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Original article

Evaluation of the anti-inflammatory properties of *Ascophyllum nodosum*-derived fucoidan in dogs with canine chronic inflammatory enteropathy – results from an *ex vivo* study

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Abstract

Like terrestrial plants, algae contain a vast number of biofunctional compounds, some of which may affect inflammatory and immune responses in animals. As such, algal bioactive components might be useful in the treatment of canine chronic inflammatory enteropathy (CIE), where persistent inflammation underlies clinical signs. In this investigation, we studied the effects of *Ascophyllum nodosum*-extracted fucoidans (ANFE) on cytokine gene and protein expression and the morphology of intestinal tissue explants from CIE dogs. Duodenal biopsies from 22 dogs with CIE were incubated for 24 hours with and without ANFE, using three replicate samples per treatment from each animal. Tumor necrosis factor alpha, interferon gamma, and interleukin-15 proteins were measured in culture supernatants by enzyme-linked immunosorbent assay, whereas relative expression of the genes for interleukin-1 beta, interleukin-6, tumour necrosis factor alpha, interferon gamma, interleukin-15, interleukin-10, forkhead box protein P3, and indoleamine-pyrrole 2,3-dioxygenase-1 was assessed through real-time qPCR. Inflammatory cell infiltrate and mucosal integrity were evaluated by light and transmission electron microscopy. Cytokine protein concentrations were all below the detection limit, and no differences were found in terms of morphological features between the groups with and without ANFE. With respect to transcriptomic data, however, mRNA levels of the pro-inflammatory genes *TNFA* and *IL15* were significantly higher ($p<0.05$) in the group without ANFE. In conclusion, while failing to improve morphological outcomes, ANFE supplementation may be associated with an overall positive effect on intestinal phlogosis.

Keywords: chronic inflammatory enteropathy, cytokine, dog, organ culture, phycochemical, sulphated polysaccharides

Introduction

Chronic inflammatory enteropathy (CIE) is a collective term applied to a group of idiopathic diseases of dogs. These diseases are characterised by persistent or recurrent gastrointestinal (GI) signs and histopathological evidence of intestinal mucosal inflammation of primary origin (Dandrieux 2016, Heilmann et al. 2017). Four subclasses of CIE can be identified according to the clinical response to treatment, viz food-responsive (FRE), antibiotic-responsive (ARE), immunosuppressive-responsive (IRE), and nonresponsive (NRE) enteropathy (Dandrieux 2016).

Like human idiopathic inflammatory bowel disease (IBD), with which it might share the same aetiology, CIE pathogenesis likely involves the breakdown of immune tolerance to innocuous luminal antigens (e.g., dietary, microbial) due to dysregulated immunity, altered intestinal barrier function, imbalanced gut microbiota composition, or any interplay of these (Allenspach 2011, Yogeshpriya et al. 2017). At the intestinal mucosal level, immunological hyper-reactivity manifests as a chronic inflammatory state, characterised by a mixed T-helper Th-1/Th-2 cytokine response as well as increased cellular infiltration of the lamina propria with leukocytes, mainly plasma cells and lymphocytes, but also eosinophils and neutrophils (Simpson and Jergens 2011, Heilmann and Suchodolski 2015).

Extensive research suggests that the phlogistic process can promote self-perpetuation of CIE by directly influencing the alleged pathogenic factors. For instance, evidence from *in vitro* studies showed that inflammation-driven production of selected cytokines, such as tumour necrosis factor (TNF)-α and interferon (IFN)-γ, together with reactive oxygen species (ROS), disrupts the structure and induces remodelling of apical multiprotein adhesive complexes (i.e., tight junctions), resulting in increased antigenic exposure to the mucosal immune system (Rao 2008, Michielan and D'Incà 2015). Furthermore, micro-environmental changes associated with the inflammatory response are thought to cause intestinal dysbiosis by promoting the bloom of facultative anaerobic bacteria, such as *Escherichia coli*, while reducing the abundance of beneficial, strictly anaerobic populations (Zeng et al. 2017).

Accordingly, the achievement of inflammation control constitutes a primary goal for CIE management. In clinical forms of CIE requiring medical treatment, the established therapy contemplates the use of several immunosuppressive drugs (e.g., corticosteroids, cyclosporine A, azathioprine, and chlorambucil), which exert beneficial effects via the dampening of immune-mediated phlogistic reactions. In spite of their high therapeutic effectiveness (Makielski et al. 2019), these pharmacological agents may cause major adverse side effects in treated dogs (Viviano 2013). The clinical need to improve treatment tolerability, and so the animal's quality of life, has catalysed the quest for novel anti-inflammatory candidates, especially among nutraceuticals.

Sulphated fucans (also known as fucoidans) are naturally occurring polysaccharides that are widely distributed in the cell-wall matrix and intracellular spaces of brown algae (Wijesinghe and Jeon 2012, Zaporozhets et al. 2014). These biopolymers are generally characterised by a sophisticated chemical structure based on substantial percentages of sulphate ester groups and L-fucose, with minor amounts of other monosaccharides (e.g., mannose, xylose, and rhamnose), uronic acid, and proteins (Wang et al. 2019). Structural complexity is likely accountable for the broad array of bioactivities displayed by fucoidans, such as anti-viral (e.g., anti-canine distemper virus; Trejo-Avila et al. 2014), anti-protozoal (e.g., anti-leishmaniasis; Kar et al. 2011), anti-cancer (Atashrazm et al. 2015), prebiotic (O'Sullivan et al. 2010), and anti-coagulant (Ustyuzhanina et al. 2013) capacities. Moreover, fucoidans possess strong anti-inflammatory properties, as elegantly reviewed by Phull and Kim (2017). In brief, proposed anti-inflammatory mechanisms encompass: free radical formation inhibition as well as scavenging (Do et al. 2010, Waraho et al. 2011, Phull et al. 2017); modulation of mitogen-activated protein kinase-family gene expression (Che et al. 2017) with ensuing downregulation of the pro-inflammatory transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Ryu and Chung 2015); and blockade of the endothelial adhesion molecules (i.e., selectins) involved in leukocyte extravasation (Bevilacqua and Nelson 1993). With special reference to the intestinal epithelium, fucoidans may also reduce phlogistic stimuli by directly inducing the expression of some tight-junction proteins (Iraha et al. 2013).

The ability of fucoidans to alleviate inflammatory processes has been tested both *in vitro* and *in vivo*. The albumin denaturation assay and the human red blood cell (HRBC) membrane stabilisation method are two benchtop techniques commonly used to predict the anti-inflammatory significance of drugs or plant extracts. In a study by Obluchinskaya et al. (2022), fucoidans extracted from five species of brown seaweed, including *Ascophyllum nodosum*, inhibited protein denaturation and enhanced HRBC membrane stabilisation in a concentration-dependent fashion. An experiment investigating the biological properties of *Colpomenia sinuosa* – and *Sargassum prismaticum*– isolated fucoidans yielded similar results (Atya et al.

2021). *In vivo*, the administration of fucoidans to rats, either as an intraperitoneal injection (Hadj Ammar et al. 2015) or as a topical ointment (Obluchinskaya et al. 2021), dose-dependently lessened carrageenan-induced paw oedema, which is a well-established model of inflammation. Furthermore, Hu et al. (2014) reported that intrathecal infusion of fucoidan was capable of attenuating mechanical allodynia and thermal hyperalgesia induced by spinal nerve ligation in rodents.

In view of the aforementioned functional attributes, fucoidans may be a valuable aid in the management of chronic GI disorders. Preliminary research in murine models of IBD seem to corroborate this hypothesis. In a controlled study by Matsumoto et al. (2004), diet supplemented with *Cladosiphon okamuranus*-derived fucoidan reduced the disease activity index and suppressed in colonic epithelial cells the mRNA expression of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and toll-like receptor (TLR)-4 in dextran sulphate sodium (DSS)-induced colitic mice. Similarly, Wang et al. (2020) reported prevention of colonic shortening, alleviation of colonic tissue damage, and promotion of an anti-inflammatory cytokine profile upon administration of fucoidan from *A. nodosum* to C57BL/6J mice with chronic antibiotic-induced colitis. On top of that, fucoidan treatment was also capable of partially reversing the alteration of gut microbiota. In dogs affected by CIE, the clinical and microbiological effects of a 30-day dietary supplementation with 4% powdered *A. nodosum* (as-is basis) were evaluated by Isidori et al. (2021). While no improvement was observed in canine IBD activity index (CIBDAI) scores or microbial richness following *A. nodosum* supplementation, an increase in faecal acetic acid concentration was noted, indicating possible benefits for hindgut fermentation. However, since the *A. nodosum* powder contained various bioactive compounds beyond fucoidans, the specific contribution of fucoidans to these effects remains unclear.

To address this knowledge gap regarding the biofunctional effects of fucoidans, the present *ex vivo* investigation was carried out to explore the anti-inflammatory and immunoregulatory actions of an *A. nodosum* fucoidan extract, hereafter referred to as ANFE, on duodenal mucosal explants from dogs with CIE and to assess these actions at morphological, proteomic, and transcriptomic levels.

Materials and Methods

This study was reviewed and approved by the Bioethics Committee of Perugia University (protocol code 111983; date of approval: 14/12/2020).

Animals

A total of 22 dogs with a final diagnosis of CIE were selected for this experiment. Patients were admitted for referral to the University Veterinary Teaching Hospital (OVUD) in Perugia, Italy, due to vomiting and/or diarrhoea endured for more than three weeks. To rule out other causes of chronic GI signs, all animals were subjected to an accurate anamnestic investigation, thorough physical examination, copro-parasitological tests (centrifugal faecal flotation with zinc sulphate solution for helminth eggs, immunofluorescence assay for *Giardia duodenalis* cysts and *Cryptosporidium* spp. oocysts), complete blood count, extended serum biochemistry profile, and abdominal ultrasonography and gastro-duodenoscopy with histological assessment of intestinal endoscopic resection specimens. Additional diagnostic procedures (e.g., urinalysis, serum folate and cobalamin titration, trypsin-like immunoreactivity assay, basal cortisol, and/or adrenocorticotropic hormone stimulation test) were performed on a case-sensitive basis at the treating clinician's discretion.

Data regarding signalment, body weight, CIBDAI (Jergens et al. 2003), and histopathological diagnosis of each study participant are detailed in Table 1. The mean age of dogs was 69.8±51.1 months (12-156 months), mean body weight was 21.5±11.5 kg (1.9-38.0 kg), and mean CIBDAI was 6.1±2.4 (2-11).

Endoscopic procedure and histological examination

Any previous anti-inflammatory/immunosuppressive treatments were discontinued 2-4 weeks before the endoscopic exam. Each dog underwent pre-procedural 12-hour food-fasting and 6-hour water-fasting.

The upper GI endoscopy was performed on all animals under general anaesthesia. Prior to induction, premedication was administered with medetomidine (5-10 µg/kg intramuscularly). Anaesthesia was induced with intravenous propofol (3-6 mg/kg) and maintained with isoflurane delivered in 100% oxygen. The endoscopic examination was conducted using a 6 mm diameter × 1050 mm video endoscope (Pentax EG-1840) connected to a Pentax EPM-3300 video processor (Pentax Corp., Tokyo, Japan). The same, highly trained endoscopist (FR) performed all procedures in keeping with the technique described by Spillmann (2013).

The proximal duodenal lumen was gently irrigated with warm physiological saline prior to intestinal mucosa sampling by means of a flexible, through-the-endoscope, pinch biopsy forceps, with 1.8 mm alligator cups (Precisor Pediatric Coated Disposable Biopsy Forceps; Conmed Corporation, Utica, NY). At least four duodenal endoscopic specimens were used for

Table 1. Summary characteristics of the enrolled dogs.

Patient ID	Breed	Age (months)	Sex	Neutering Status	Weight (kilograms)	Histopathological diagnosis	CIBDAI	Used for
1	Zwergpinscher	108	Female	Intact	6.5	Moderate-to-severe lymphocytic-plasmocytic duodenitis	4	GPE
2	German Shepherd Dog	36	Female	Intact	19.0	Moderate lymphocytic-plasmocytic duodenitis	9	GPE
3	Chihuahua	24	Male	Intact	1.9	Moderate lymphocytic-plasmocytic and eosinophilic duodenitis	5	GPE
4	Jack Russell Terrier	36	Female	Intact	7.1	Severe lymphocytic-plasmocytic duodenitis	5	GPE
5	German Shepherd Dog	24	Female	Intact	25.0	Moderate-to-severe lymphocytic-plasmocytic duodenitis	8	GPE
6	Jack Russell Terrier	156	Female	Neutered	5.4	Severe lymphocytic-plasmocytic and eosinophilic duodenitis	4	GPE
7	Boxer	132	Male	Intact	35.5	Mild lymphocytic duodenitis	4	GPE
8	Mixed-breed	36	Male	Intact	10.0	Moderate lymphocytic-plasmocytic and eosinophilic duodenitis	4	GPE
9	Golden Retriever	144	Female	Neutered	31.3	Severe lymphocytic-plasmocytic duodenitis	5	GPE
10	Boston Terrier	12	Male	Intact	6.5	Moderate lymphocytic-plasmocytic duodenitis	8	GPE
11	Mixed-breed	72	Male	Neutered	25.9	Severe lymphocytic-plasmocytic duodenitis	4	GPE
12	Mixed-breed	84	Male	Neutered	34.0	Moderate lymphocytic-plasmocytic duodenitis	5	GPE
13	Italian Corso Dog	60	Male	Neutered	38.0	Moderate lymphocytic-plasmocytic duodenitis	7	GPE
14	German Shepherd Dog	24	Male	Intact	24.0	Moderate-to-severe lymphocytic-plasmocytic duodenitis	8	GPE
15	Kurzhaar	24	Male	Intact	19.8	Moderate lymphocytic duodenitis	9	GPE
16	Mixed-breed	144	Female	Neutered	5.6	Moderate lymphocytic-plasmocytic duodenitis	8	GPE
17	Yorkshire Terrier	60	Male	Intact	5.0	Moderate lymphocytic-plasmocytic duodenitis	11	GPE
18	German Shepherd Dog	36	Female	Intact	23.0	Moderate lymphocytic-plasmocytic duodenitis	7	GPE
19	Jack Russell Terrier	36	Male	Intact	7.7	Moderate lymphocytic-plasmocytic duodenitis	2	MT
20	Golden Retriever	156	Male	Intact	31.0	Severe lymphocytic-plasmocytic and neutrophilic duodenitis	4	MT
21	French Bouledogue	12	Male	Intact	12.2	Moderate lymphocytic-plasmocytic and eosinophilic duodenitis	9	MT
22	Jack Russell Terrier	120	Female	Intact	6.0	Severe lymphocytic-plasmocytic duodenitis	4	MT

CIBDAI=Canine IBD activity index; GPE=gene and protein expression; MT= morphological traits.

Table 2. Main physico-chemical attributes of the fucoidan used in the study.

Item*	
Molecular weight (kDa)	1,300
Protein content (% Eq BSA)	4.4
Sulphate content (% Eq SO_3)	12.5
Sulphate residue position	C-2, C-4
Monosaccharide composition (%)**	
Fucose	37.8
Rhamnose	0.3
Galactose	8.4
Glucose	1.4
Mannose	2.9
Xylose	28.0
Galacturonic acid	0.4
Glucuronic acid	8.7

* Data provided by Elicityl SA

** Data obtained via High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection.

histopathological confirmation and grading of CIE according to the World Small Animal Veterinary Association guidelines for the evaluation of GI inflammation in companion animals (Washabau et al. 2010). The grading was performed by a single board-certified pathologist (EL) who was blinded regarding the history, clinical signs, and endoscopic observations of the dogs.

Six additional intestinal biopsies were collected from the same anatomical region and immediately transferred to the laboratory in a transport solution phosphate buffered saline (PBS) supplemented with 200 IU/mL of penicillin and 200 IU/mL of streptomycin (Euroclone, Milan, Italy) for further processing.

Ex vivo culture of duodenal biopsies

Organ culture of mucosal specimens was performed with a modified protocol derived from Vitale et al. (2017). Briefly, six duodenal pinch biopsies from each dog were rinsed three times with fresh washing solution consisting of PBS supplemented with 200 IU/mL of penicillin and 200 µg/mL of streptomycin (Euroclone, Milan, Italy). The tissues were then placed in 24-flat-bottom multiwell plates onto the upper compartment of 8 µm pore diameter cell culture inserts (ThinCert; Greiner Bio-One GmbH, Kremsmünster, Austria), with the luminal side facing upwards. Three of the six samples were used as controls and incubated in a maintenance medium (MM) composed of 80% RPMI 1640 (EuroClone, Milan, Italy), 15% foetal bovine serum (EuroClone, Milan, Italy), 2 mM of L-glutamine, 100 IU/mL of penicillin, 100 µg/mL of streptomycin, and 10 µg/mL of recombinant human insulin (SERVA Electrophoresis GmbH, Heidelberg, Germany). The other three samples were cultured in MM supplemented with purified ANFE (FUC400; Elicityl Oligotech, Crolles, France) with the physico-chemical characteristics shown in Table 2. An ANFE inclusion level of 75µg/mL was chosen based on previous research (Jiang et al. 2010, Kim et al. 2012, Ryu and Chung 2015, Shu et al. 2015). In the control samples, the ANFE was replaced by an equivalent volume of PBS.

All samples were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. At the end of the incubation period, tissue specimens from 18 subjects (see Table 1) were pooled, homogenised in TRIzol (Invitrogen, Monza, Italy) per the manufacturer's instructions, and stored at -80°C for gene transcription analysis. In like manner, tissue culture supernatants from each animal were harvested, pooled in a single aliquot, centrifuged at 1000 *g* for 15 minutes at 4°C to remove particulate material, and frozen at -20°C until an enzyme-linked immunosorbent assay (ELISA) was performed for cytokine quantification. The biopsy samples of the remaining animals (n=4) were used for light microscopy (LM) and for transmission electron microscopy (TEM) and processed accordingly (see below).

Quantification of cytokine mRNA transcription in the *ex vivo* duodenal biopsies

RNA extraction was performed from Trizol-homogenised tissue samples with the PureLink™ RNA Mini Kit (ThermoFisher Scientific, Waltham, Massachusetts,

Table 3. Primer combinations and accession numbers of the tested genes.

Gene	Primer Forward	Primer Reverse	Product Size	Accession Number
GUSB	GTGCTGGATCAGAAACGCAA	CTTGGTTGTCTCTGGCGA	134	NM_001003191.1
SDHA	GTTCGTTCAGTTCCACCCCA	CTCTGGATGCCAGGTCCTTT	149	XM_535807.6
IL1B	CTGATGGCCCTGGAAATGTG	TGGGAGACTTGCAACTGGAT	88	NM_001037971
IL6	GTCTACCACTCACCTCTGCA	GCCTCTTTGCTGTCTTCACA	290	NM_001003301
TNFA	CCACCACACTCTTCTGCCT	CTTGTCACTTGGAGTTCGAG	140	NM_001003244
IFNG	CGTGATTTTGTGTTCTTCTGGC	CCGATACATCTGGATTACTTGCA	101	NM_001003174
IL15	GGCAGGACGTGATACTTGATTT	GCGAGATAACACCTAACTCCAG	150	NM_001197188
IL10	GAGAACCACGACCCAGACAT	TCTCACAGGGCAGAAATCGG	160	NM_001003077
FOXP3	GTCTTCGAGGAGCCAGAGGA	GCACCCAGCTTCTCCTTCTC	146	NM_001168461.1
IDO1	GTCTGCACCGAGCCCATAAA	TGGAGTTGCCTTTCCAACCA	107	XM_532793.6

GUSB – Canine beta-glucuronidase, *SDHA* – Succinate dehydrogenase complex subunit A, *IL1B* – Interleukin-1 beta, *IL6* – Interleukin-6, *TNFA* – Tumour necrosis factor alpha, *IFNG* – Interferon gamma, *IL15* – Interleukin-15, *IL10* – Interleukin-10, *FOXP3* – Forkhead box protein P3, *IDO1* – Indoleamine-pyrrole 2,3-dioxygenase-1.

USA) following the manufacturer's protocol and adding a DNAse digestion step to remove residual genomic DNA. Subsequently, extracted RNA was evaluated for purity and quantity by a NanoDrop2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and 1.2% denaturing agarose gel electrophoresis. An amount of 400 ng of RNA from each sample was reverse-transcribed with SuperScript™ IV VILO™ Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. Canine beta-glucuronidase (*GUSB*) and succinate dehydrogenase complex subunit A (*SDHA*) were adopted as reference genes for real-time qPCR reactions based on available literature (Peters et al. 2007, Osada et al. 2017). Primers for the genes interleukin-1 beta (*IL1B*), interleukin-6 (*IL6*), tumour necrosis factor alpha (*TNFA*), interferon gamma (*IFNG*), interleukin-15 (*IL15*), interleukin-10 (*IL10*), forkhead box protein P3 (*FOXP3*), indoleamine-pyrrole 2,3-dioxygenase-1 (*IDO1*), and *SDHA* were designed based on available gene sequences by using Primer-BLAST suite (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), whereas the primer for *GUSB* was adopted from Porcellato et al. (2020). Whenever possible, primer pairs were designed to include exon-exon boundaries to avoid amplification of residual genomic DNA. Information regarding primer as well as gene sequence accession numbers is in Table 3.

The real-time qPCR reaction was carried out with 5 µL of a 10-fold diluted cDNA and SsoFast™ EvaGreen® Supermix, (BioRad, Hercules, California, USA). Amplification was executed in a CFX96 Touch™ instrument (BioRad, Hercules, California, USA) with the following protocol: 3 minutes at 95°C, followed by 45 cycles of 10 seconds each at 95°C and 15 seconds each at 60°C. Each reaction was run in triplicate with the insertion of an appropriate negative control, with the fluorescence being detected at the end of each cycle. The melting curve was estimated between 60 and 95°C with incremental steps of 0.5°C. To calculate relative gene expression, the CFX maestro software (ver. 4.1-BioRad, Hercules, CA, USA) was applied using the ΔΔCq method.

Cytokine protein quantification in organ culture supernatants

Cytokine concentrations were measured in tissue culture supernatants by using commercially available canine-specific ELISA kits. Assay systems were used for TNF-α (ECTNF; Thermo Fisher Scientific), IFN-γ (CAIF00; R&D Systems), and IL-15 (MBS741916_ Sandwich; MyBiosource). Cytokine standards and undiluted culture supernatants were tested in each 96-well ELISA plate, following the manufacturer's protocol. All cytokine assays were carried out using duplicate samples, results of which were averaged. The mean minimal detectable dose was 2 pg/mL for canine TNF-α, 25 pg/mL for IFN-γ, and 1 pg/mL for IL-15, as stated by the products' datasheet. The absorbance of each sample was read at 450 nm on a microplate reader (Infinite 200 PRO, Tecan, Switzerland) for all cytokines.

Histopathological and ultrastructural examination of the *ex vivo* duodenal biopsies

For LM examination, biopsy samples were fixed in 10% buffered formalin, dehydrated in a graded series of alcohol, and embedded in paraffin. Sections of 4 µm thickness were stained with haematoxylin and eosin.

For TEM observations, tissue samples were fixed with 2.5% glutaraldehyde in 0.1 M PBS at pH 7.3 for

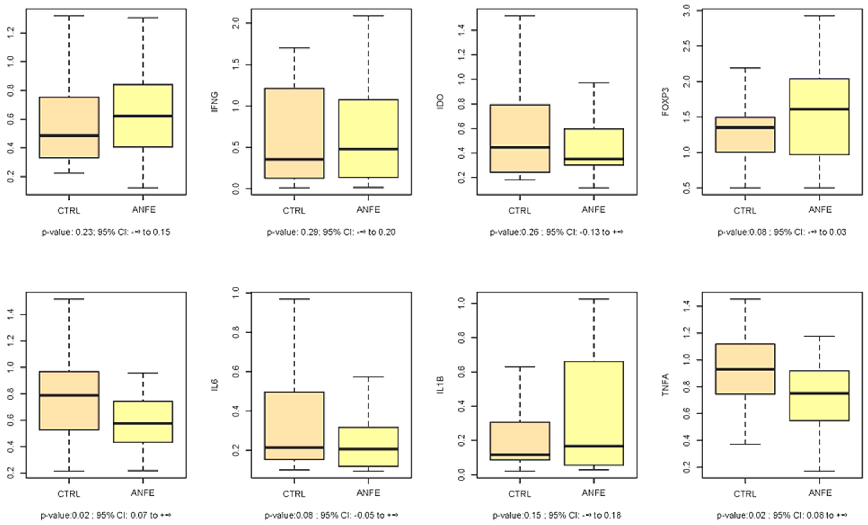

Fig. 1. Boxplot of relative cytokine gene expression between Control (CTR; orange-coloured) and Treated (ANFE; yellow-coloured) dog groups. Data are expressed as $2^{-\Delta\Delta Cq} \pm 1$ standard error.
IL1B: Interleukin-1 beta; *IL6*: Interleukin-6; *TNFA*: Tumour necrosis factor alpha; *IFNG*: Interferon gamma; *IL15*: Interleukin-15; *IL10*: Interleukin-10; *FOXP3*: Forkhead box protein P3; *IDO1*: Indoleamine-pyrrole 2,3-dioxygenase-1.

2 hours at room temperature. The samples were subsequently washed twice in PBS, postfixed in 1% osmium tetroxide, dehydrated in graded ethanol up to absolute, pre-infiltrated in propylene oxide, and embedded in Epoxy resin. Sections of 90 nm thickness were mounted on 200-mesh copper grids, stained with uranyl acetate, and examined under a Philips EM 208 equipped with a digital camera (CUMEF – Perugia).

Statistical analysis

The sample size for each group was estimated based on the following assumptions: a 1:1 ratio between the control and ANFE-treated groups; an expected effect size (Cohen's d) of 0.5 (i.e., a medium effect size); a significance level (Type I error, α) of 5%; and a statistical power of 80% (Type II error, β = 20%). Based on these assumptions, the required number of samples per group for each comparison was at least 27.

The gene expression data were analysed using R Core Team (2021). Before analysis, the distribution of each gene's expression levels was assessed for normality using the Shapiro-Wilk test. Upon confirming that the data were not normally distributed, the values were log-transformed (log base 2) to normalise the distributions. Subsequently, a one-tailed Student's t-test was employed to evaluate differences between the two groups. P-values less than 0.05 were considered statistically significant, while tendencies were noted for $0.05 \leq p < 0.1$

To facilitate interpretation of the results, the data were retrotransformed by raising 2 to the power of the transformed values to revert to the original scale. Confidence intervals were then calculated for the retrotransformed data to provide estimates on the original scale.

Results

Relative cytokine gene expression

The relative expression distribution of *TNFA*, *IFNG, IL1B*, *IL6*, *IL10*, *IL15*, *FOXP3*, and *IDO1* transcripts in the control and ANFE-treated duodenal biopsies are shown in (Fig. 1).

The reference genes exhibited relatively high stability with M values below the accepted limit of 1.5 (Vandesompele et al. 2002). Differences in gene expression were reached only for *TNFA* (p=0.015) and *IL15* (p=0.016), which showed a lower transcription level in ANFE-treated samples. Moreover, the ANFE-treated group was characterised by a tendency for lower *IL6* (p=0.08) and higher *FOXP3* (p=0.08) mRNA expression.

Cytokine ELISA assays

TNF-α, IFN-γ, and IL-15 quantification in tissue culture supernatants was not possible as ELISA results were below the lower limit of detection in the control and ANFE-treated groups.

Histopathological and ultrastructural examination

In the LM observations, all the samples showed various degrees of alteration that have been interpreted as predictable changes in tissue samples outside the body. Also, the ultrastructural examination of control and ANFE-treated samples revealed degenerative changes non-homogeneously distributed throughout the samples. The preserved areas displayed normal morphology of structural elements and inflammatory cells. In particular, the epithelial barrier was characterised by

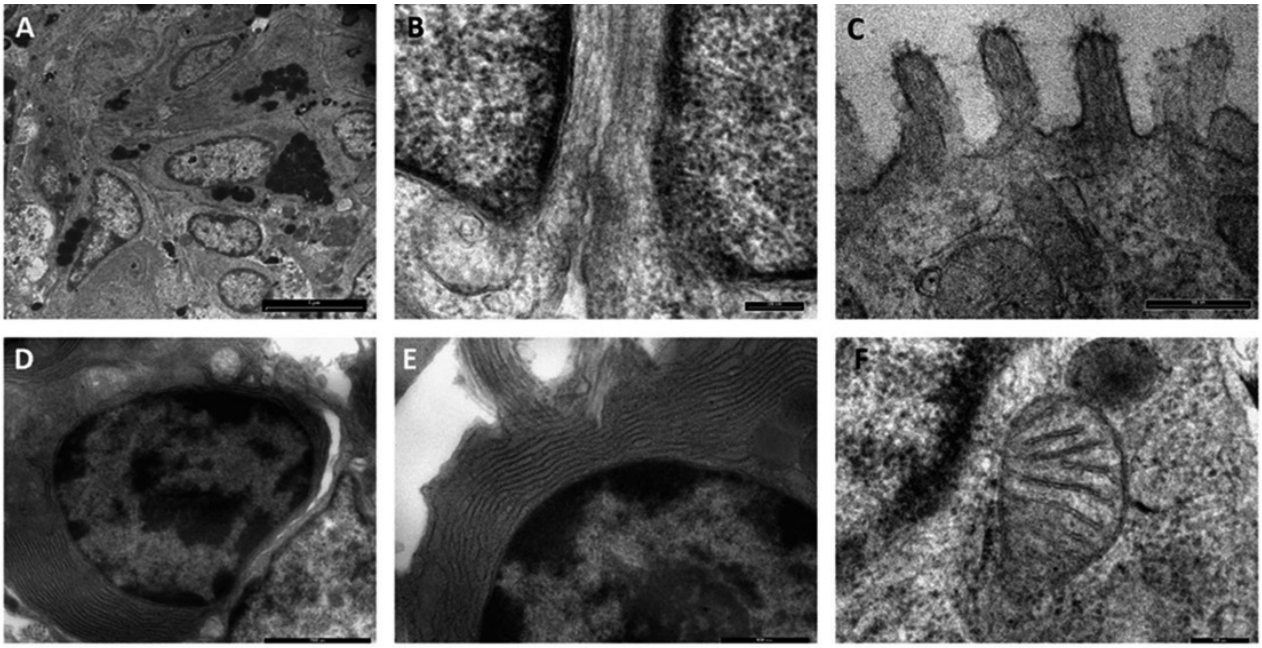

Fig. 2. Ultrastructural key features of morphologically-preserved areas in treated and untreated dog biopsy samples after 24 h *ex vivo* culture. Electron micrographs depicting the epithelial barrier (**A.**, scale bar=200 µm) without changes at the level of intercellular spaces and junctions (**B.**, scale bar=200 nm). A particular of apical specialisations (microvilli) showing typical distribution and ultrastructure (**C.**, scale bar=500 nm). Representative image showing the unchanged ultrastructure of cell organelles in both structural elements and inflammatory cells (**D.**, plasma cell, scale bar=2 µm; **E.**, a particular of plasma cell RER, scale bar=1 µm). Mitochondria with typical shape and morphology of cristae (**F.**, scale bar=200 nm).

well-preserved microvilli, and no changes were detected at the level of intercellular spaces and junctions (Fig. 2). The specimens' lamina propria appeared quite scarce. Consequently, the inflammatory cells present in the samples were too few to be evaluated and compared to pre-treatment biopsies.

Discussion

To the best of our knowledge, the present study is the first to explore the use of ANFE in a canine *ex vivo* model of CIE. Based on experimental evidence gathered in other animal species, it was hypothesised that ANFE might exert anti-inflammatory and immunomodulatory effects towards inflammatory disorders, including those of the digestive system. To this aim, a set of experiments was carried out to detect possible effects on morphology and in cytokine expression at the transcriptomic and at the protein synthesis level. To accomplish the first aim, LM and TEM analyses were performed to investigate changes in cellular infiltrate as well as significant differences in structural and ultrastructural organisation of control and ANFE-treated samples.

Concerning the expression of immunoinflammatory mediators, *TNFA*, *IFNG, IL1B*, *IL6*, *IL10*, *IL15*, *FOXP3*, and *IDO1* genes were investigated to evaluate ANFE activity on the quantity and quality of the inflammatory infiltrate. Proteins coded by *IL1B*, *IL15*, *TNFA*, and *IFNG* were particularly examined as key cytokines of the innate immunity and/or acquired Th-1 responses (Abbas et al. 2018). Conversely, the expression of *IL6* is predominantly associated with Th-2 cells, whereas the cytokine IL-10, which is endowed with immunomodulatory properties, is produced by Th-1 as well as Th-2 lymphocytes (Day 2012). *FOXP3* is a transcription factor that operates on binding certain cytokines, such as IL-10 and TGF-β, to cognate receptors on T lymphocytes. Activation of *FOXP3* stimulates the expression of a set of genes that induce the skewing of T cells toward a more immunosuppressive phenotype, namely regulatory T cells (T-regs) (Hori et al. 2003). T-regs are also capable of activating IDO1, a cytoplasmic enzyme that is involved in the catabolism of tryptophan to metabolites that suppress the function of antigen presenting cells, and thus of T lymphocytes (Fallarino et al. 2003). Moreover, IDO1 can furtherly activate T-regs (Moffett and Namboodiri 2003). FOXP3 and IDO1 have been evaluated in dogs with melanocytic tumours (Porcellato et al. 2019), as well as in canine protein-losing enteropathies (Kathrani et al. 2019).

Several studies on the gene expression of different cytokines in CIE were reviewed by Kołodziejska-Sawerska et al. (2013). These studies differ in regard to the study population, the subset of evaluated cytokine genes, and the results. The activation of *TNFA* is reported to be either increased, equally represented,

or decreased comparing healthy animals and animals with CIE. German et al. (2000) compared healthy dogs with dogs affected either by IRE or ARE and found augmented *TNFA* transcription in the latter group; notably, antibiotic therapy reduced the expression of *TNFA*, suggesting that modulation of this cytokine gene could be central to disease control. In our study, ANFE-treated duodenal explants were characterised by a lower mRNA transcription level of *TNFA* than control explants (p=0.015). This finding is in keeping with the report by Wang et al. (2022), where two ANFE with different molecular weight down-regulated the transcription of several pro-inflammatory genes, including *TNFA* and *IL6*, in bacterial lipopolysaccharide-stimulated macrophages, likely via hindrance of the TLR/NF-κB signal transduction. Accordingly, the tendency for a diminished mRNA expression of *IL6* in the ANFE-exposed biopsies is also well-aligned with the evidence provided by Wang et al. (2022) and with the long-standing knowledge that TNF-α is among the stimulators of IL-6 synthesis (Abbas et al. 2018).

IL-15 is a widespread, pleiotropic cytokine with a pivotal role in the regulation of innate and acquired immunity; its function, among others, is to protect T lymphocytes from apoptosis, thus improving T-cell function. In humans, IL-15 is highly expressed in IBD and regulates T-cell production of inflammatory cytokines; treatments against IL-15 can have therapeutic potential for human IBD (Liu et al. 2000). Moreover, probiotic therapy with *Lactobacillus jensenii* TL3927 in mice with DSS-induced colitis was able to alleviate the disease by reducing the release of various inflammatory mediators, including IL-15 (Sato et al. 2020). In CIE, the expression of IL-15 has been hardly researched (Furukawa et al. 2022); in this respect, the downregulation of *IL15* in ANFE-treated duodenal explants represents an interesting result and thus deserves further investigation.

Importantly, the differences in gene activation patterns described so far raises the question of why the mRNA levels of other major inflammatory response markers, such as *IFNG* and *IL1B*, were not affected. A possible explanation for this apparent discordance may be ascribed to the fact that intestinal mucosal biopsies contain multiple cell types, each of which has a peculiar biological response when exposed to an algal fucoidan (Park et al. 2017). As a result, the retrieved mRNA expression profile of the tested genes may not be as consistent as it usually is when cellular monolayers are employed.

The number of *FOXP3*-expressing T-regs in the intestine of dogs affected by IRE was assessed in two studies (Junginger et al. 2012, Maeda et al. 2016) that used immunofluorescence and/or immunohistochemistry; in both cases, a decrease of T-regs in diseased animals relative to healthy ones was found. In our study, the mRNA transcription properties of *FOXP3* tended to be higher (p=0.08) in ANFE-treated biopsies than in untreated samples. This observation is not unprecedented in the context of algal saccharide research. In fact, the monosaccharide L-fucose, which is the main component of fucoidans, has been shown to increase *FOXP3* gene mRNA in *Muc2*$^{-/-}$ mice with colitis, possibly via direct effects such as inhibition of macrophage and dendritic cell activation (Feofanova et al. 2022). Moreover, given their prebiotic attributes, fucoidans in an *in vivo* setting may also expand the intestinal T-reg pool by changing the composition of the gut microbiota as well enhancing the production of short-chain fatty acids (Wang et al. 2020).

It was not possible to quantify the TNF-α, IFN-γ, and IL-15 in the tissue culture supernatants as their concentrations were too low for detection by the ELISA. A similar issue was reported by Schmitz et al. (2014), where the incubation of duodenal biopsies from healthy and CIE dogs with TLR ligands and a probiotic bacterial strain did not enable cytokine quantification in supernatants in the face of significant transcriptional changes. Schmitz et al. (2014) speculated that the inconclusiveness of the ELISA was likely caused by the large amount of medium used for culturing the explants. Since an identical volume of culture medium (1 mL) was used in our study, it cannot be excluded that the lack of cytokine detection was consequential to an excessive protein dilution. In this regard, increasing the number of tissue samples per well, as done by Sauter et al. (2005), is conceivably useful, yet this was not possible in our study owing to the limited amount of biological material. It could also be hypothesised that the paucity of lamina propria affected the number of inflammatory cells present in the samples and, therefore, on the quantity of cytokines.

No differences were observed between the control and ANFE-treated groups regarding the morphological evaluation of the intestinal inflammatory infiltrate and the gut epithelial integrity of the biopsies. This could be due to the non-optimal preservation state of the tissue specimens. Indeed, signs of cellular degeneration (e.g., increased cytoplasm vacuolisation, swelling, shrivelling, pyknosis, and ghost cells) were observed multifocally in most of the sections. This finding is not unexpected and reflects the unavoidable degradation process undergoing in tissue explants whenever the assay microenvironment is not identical to the one of the organs of origin (Randall et al. 2011). In this respect, while acknowledging the existence of bettermore performing culture systems for *ex vivo* studies (Vadstrup et al. 2016), an attempt was made to improve intestinal

biopsy preservation with the Transwell method by minimising the culture duration and carefully selecting the quality of culture medium components. It should be emphasised, though, that while degenerative changes were diffuse when observed at the lower definition and magnification of LM, they appeared interspersed when assayed by TEM. For each biopsy, an alternation of areas of damaged and well-preserved cells was recognised. Additionally, another factor that could have affected the morphological appearance of the intestinal explants is the ANFE dosage employed in our study. Among the biological properties of fucoidans is their capacity to inhibit protein denaturation and to stabilise cell membranes (Obluchinskaya et al. 2022). In the context of ischemia-related cell stress following tissue explant, a higher inclusion level of ANFE could have protected against oxidative protein and lipid damage and enzyme leakage from lysosomes, thus potentially improving the survivability of the intestinal epithelium. On the other hand, although fucoidans generally display low cytotoxicity to non-tumorous cells (Lin et al. 2020), it cannot be ruled out that an increased ANFE dosage would have ultimately produced a worse overall morphological picture.

Several caveats should be noted regarding the present study. First, although it would be logical to expect changes in protein abundance being preceded by variations in relative mRNA expression, multiple regulatory processes may alter protein-mRNA correlations, as elegantly reviewed by Maier et al. (2009) and Liu et al. (2016). Accordingly, the inability to perform ELISA on supernatants and immunohistochemical analysis on tissue samples in our study precludes any definitive conclusion. Second, an open question is whether the level of biopsy deterioration had an influence on the execution of biomolecular and immunological analyses. Provocative evidence underscores that, during apoptosis, protein synthesis is inhibited, and mRNA decay is increased (Mondino and Jenkins 1995, Del Prete et al. 2002) and that the two processes seem to be linked (Bushell et al. 2004). In our study, the integrity of mRNA strands was not assessed using microchip gel electrophoresis, which is regarded to be the gold standard technique for RNA quality control (Schroeder et al. 2006). Nevertheless, it can be hypothesised that the integrity of the tested transcripts was acceptable due to the lack of abnormal banding patterns following the agarose gel electrophoresis and the high real-time qPCR performance (Sambrook and Russell 2001). Third, cytotoxicity assays of ANFE were not conducted in our study; rather, it was decided to gauge ANFE inclusion level by relying on the results of previous viability tests performed on different cell monolayers. It is still possible that ANFE cytotoxicity could have accounted for some aspects of the results. Fourth, because the biological features of fucoidans are dependent on their chemical and structural composition (Zayed et al. 2020), it can be conjectured that the use of a different ANFE might have yielded different results. Lastly, our study may have been underpowered to detect statistically significant differences between groups. The sample size was smaller than typically required to achieve adequate statistical power (e.g., 80%), with the observed power being 73.4%. As a result, there is an increased risk of Type II error (failing to reject a false null hypothesis), meaning that potential differences may exist but were not detected. This limitation arose due to recruitment challenges and the inability to access a sufficiently large participant pool within the study's timeframe.

Conclusions

This study was designed to evaluate the anti-inflammatory and immunoregulatory properties of ANFE in an *ex vivo* model of naturally occurring CIE in dogs. Overall, the results of the transcriptomic analyses suggest that sulphated fucans possess the ability to reduce intestinal inflammation, thus supporting their potential as a novel therapeutic avenue for the management of CIE. This finding reinforces interest in marine-derived polysaccharides as promising candidates for biomedical applications. Given the methodological limitations discussed, further studies under more refined experimental conditions are needed to validate and expand these preliminary findings.

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