, as it promoted a reduced colonic expression of cell proliferation-related biomarkers, namely NF-κB and Ki-67 (Briata et al., 2021). Mirtoselect® is a product made of bilberry extract and that contains as its main bioactive components C3G and D3G (Indena, 2018), both of which are also relevant compounds found in jaboticaba's polyphenolic composition, as shown previously.

3.4. Histopathology

Histology images confirmed what was previously observed macroscopically. Animals that consumed FDJP did not present adenoma or carcinoma, while three out of four mice in the *control cancer* group develop medium to large adenocarcinoma tumors (Fig. 3). Two out of four mice from the *control cancer* group had extensive adenocarcinoma tumors reaching even the colonic submucosa. By checking the score results, it was possible to notice that FDJP also completely stopped excessive inflammatory infiltrate, edema, crypt distortion, and goblet cell depletion (Table 2). Colonic vascularization was the solo score parameter that appeared to not correlate well with the results found. Fibrosis and necrosis were not detected in the slides from either *cancer* group. Recently, do Carmo et al. (2021) revealed that the ellagitannins isolated from jaboticaba seed can markedly reduce histology damage in mice with 1,2-dimethylhydrazine-induced aberrant crypt foci by decreasing intraepithelial lymphocytes and lamina propria cellularity. Our study, however, appears to be the first one to apply an extended histology evaluation to a robust model of CRC. Other investigations by da Silva-Maia et al. (2019) and Nascimento et al. (2021) have found improved histopathology parameters on rodents with chemically-induced colitis by using an anthocyanin-rich extract or powder from jaboticaba. In these studies, the treatments reduced colonic crypt damage, edema, and/or immunological infiltrate, thus regulating inflammatory-related markers and/or epithelial barrier function (da Silva-Maia et al., 2019; Nascimento et al., 2021).

The histology results indicate the power of jaboticaba in completely averting the inflammatory process leading to adenocarcinoma

formation, being necessary to address some of the probable mechanisms behind it.

3.5. Proinflammatory markers

The levels or expression of inflammatory mediators were verified in order to understand the mechanisms behind FDJP action on mitigating CRC. IL-1β, COX-2, and p–NF–κB p65 were significantly increased ($p < 0.05$) due to tumors being present in the colon. A tendency ($p = 0.061$) for iNOS increased expression was also noticed in the same experimental group (Fig. 4). Animals that consumed FDJP showed significantly reduced IL-1β levels and COX-2 expression, and a high tendency towards a decreased expression of iNOS ($p = 0.052$). The expression of p–NF–κB was not significantly different between *control cancer* and *jaboticaba cancer* groups, however, animals in the *jaboticaba cancer* group showed a p–NF–κB relative intensity similar to the *control* group, indicating a probable effect (Fig. 4). TNF-α levels were not changed by either the CRC model or the treatment (Fig. 4).

IL-1β is indirectly linked with COX-2 induction in colon cancer cells. This cytokine is said to help promote CRC progression via activating members of the NF-κB pathway, which eventually leads to the expression of the COX-2 enzyme. Those members include *c*-Jun N-terminal kinase, extracellular signal-regulated kinases, p38, and the NF-κB signaling (Duque et al., 2006; Liu et al., 2003). When activated, COX-2 is capable of mediating inflammatory processes through the conversion of arachidonic acid into prostaglandins, metabolites that indirectly modulate cell adhesion, growth, and differentiation (Tsujii & DuBois, 1995). In up to 85% of CRC cases, the expression of COX-2 is increased and studies show that this alteration is capable of promoting the tumorigenesis of the intestinal epithelial cell, through processes such as resistance to apoptosis, cell adhesion to extracellular matrix proteins, and mainly angiogenesis (Marnett & DuBois, 2002; Wang & Dubois, 2010). Additionally, studies suggest that the product of iNOS activity, nitric oxide (NO), would also be responsible for the increase in COX-2 activity. A blockage in the excessive levels of COX-2, iNOS, and NO has been studied as a possible solution for the control of CRC (Janakiram & Rao, 2012; Koehne & Dubois, 2004; Tsujii & DuBois, 1995).

Considering previous pre-clinical investigations done with jaboticaba on CRC, overall the fruit and its bioactive compounds have been able to reduce cell proliferation, regulate the cell cycle, increase apoptosis – *in vitro* -, decrease inflammation, and regulate the gut microbiota – *in vivo* -, the latter which have been deeply associated with the pathogenesis of CRC (Nascimento et al., 2022). Particularly, our study is the first to indicate an action of dietary FDJP on inflammation-linked CRC and the NF-kB pathway. Besides FDJP, other anthocyanin-rich fruit products have also been able to partially block the axes cytokine/NF-kB or NF-kB/COX-2 on CRC. These include the anthocyanin fraction of cocoplum (*Chrysobalanus icaco*) (Venancio et al., 2017), a fruit native to tropical America, and the freeze-dried powder of black raspberry (Pan et al., 2018).

4. Conclusions

In conclusion, the jaboticaba fruit peel, a rich source of anthocyanins (C3G, D3G) and phenolic acids (mostly gallic and ellagic), represents a promising product intending the blockage of adenocarcinoma formation, a result which has only been seen before in studies with turmeric's curcumin. FDJP appears to be a safe product capable of acting on inflammation-driven CRC by reducing the activation of proinflammatory-related molecules, mostly IL-1β and COX-2, thus probably acting against NF-kB induction. Further studies with other animal models of CRC and especially with humans are mandatory to confirm the potential of jaboticaba peel against CRC.

Author contributions

RPN: writing - original draft, data curation, formal analysis, investigation, methodology; JSR: investigation, methodology; GP: investigation, methodology; AMTMM: writing - review & editing, investigation; MFS: writing - review & editing, investigation; APFM: writing - review & editing, investigation; GCFJ: writing - review & editing, investigation; RGB: writing - review & editing, investigation; MCPAS: writing - review & editing, investigation; APRP: writing - review & editing, investigation; JAP: writing - review & editing, investigation; CARM: writing - review & editing, investigation; MRMJ: writing - review & editing, funding acquisition, project administration, resources, supervision. All authors read and approved the final version.

Declarations of interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102578.

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