Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
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Cite this: *Food Funct.*, 2024, **15**, 2314

Gut microbiota modulation and effects of a diet enriched in apple pomace on inflammation in a DSS-induced colitis mouse model†

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Certain types of soluble dietary fibre, such as pectin and pectic oligosaccharides from different sources, have demonstrated protective effects against inflammation in DSS-induced colitis mouse models. In this work, we have evaluated the impact of a diet enriched in apple pomace (AP-diet), an agricultural by-product with a significant content of pectin and that previously demonstrated prebiotic properties in human fecal batch fermentation models, on the gut microbiota composition, intestinal damage and inflammation markers in a DSS-induced colitis model. We found that the apple pomace enriched diet (AP-diet), providing a significant amount of pectin with demonstrated prebiotic properties, was associated with a slower increase in the disease activity index, translating into better clinical symptomatology of the animals. Histological damage scoring confirmed less severe damage in those animals receiving an AP-diet before and during the DSS administration period. Some serum inflammatory markers, such as TNFα, also demonstrated lower levels in the group receiving the AP-diet, compared to the control diet. AP-diet administration is also associated with the modulation of key taxa in the colonic microbiota of animals, such as some *Lachnospiraceae* genera and *Ruminococcus* species, including commensal short chain fatty acid producers that could play a role in attenuating inflammation at the intestinal level.

Received 6th October 2023,
Accepted 15th January 2024

DOI: 10.1039/d3fo04277d

rsc.li/food-function

1. Introduction

Inflammatory bowel disease (IBD) comprises a spectrum of multifactorial disorders, such as ulcerative colitis (UC) and Crohn's disease (CD), characterized by inflammation and ulceration of the gastrointestinal tract. Their incidence is increasing globally, influenced by a multitude of environmental and lifestyle factors associated with westernized lifestyle, including low fibre diets.[1] Numerous investigations have described gut microbiome disbalances in CD patients, including the overall reduction of gut microbiota diversity[2] and underrepresentation of certain commensals and butyrate producers such as *Faecalibacterium, Roseburia*, or *Akkermansia*.[3–6] Indeed, some of these microbial groups have been demonstrated to confer protection against intestinal inflammation,[5] and there exists interest in developing strategies to promote their representation within the human gut.[7,8]

Diet has been recognized to play a key role in the prevention and management of IBD,[1] and high fibre diets have been associated with reduced risk of developing CD.[9] However, there are no general recommendations regarding dietary fibre in IBD management, as its consumption has been related to inflammation exacerbation in some patients. Recent research has suggested that the different responses of IBD patients to dietary fibre may relate to basal differences in their gut microbiota composition and, consequently, in their ability to metabolize the fibre that reaches the intestinal environment.[10] Furthermore, dietary fibre comprises a vast variety of oligosaccharides and polysaccharides with diverse chemical and structural compositions, influenced by its vegetable source and extraction process, and it ultimately affects its physical–chemical (*e.g.* solubility) and bioactive properties, including its pattern of fermentation by specific gut microbes. Notably, fibre structures comprise most of the recognized and emerging prebiotics investigated to date, due to their capacity to be selectively used by the microbiota, conferring health benefits.[11]

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† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d3fo04277d

Some investigations have pointed to the beneficial effects of particular prebiotic fibres and the metabolites produced during their fermentation by the gut microbiota in IBD patients. Fibres from fruits generally exhibit protective effects against CD, as opposed to those from whole grains and legumes.[10] Administration of pectins, which are abundant fibre structures in some fruits, have demonstrated amelioration of inflammation and gut microbiome biomarkers in experimental models of colitis,[12,13] enhanced the effects of fecal microbial transplantation in UC patients,[14] and demonstrated the capacity to strengthen the gastrointestinal immune barrier through affecting the gut microbiota and intestinal immune cells.[15]

In previous investigations, we reported a physical–chemical characterization of apple pomaces from monovarietal apple ciders, demonstrating that they are a good source of pectin with appealing properties.[16] Furthermore, we demonstrated *in vitro* the modulatory potential of some of these apple pomaces on the gut microbial populations from healthy subjects and IBD patients.[17] Besides, we confirmed through *in silico* analyses that the gut metagenomes from IBD patients retain the capacity to potentially metabolize prebiotic pectins.[18] Hence, in this work, we investigated *in vivo* the effect of a diet supplemented with apple pomace on the gut microbiome architecture, disease index and inflammatory markers in a mouse model of DSS-induced colitis.

2. Materials and methods

2.1. Diet, animals, experimental designs and samples collected

2.1.1. Diet. Apple pomace resulting from the extraction of juice for cider production was supplied by the Regional Agrifood Research and Development Service (SERIDA) (Asturias, Spain). The apple pomace was obtained from the PDO Perico apple cider variety. It was lyophilized, ground milled and stored at 4 °C until use. The main composition characteristics of the apple pomace used have been previously described[16] and are summarized in ESI Table S1.† Animal diets for all the experimental groups were provided in pellet forms by Envigo RMS (Spain). The standard diet was Teklad Global Diet 2014 (control-diet), while the apple pomace enriched diet (AP-diet) was prepared by the same rodent diet provider (Envigo) by adding 10% lyophilized and milled apple pomace to the feed preparation. The standard and AP-diet compositions are provided in ESI Table S2.†

2.1.2. Animals. A total of 40 mice (C57BL/6NRj, male 6–8 weeks) provided by Janvier Labs (Le Genest-Saint-Isle, France) were maintained in the Animal Facilities at the University of Oviedo (authorized facility No. ES330440003591). All animal experimentation was conducted in accordance with the Spanish guidelines for the care and use of laboratory animals, and the protocols were approved by the Ethics Committee of the Principality of Asturias (authorization code PROAE 26/2020, August 2020).

2.1.3. Experimental design and disease evolution monitoring. Following a one-week adaptation at the animal facility, in an environmentally controlled room and provided with standard food and water *ad libitum*, the mice were randomly assigned to 4 groups (n = 10 animals per group, distributed in two cages with 5 animals each). Cohorts 1 and 2 were fed universal feed (2014 Teklad Global 14% Protein Rodent Maintenance Envigo, Barcelona, Spain) while cohorts 3 and 4 were fed with the same basal 2014 Teklad Global diet enriched with 10% lyophilized and milled apple pomace (Fig. 1).

Following two weeks of administration of the corresponding diets to each cohort, cohorts 2 and 4 received 2.5% DSS (colitis grade, MP Biomedicals, LLC, France) in the drinking water until sacrifice (Fig. 1). Intake and volume of drinking water consumed, weight loss, stool frequency and consistency, and the presence of occult blood in stool were monitored daily (Fig. 1). The food and water ingested were estimated during the entire experiment by the amounts consumed by the five mice in each cage. The three Rs principles were considered at all stages of experimentation. A dose of DSS 2.5% in the drinking water was chosen to minimise pain and suffering and alter the welfare of the animals as little as possible. A pilot study was performed by adding this concentration of DSS to the drinking water (n = 5), confirming the establishment of DSS-induced colitis. A disease activity index (DAI) was estimated following the criteria previously established in the literature: [reduced body weight + stool consistency + presence of bleeding in stool]/3 (ESI Table S3†).[19] DAI values ≥1.5 were considered signs of ulcerative colitis. The decision to sacrifice the mice during the experimental period was based on DAI ≥ 3, a weight loss greater than 20%, and/or clinical signs of animal suffering. According to the facility veterinarian, the hunched posture, decreased activity and response to external stimulation, and detachment from the group, which may reflect pain and suffering, lead to the decision to euthanize the mouse or treat it with analgesics (option ruled out).

2.1.4. Samples collected. Three pools of five fecal samples were collected from all cages on days 1, 3, 7, 15 (corresponding to the first two weeks of dietary intervention), 17 and 21 (corresponding to the period of development of DSS-induced colitis) for determination of fecal microbiota composition and short chain fatty acids (SCFAs). At the end of the experiment, the animals were euthanized using CO_2 to obtain a blood sample for the determination of markers of inflammation in serum. After proceeding to euthanasia, the intestine was sectioned from the animals to determine the colon length, as a macroscopic indicator of intestinal inflammation. Likewise, samples of intestinal content in the colon were collected and preserved at −80 °C until processing to determine the microbial composition and SCFA profile.

2.2. 16S rRNA gene sequencing and data analysis

DNA from fecal samples and colonic contents was isolated by using the Power Soil ProKit (Qiagen) and following the modifications previously described.[17] Partial 16S rRNA sequencing

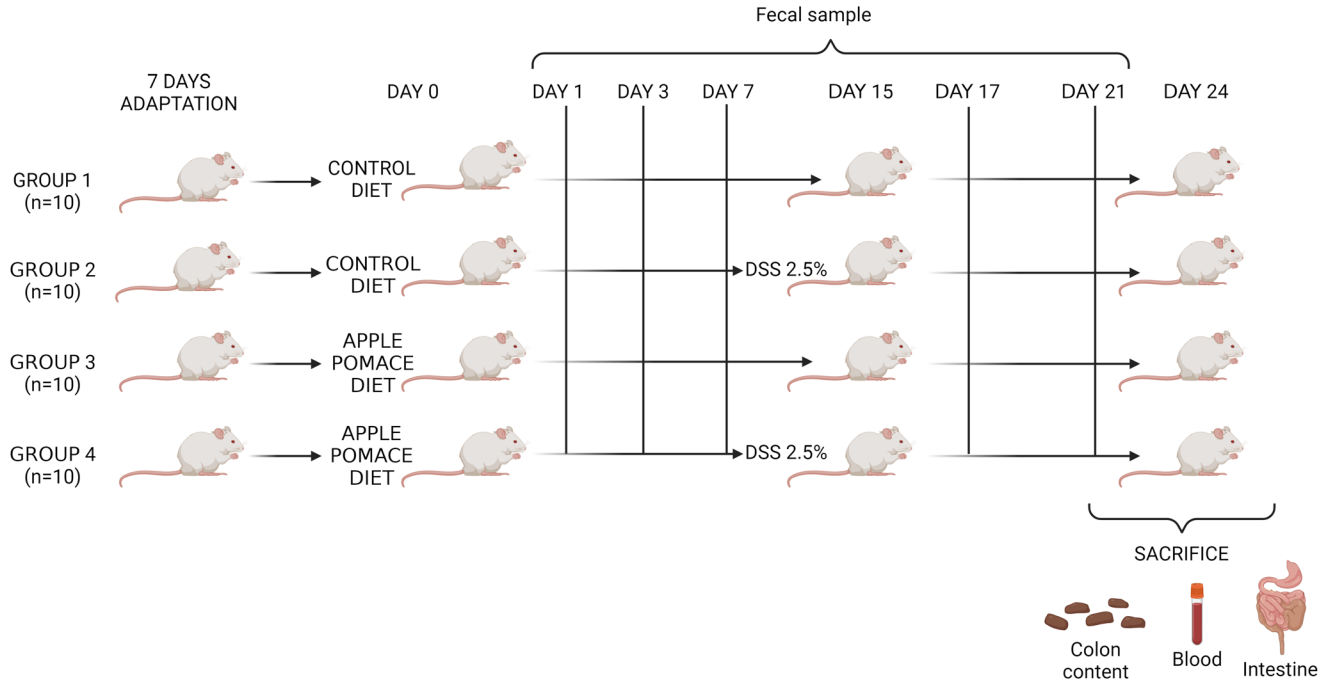

Fig. 1 Schematic representation of the animal experimentation design. A total of 40 mice (C57BL/6NRj, male 6–8 weeks) were randomly assigned to four groups (n = 10 animals per group), corresponding to the following treatments: control-diet with no colitis induction (group 1); control diet with DSS-induced colitis (group 2); apple pomace enriched diet (AP-diet, containing 10% apple pomace) with no colitis induction (group 3); and AP-diet with DSS-induced colitis (group 4). Sacrifice point for some of the animals receiving DSS was established earlier, due to the evolution of their DAI and clinical signs.

was performed on 72 fecal samples, corresponding to 3 pools of fecal samples per cage collected at different times (days 1, 15 and 21) from each animal of the groups, and the colon content of each animal (n = 40). The V3–V4 region was sequenced by using the primers 16S-ProV3V4-forward (CCTACGGGNBGCASCAG) and 16S-ProV3V4-reverse (GACTACNVGGGTATCTAATCC) on an Illumina MiSeq instrument in the Sequencing Facilities of Instituto de Parasitología y Biomedicina "López Neyra". Sequence reads were quality filtered and the resulting ones were processed using a personalized script of QIIME2 v.2021.8 software[20] matched by pair-ends. Quality control filtering was performed, keeping sequences with a mean sequence quality score >20 and a length between 140 and 400 bp. Raw sequencing data generated have been deposited in the Short Reads Archive of the NCBI under accession number PRJNA995428.

2.3. Short chain fatty acids

The major SCFAs were analyzed in cell free-supernatants from the colon content. The samples were diluted 1 : 10 in PBS and centrifuged. The supernatants were collected and supplemented with an internal standard solution (2-ethylbutyric acid 1.05 mg mL^{-1}, Sigma, St Louis, USA), acidified with a 20% v/v formic acid solution and extracted with methanol. Then the supernatants were centrifuged and filtered. The samples were used for SCFA separation and quantification by GC in equipment composed of a 6890NGC injection module (Agilent Technologies Inc., Palo Alto, CA, USA) connected to an FID.

2.4. Inflammatory marker analysis of serum samples

Blood was extracted after the sacrifice of all mice and left in RT for 2 h; after this time, the samples were centrifuged at 1000g for 15 min and the supernatant was transferred to a new tube and stored at −20 °C until use. A Mouse IL10 (Interleukin10) ELISA Kit (Cat: ELK1143, ELK Biotechnologies) and a Mouse TNFα (Tumor Necrosis Factor Alpha) ELISA Kit (Cat: ELK1287, ELK Biotechnologies) were used for this analysis following the manufacturer's instructions.

2.5. Myeloperoxidase (MPO) activity

MPO activity was measured following previously described procedures[21] with slight modifications using distal and proximal colon tissue fragments of each mouse. The samples were removed from −80 °C and placed on ice. First, the samples were homogenized with a pestle in HTAB (hexadecyltrimethylammonium bromide) buffer and then, they were transferred to a new tube with silica beads (1 mm) and homogenized in a Fisherbrand™ Homogenizer Bead Mill 24 by employing 4 cycles of alternating 30 s on and 30 s off, maintaining the samples on ice in between the cycles. Then the samples were frozen at −80 °C for 1 h. After this time, the samples were placed on ice and centrifuged at 13 400g, for 6 min at 4 °C.

The final steps and quantifications were according to the described procedures.[21]

2.6. Histological analysis

Histological analysis was carried out by the Biobank and Microscopy Service from the Health Research Institute of the Principality of Asturias (ISPA). Distal and proximal colonic tissues were processed and fixed in paraffin. Then, 3-micron sections were made using a microtome (Thermo Scientific HM355S) and were stained with hematoxylin and eosin. The microscopic study was carried out with a double observer. Histological lesions were scored considering the depth of lesions, inflammation (based on the length of damaged mucosa and qualitative estimation of the density of cellular infiltrates), and the presence of lymphoid hyperplasia, crypt ectasia, edema, erosion and ulceration. To integrate the results, a score similar to that used for the grading of ulcerative colitis lesions in humans was made (Table S1†).[22]

2.7. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics v.28.0.1 (IBM, Armonk, NY, USA). The goodness of fit to normal distribution was analyzed with the Shapiro–Wilk test. When the distribution of variables was skewed, the natural logarithm of each value was used in the statistical test. Box-and-whisker plots were used to represent the data, and the changes between groups were analyzed by the non-parametric Mann–Whitney U test. In the case of normal distribution, the values were represented as the mean ± the standard error of the mean (SEM). One- or two-way analysis of variance (ANOVA) was followed up with a *post-hoc* test using the Bonferroni adjustment for multiple comparisons among variables. A paired or independent *t*-test was used to analyze statistical differences between groups. The days at which 50% of the DAI score was elicited (DAI_{50}) were determined by a sigmoid fitting. Two-tailed probability values of $p \leq 0.05$ were considered significant in all cases.

3. Results

3.1. Food and fluid intake

During the adaptation period, the mice following the control diet (4 cages, n = 20 mice) or AP-diet (4 cages, n = 20 mice) showed an average intake of food of 0.135 (SEM: 0.0035, n = 4 cages) and 0.143 (SEM: 0.0064, n = 4 cages) grams per gram weight of the mouse per day, respectively, and drank an average of 0.182 (SEM: 0.0138, n = 4 cages) and 0.16 (SEM: 0.007, n = 4 cages) ml per gram mouse weight per day of water, respectively. An independent *t*-test showed no significant differences in food and water intake between both groups of diets. Neither were differences observed by the one-way ANOVA in the food and water intake after the adaptation period when comparing the four groups of mice on both types of diet, treated or not with DSS 2.5% (Fig. 2A and B).

Following the criteria for the sacrifice of the animals, the average number of days in which they were euthanized, after the administration of DSS 2.5%, was significantly lower for those of the control diet, 6.9 (SEM: 0.1) days, compared to those of the AP-diet, 9 (SEM: 0.36) days ($p < 0.001$, independent *t*-test).

3.2. Macroscopic indicators of disease

The weights of the animals at day 0 (after the one-week acclimation period) were 22.13 ± 0.24 g in the cohorts receiving the control diet (n = 20) and 22.92 ± 0.25 g (n = 20) in the cohorts that received the AP-diet (p = 0.032, *t*-test for independent samples). During the housing period, the mice of both groups increased significantly in weight, with respect to the initial value, from day 4 (ANOVA of a factor of repeated measures) ($p < 0.001$ and p = 0.05, respectively), without any significant differences between the weight of both groups of mice from day 4 (ANOVA of two factors (days of confinement and diet group) of repeated measures) (Fig. 2C).

The administration of 2.5% DSS, starting on day 15, limited weight gain in both cohorts, albeit that in the cohort receiving the standard diet, this effect was observed from day 2 of DSS administration, while in the cohort receiving the bagasse-enriched diet, the weight loss was observed from day 6 (Fig. 2D).

The disease activity index (DAI) was calculated during the experimental period to evaluate the disease progression. Overall, among the animals receiving DSS, the evolution of DAI was delayed in the groups receiving the AP-diet. Two-way ANOVA showed that the DAI score significantly increased on day 4 in the AP-diet group (p = 0.007), while this occurred on day 5 ($p < 0.001$) in the control diet. The curve fitting showed that the DAI_{50} for the AP-diet was on day 6 (SEM: 0.26) compared to those receiving the standard control diet on day 5.5 (SEM: 0.13) ($p < 0.001$). No significant differences were observed between diet types regarding the days (Fig. 2E).

3.3. Evaluation of intestinal damage

Colon length has been inversely related to intestinal inflammation in UC models, colon length shortening being an established biomarker of increased intestinal inflammation in models of colitis.[23] In this work, a significant colon length shortening was observed in the DSS groups, compared to their respective control groups. Similar results were observed in terms of colon shortening following DSS exposure for the groups fed with the standard diet and those fed with the apple pomace enriched diet (Fig. 3). The administration of the apple pomace enriched diet *per se* did not induce any colon length shortening in the animals not receiving DSS.

Histological examination of distal and proximal tissue sections was performed to compare the severity of the intestinal damage across the four groups of animals in the intervention. Almost all DSS-treated animals showed erosive inflammatory lesions. In all cases, the inflammatory infiltrate was mixed with polymorphonuclear and mononuclear cells, which is indicative of an active and acute lesion (Fig. 4A). In relation to the groups receiving different diets, animals from group 2 (control diet + DSS) were characterized as having exclusively distal pathology, with minimal or absent proximal lesions. The

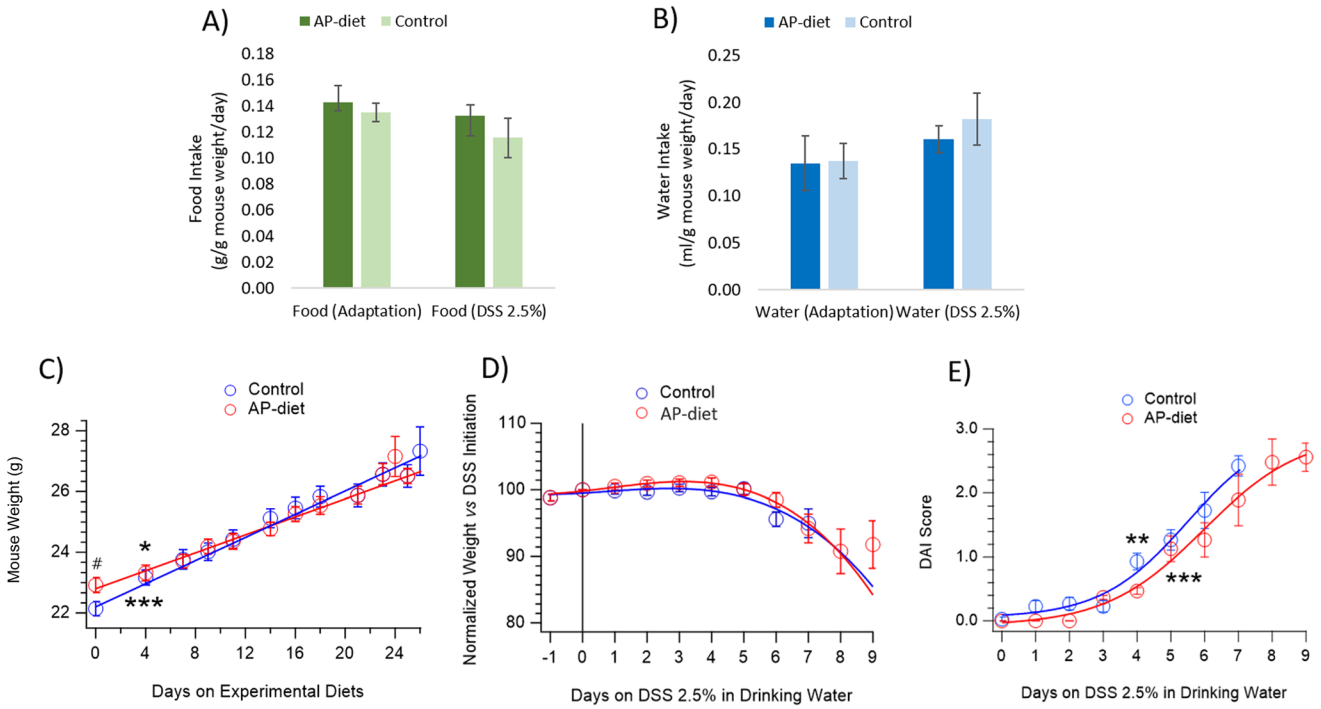

Fig. 2 Food (A) and water (B) intake of the different groups of animals during the first 2 weeks of the diet adaptation period (adaptation, n = 10 animals per group) and during the DSS administration period from day 15 to sacrifice (DSS 2.5%, n = 10 animals per group). AP-diet: apple pomace enriched diet. (C) Weight evolution in healthy animals receiving either the control or AP-diet and those not receiving any DSS administration along the experimental period (n = 10 animals per group). $^{\#}p$ = 0.032, using a t-test for independent samples at day 0, comparing the two groups of mice for the two types of foods. $^{*}p$ = 0.05 and $^{***}p < 0.001$, by comparing day 4 *vs.* day 0 in the control and AP diets, respectively. (D) Normalized weight gain and loss, represented as the percentage of loss referred to the weight of the animals on day 0 (prior to initiating DSS administration) (n = 10 animals per group). (E) Evolution of the disease activity index (DAI) in DSS receiving animals (n = 10 animals per group) determined according to the Cooper scoring system[19]. $^{**}p$ = 0.007 and $^{***}p < 0.001$, by Bonferroni test adjustment for multiple comparisons regarding day 0.

degree of inflammation ranged from moderate to severe, clearly predominating erosion over ulceration. The inflammatory infiltrate was notable in the injured areas, highlighting the acute cellular inflammatory component. This group of animals also showed the maximum degree of reactive lymphoid hyperplasia (Fig. 4B). In contrast, in group 4 (apple pomace enriched diet + DSS), although pathology was apparent in both proximal and distal regions, there was a lower cellular inflammatory component associated with the lesions, ranging from mild to moderate, as well as the absence of edema. There is an increase in the fibroblastic component in the lesion bed and the submucosa affected by inflammation, together with re-epithelialization phenomena (Fig. 4C).

In order to facilitate the comparison of the type and severity of the lesions observed at the histological level, these were scored according to previously described procedures, using the scoring levels established in Table 1. No damage was observed in the mice without colitis induction, obtaining a score of 0 in all of them, irrespective of the diet. Comparing the distal sections of the colon of the mice in which 2.5% DSS was added to the drinking water, significant differences (p = 0.023) were observed between cohorts at the level of intestinal tissue damage, with greater scoring in group 2 (standard diet + DSS) as compared to group 4 (AP-diet + DSS) (Fig. 4D). This observation suggests that the severity of the intestinal inflammation is weaker in animals receiving the AP-diet.

3.4. Intestinal and serum inflammatory markers

Intestinal MPO activity, determined as an indication of neutrophil accumulation in colonic mucosa,[24] did increase significantly during DSS administration in both animals receiving the control diet and AP-diet. No differences in MPO levels were encountered among the DSS-treated animals fed with the control diet and those fed with the AP-diet (Fig. 5).

In relation to serum levels of inflammatory markers, serum levels of IL10 and TNFα were determined as an indication of the inflammation level in the animals. The levels of IL10 were reduced in the DSS-treated groups, as compared to their respective control groups, and the same tendency occurred with both diet types. Comparison of the DSS-treated animals from the two diets revealed significant differences (p = 0.043) with higher IL10 levels in group 2 (control diet) as compared to group 4 (AP-diet).

Remarkably, in the healthy control groups, IL10 levels did not show any statistically significant differences between the two types of diet (Fig. 5). IL10 has been recognized as one of the most important anti-inflammatory cytokines regulating mucosal immune responses in the gut[25] and has been

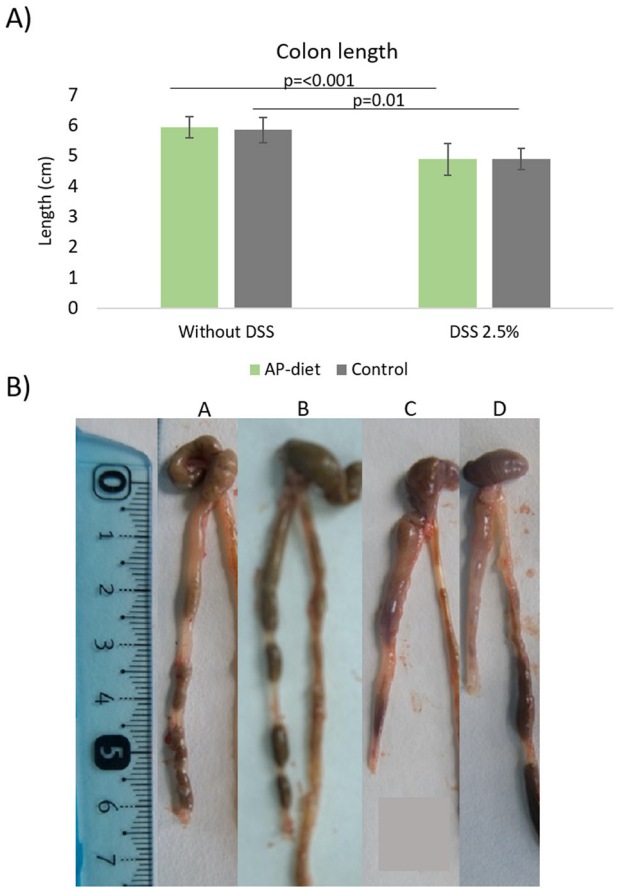

Fig. 3 (A) Colon length of the different groups of animals. The statistical analysis performed was the non-parametric Mann–Whitney U test. (B) Representative image of the colon length of each of the groups; A: AP-diet (apple pomace enriched diet) without the DSS treatment; B: control diet without the DSS treatment; C: apple pomace diet with DSS treatment and D: control diet with DSS treatment.

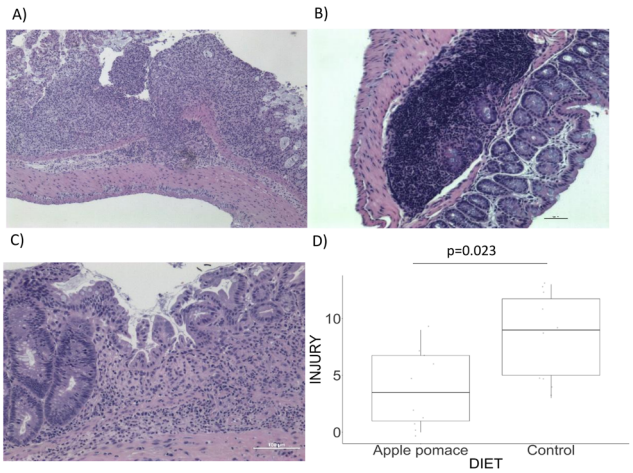

Fig. 4 Representative microscopic images of intestinal tissues analyzed from the DSS-treated animals showing different indications of histological damage: (A) grade 3 mixed inflammation; (B) moderate lymphoid hyperplasia; and (C) re-epithelialization. (D) Histological score of inflammation in the groups receiving DSS treatment (distal region), determined according to Geboes *et al.* (2000).[22] The statistical analysis performed was the non-parametric Mann–Whitney U test (n = 10 animals per group).

Table 1 Scoring system used to evaluate disease severity from histological examination of intestinal tissue, according to Geboes *et al.* (2000)[22]

Score	Observation
Grade 0	Minimal changes
Grade 1	Chronic inflammatory infiltrate, 0–3
Grade 2a	Eosinophils presence
Grade 2b	Neutrophils presence, 0–3
Grade 3	Intraepithelial neutrophils, 0–3
Grade 4	Crypts destruction, 0–3
Grade 5	Erosion or ulceration: 0 to 3 erosion, 4 ulceration

reported to protect against intestinal damage in models of colitis.[26] Several probiotic or prebiotic intervention studies have reported increased expression of IL10 associated with reduced disease severity.[27–30] Certain types of pectin, previously proposed as one of the main bioactive components exhibiting prebiotic properties in apple pomace,[17] have been reported to be capable of conferring protection against intestinal inflammation in DSS-induced colitis models, generally in association with increased IL10 expression either at the systemic or intestinal level.[31] In our work, DSS administration did not significantly affect serum IL10 levels in the animals receiving the standard diet, but animals receiving DSS and the apple pomace enriched diet showed lower IL10 serum levels. While these results appear to contradict those already described, it is not the first time that conflicting results regarding the role of IL10 in inflammation in colitis are presented. Some works have already reported that colitis patients have increased IL10 production, yet the specific physiological consequences of such elevation may be highly influenced by the presence of other cytokines in the local environment.[32]

TNFα is a proinflammatory cytokine whose levels generally appear increased in both serum and colonic contents in DSS-induced colitis models.[33,34] Besides, administration of probiotics or prebiotics demonstrating alleviation of colitis symptoms in such models is generally accompanied by an attenuation of TNFα production in DSS-treated animals.[30,33–35] In our experimental groups, no statistically significant differences in TNFα were detected between the cohorts with DSS (p = 0.052). Remarkably, in animals receiving the standard diet, administration of DSS resulted in a significant increase in TNFα (p < 0.001), while in the animals receiving the AP-diet, administration of DSS did not increase the serum levels of TNFα, suggesting that DSS-induced inflammation is reduced in animals fed with the AP-diet (Fig. 5).

These results are consistent with the intestinal tissue damage described above (intestinal and serum inflammatory markers) and the evolution of the DAI in all the experimental groups (evaluation of intestinal damage and Fig. 5) and support the contribution of the AP-diet in ameliorating some inflammatory indicators in the DSS-induced colitis model.

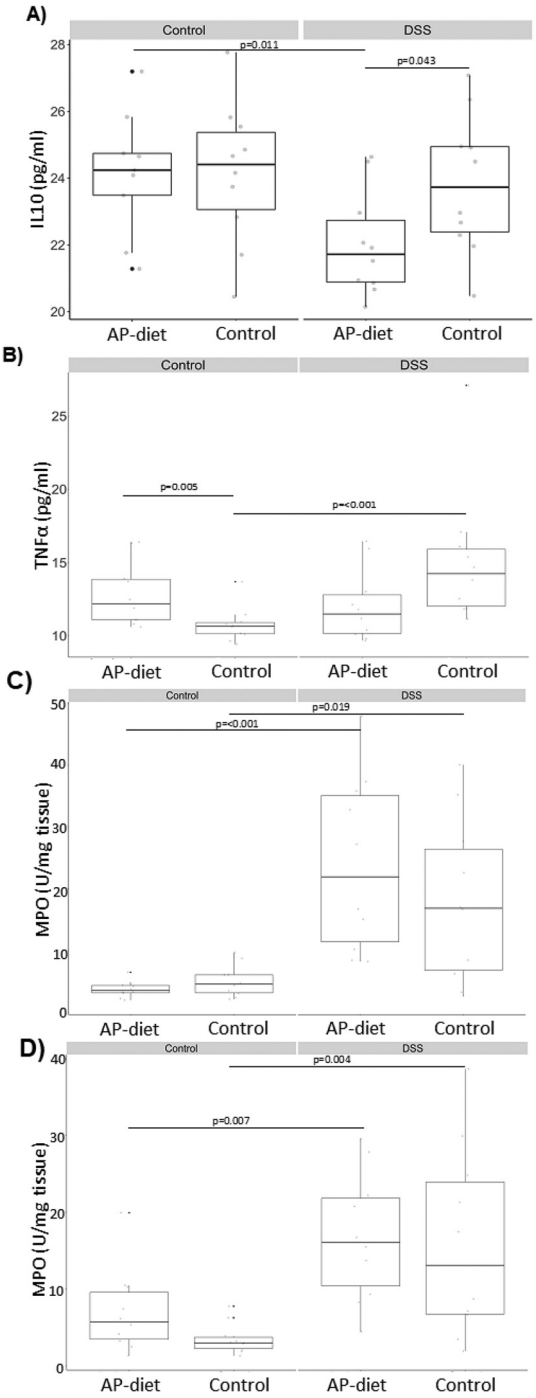

Fig. 5 (A) IL10 levels determined in serum. (B) TNFα levels determined in serum. (C) Myeloperoxidase (MPO) content in distal intestinal tissue and in proximal intestinal tissue (D). Non-parametric Mann–Whitney U test was used to analyse differences among groups (n = 10 animals per group).

3.5. Gut microbiome

The possible influence of the AP-diet on gut microbiota modulation was analyzed by means of 16S sequencing of fecal samples, collected along the experimental period, and colon contents collected at sacrifice. Concerning colon contents, most alpha diversity coefficients, determined to measure the variability of genera within samples, showed similar trends (ESI Fig. S1†). In the particular case of the Chao1 index, healthy and DSS groups following control diets (31.6 ± 1.4 and 30.5 ± 1.0) and AP-diets (32.9 ± 0.3 and 30.0 ± 0.9) showed similar values. The healthy group following the AP-diet showed alpha diversity estimators significantly ($p > 0.05$) different from the rest of the groups, including a higher Chao1 index, likely reflecting a higher number of distinct genera in samples from this group.

Colonic content diversity among samples and groups was estimated through Bray–Curtis dissimilarity metrics (ESI Fig. S2†). The DSS group following control diets showed significantly higher ($p < 0.05$) beta-diversity coefficients than the healthy group following AP-diets. These differences might be due to a higher intragroup variability according to alpha diversity analysis (ESI Fig. S1†) and could be associated with the range of differential clinical manifestations of these animals at the end of the experimental period (see sections 3.3. and 3.4). The Bray–Curtis dissimilarity metric was also used to cluster these microbiota samples (ESI Fig. S2B†). Samples corresponding to healthy or DSS animals generally clustered in different branches of the hierarchical tree, yet both sample groups, were not completely discriminated likely due to the high interindividual variability of microbiota profiles. In relation to animals receiving control or AP-diets, no clear clustering between groups was observed, neither in the healthy group nor in the DSS-receiving animals.

An additional principal coordinates analysis (PCoA) was computed to further characterize these differences in microbial communities among colonic content samples (Fig. 6A). Interestingly, samples from healthy groups following both diets were grouped together and were discriminated from the DSS group following control diets. In contrast, some samples from the DSS group following AP-diets showed similar profiles to those of the healthy groups, revealing the positive influence of apple pomace supplementation and its gut microbiota modulatory properties in the context of DSS-induced colitis.

To further characterize the microbiota modulatory properties of apple pomace, significant differences between groups were determined. Statistical methods designed for microbiome analysis (ANCOM and LEfSe) were performed. To assess the impact of diet on gut microbiota, differences between samples taken at 0 and 15 days of dietary intervention were calculated (ESI Fig. S3†). Along with the dietary intervention, prior to DSS administration, both the control diet and AP-diet led to an increase in *Bifidobacterium, Muribaculum, Enterohabdus*, and *Erysipelatoclostridium* and a reduction in *Lactobacillus* (ESI Fig. S3A and B†).

With regard to the healthy groups, AP-diets resulted in higher abundances of *Blautia, Alloprevotella* and *Muribaculaceae* compared to those following control diets after 24 days of intervention (ESI Fig. S3C and D†). An increment in *Roseburia* was also observed in colonic content samples of mice following AP-diets compared to the control diet group.

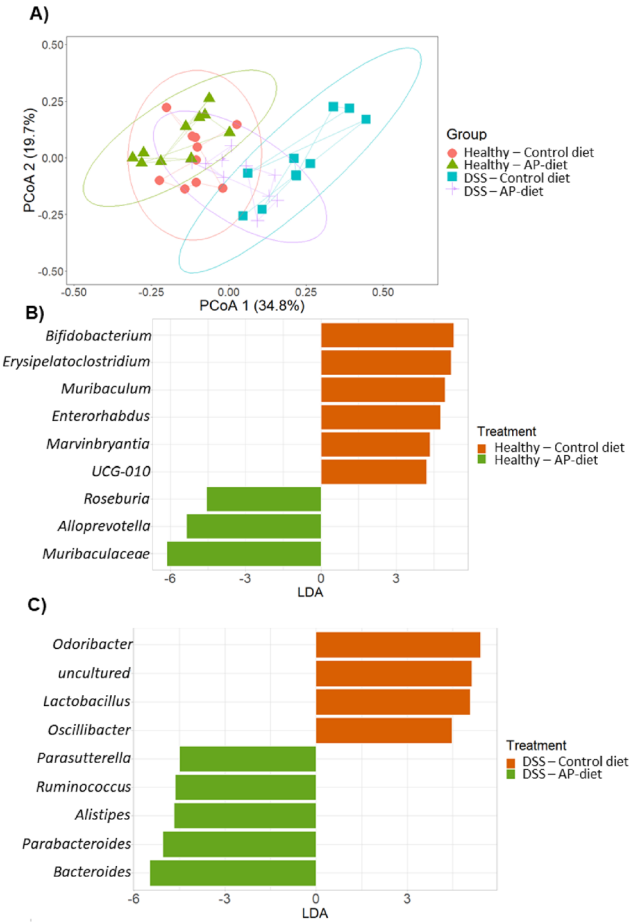

Fig. 6 (A) Principal coordinates analysis (PCoA) of colonic content samples of mice following control and apple pomace-enriched diets. These samples correspond to healthy and DSS-induced colitis groups. Therefore, a total of 4 groups (n = 10 animals per group) are represented. Samples from healthy groups following both diets were grouped together and were discriminated from the DSS group following control diets. In contrast, some samples from the DSS group following diets enriched in pomace showed similar profiles to those of healthy groups, revealing the positive influence of apple pomace supplementation and its gut microbiota modulatory properties in the context of DSS-induced colitis. PC: principal coordinate. The percentage of variance explained by each PC is indicated in the axis. (B and C) Linear discriminant analysis (LDA) scores of differentially abundant microbial genera among colonic content samples of mice following control and pomace-enriched diets. These samples correspond to the healthy (B) and DSS-induced colitis (C) groups. Therefore, a total of 4 groups (n = 10 animals per group) are compared. As can be seen, an increment in *Roseburia* was observed in colonic content samples of healthy mice following pomace-enriched diets compared to control diets. Apple pomace-enriched diets also promoted the growth of *Ruminococcus* and *Parasutterella* in colonic content samples compared to control diets in the DSS group.

Other genera that were promoted throughout the administration of AP-diets include *Bifidobacterium* and the *Lachnospiraceae* NK4A136 group (Table 2).

In contrast, in the DSS group, the AP-diet led to higher abundances of *Alistipes, Parabacteroides* and *Oscillibacter* compared to the control diets (ESI Fig. S3D†). AP-diets also promoted *Ruminococcus* and *Parasutterella* in colonic content samples compared to control diets (Fig. 6C). Other genera that were stimulated throughout the administration of AP-diets in the DSS group include *Akkermansia, Parabacteroides* and novel genera *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG-006 (Table 2).

The influence of characteristic taxa on inflammatory markers was investigated through calculation of Pearson correlation coefficients between microbial genera were found showing significant differences among intervention groups' SCFA levels, inflammatory markers and DAI scores (Fig. 7). Positive correlations between novel *Lachnospiraceae* genera including the *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG 006 and SCFA levels were found. Associations between microbial genera and inflammatory markers included positive correlations between *Roseburia* and IL10 levels and between *Odoribacter* and an uncultured genus and TNFα levels. In contrast, the *Eubacterium coprostaligenes* group, *Muribaculum, Roseburia* and novel *Ruminococcaceae* genus UCG 010 correlated negatively with MPO levels in both proximal and distal colon. Finally, histological scores of inflammation in the distal colon were positively correlated with *Odoribater*, according to previous literature,[36] and negatively correlated with *Alloprevotella* and *Muribaculum*.

4. Discussion

The present work shows that a diet enriched in apple-pomace, an agricultural by-product with a demonstrated content of complex carbohydrates and polyphenols with potential prebiotic properties,[16,17] may exert a protective effect *in vivo* against intestinal damage induced by DSS in mice. This would open the way to further research aimed at facilitating the development of bioactive and functional ingredients from agricultural by-products, without the need to use highly purified extracts.

Prebiotics have been scarcely explored as preventive strategies to protect against intestinal inflammation in IBD patients, mainly due to the fact that diets rich in non-fermentable fibres have been associated with worsening of symptomatology and even with triggering flare-ups in certain IBD patients. Specifically, this effect has been observed with B-glucans (inulin and FOS),[37,38] although conflicting results have been presented in preclinical models.[39] Regardless of this, with the exception of intestinal obstruction, several authors agree that there is no evidence that fibre intake should be restricted in most IBD patients[40,41] and warrant further investigation into the definition of the role of well-defined prebiotic fibres in IBD management.

In this regard, although some preclinical and clinical studies using other prebiotics and/or fibre supplementation reported inconsistent success in ameliorating IBD, certain beneficial effects of defined prebiotic fibres on specific markers of the disease have been reported. For instance, in DSS-induced colitis models, fermented barley and soybean mixtures enhanced intestinal barrier function ameliorating disease pro-

Table 2 Abundances of microbial genera found in fecal and colonic content samples of mice following apple pomace enriched diets. Fecal samples were taken at 15 and 24 days of intervention, while colonic content samples were collected at sacrifice and at the end of the experimental period, and they correspond to the healthy (n = 10 mice) and DSS-induced colitis (n = 10 mice) groups. Mean abundances and standard deviations (SD) of microbial genera are compared. These genera showed statistically significant differences ($p < 0.05$ and $p_{adj} < 0.25$) determined by ANCOM and LEfSe methods at different intervention times including (i) *Bifidobacterium* and *Lachnospiraceae* NK4A136 in the healthy group and (ii) *Akkermansia*, the *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG-006 in the DSS-induced colitis group

	15 Days		24 Days		Colonic content	
	Mean	SD	Mean	SD	Mean	SD
Healthy group: pomace-enriched diet						
Bacteroides	1.31	0.31	1.97	0.49	1.83	0.91
Bifidobacterium	1.21	1.39	3.41	2.30	4.75	3.90
Candidatus Saccharimonas	2.65	1.12	0.67	0.21	0.57	0.41
Colidextribacter	0.41	0.22	0.55	0.37	0.62	0.31
Dubosiella	5.13	4.33	1.34	0.46	0.99	0.97
Enterorhabdus	2.30	0.23	1.44	0.34	1.30	0.42
Erysipelatoclostridium	1.59	1.04	0.77	0.51	0.77	0.70
Faecalibaculum	0.09	0.08	0.43	0.63	1.27	1.74
Lachnospiraceae NK4A136 group	3.29	0.64	5.21	4.02	8.99	5.83
Muribaculaceae	33.28	2.85	41.74	5.92	36.53	5.55
Parabacteroides	0.28	0.12	0.32	0.07	0.39	0.31
Rikenellaceae RC9 gut group	0.70	0.20	0.28	0.26	0.19	0.13
Ruminococcus	0.07	0.06	0.17	0.11	0.27	0.27
Uncultured	0.41	0.09	0.63	0.58	1.03	1.00
DSS group: pomace-enriched diet						
[Eubacterium] coprostanoligenes group	2.15	1.31	1.24	0.51	1.59	0.79
Akkermansia	10.74	5.93	20.31	7.20	21.66	5.23
Bifidobacterium	7.83	8.60	4.68	1.84	2.01	3.01
Blautia	0.17	0.18	0.05	0.04	0.15	0.11
Candidatus Saccharimonas	1.40	1.54	1.20	0.97	0.74	0.98
Clostridia UCG-014	4.10	1.85	9.36	2.66	6.04	4.10
Colidextribacter	0.30	0.19	0.54	0.10	0.60	0.19
Dubosiella	3.09	1.98	2.31	0.94	1.98	3.68
Enterorhabdus	2.45	1.79	0.68	0.24	0.44	0.09
Erysipelatoclostridium	1.55	1.27	0.72	0.39	2.26	0.99
Faecalibaculum	0.08	0.09	0.35	0.37	0.85	1.39
Lachnoclostridium	1.53	0.93	0.95	0.54	1.29	0.89
Lachnospiraceae NK4A136 group	3.73	0.61	6.84	1.91	9.23	4.10
Lachnospiraceae UCG-006	0.35	0.33	1.46	0.51	3.14	3.61
Lactobacillus	6.11	6.34	1.80	1.13	3.27	2.69
Marvinbryantia	0.12	0.14	0.01	0.01	0.04	0.03
Muribaculaceae	37.09	10.13	23.64	3.46	16.87	7.75
Muribaculum	2.48	0.85	0.63	0.41	0.28	0.30
Odoribacter	0.20	0.11	0.69	0.67	1.10	1.52
Oscillibacter	0.25	0.27	0.88	0.34	1.01	0.66
Other	13.20	6.78	20.02	5.50	21.68	6.44
Parabacteroides	0.67	0.40	1.07	0.26	2.59	2.05
Roseburia	0.12	0.19	0.00	0.00	0.00	0.00
Ruminococcus	0.07	0.03	0.44	0.10	1.12	1.09
UCG-010	0.19	0.11	0.16	0.06	0.08	0.08

gress;[42] and pectin from different sources ameliorated inflammation signals[12,43] and enhanced barrier function.[31] Remarkably, pectin encompasses a wide diverse group of polysaccharides which are recognized as emergent prebiotics. Besides, their specific chemical and structural composition has been linked to variations in their bioactive and functional properties.[12,44]

In a prior work, we have reported *in vitro* gut microbiota modulatory properties of apple pomace from selected apple varieties and of their derived pectins,[17] supporting a beneficial modulation of beneficial commensal species in human fecal batch cultures. Besides, a prior *in silico* study based on comparative analyses of predicted carbohydrate utilizing activities in the gut microbiome of healthy *vs.* IBD patients revealed that despite the important gut microbiota shifts encountered in IBD patients, pectin degraders are still represented in most metagenomes.[18] Thus, these results encouraged us to evaluate *in vivo* the potential beneficial effects of pectin-rich apple pomace as a dietary ingredient. To this end, a basal rodent diet was supplemented with 10% dried apple pomace, an amount that has already been reported to confer metabolic effects in other rodent models[45] and which, in our case, is expected to provide sufficient pectin components to exhibit effects on the microbiome[46] (ESI Table S1†).

Tolerance of IBD patients to certain prebiotics administration, especially when referring to complex non-digestible carbohydrates, depends on the basal microbiota configuration of the patients and, thus, on their capacity to appropriately

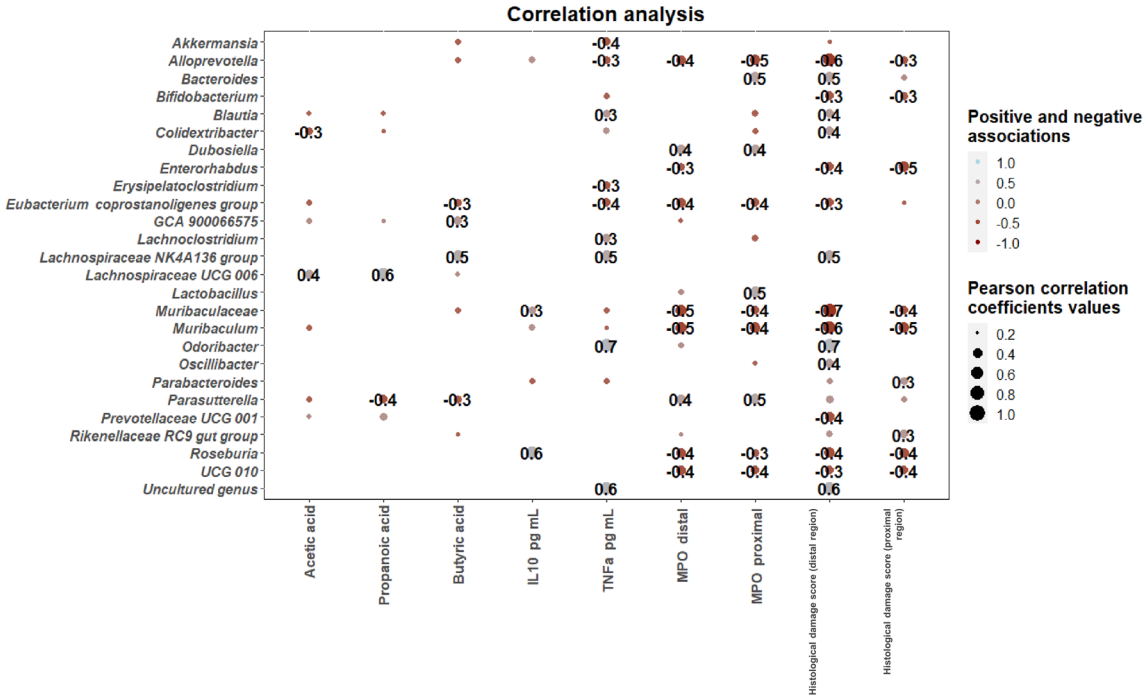

Fig. 7 Correlation heatmaps showing the associations ($p < 0.05$) between microbial genera determined in colonic contents (collected at the time of sacrifice) showing significant differences among intervention groups and short-chain fatty acid (SCFA) levels, biochemical parameters and inflammatory markers. Blue and red dots indicate positive and negative correlations expressed as Pearson correlation coefficients. Colour intensity and dot size are in proportion to magnitude. Positive correlations between novel *Lachnospiraceae* genera including the *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG 006 and SCFA levels were found. Associations between microbial genera and inflammatory markers included positive correlations between *Roseburia* and IL 10 levels and positive correlations between *Odoribacter* and an uncultured genus and TNFα levels.

metabolize the provided prebiotics.[10] Indeed, while some fibre interventions have demonstrated the capacity to ameliorate defined IBD biomarkers, fibre supplementation alone has demonstrated a poor capacity to restore the characteristic IBD gut microbiome disbalances.[47] The present work demonstrates the gut modulatory potential of an AP-diet in a DSS-induced colitis mouse model. Apple pomace selectively stimulated bacterial groups that are associated with a healthy gut microbiota composition, including *Akkermansia, Parabacteroides* and the *Lachnospiraceae* NK4A136 group and *Lachnospiraceae* UCG-006. In addition, these novel *Lachnospiraceae* genera showed positive associations with SCFA levels, known to exert several health benefits in the context of IBD.[48] It should be noted that these taxa have been proposed as emerging probiotics associated with specific health benefits.[49–51] Specifically, *Lachnospiraceae* members involve cell wall polysaccharide degraders and butyrate producers[52–54] that promote the production of anti-inflammatory cytokines[55] and may contribute to the amelioration of intestinal inflammatory conditions.[56] In our work, the gut microbiota of mice suffering from colitis following the AP-diet showed profiles more similar to those of healthy mice, in association with amelioration of some inflammatory markers, highlighting the potential of apple pomace fractions to restore the gut ecosystem and ameliorate colitis symptoms. In this regard, previous studies dealing with fecal fermentation experiments of apple pomace and pectin fractions reported similar increases in the abundance of *Lachnospira* members that could be attributed to the neutral sugar content of polysaccharide chains.[17] Similar associations between SCFA levels and the abundance of these taxa were also reported[17] in agreement with the present study.

5. Conclusions

Our results demonstrate a significant contribution of AP-diet to reduce intestinal inflammation in a DSS-induced colitis model, reflected by a delayed increase of DAI, reduced promotion of pro-inflammatory TNFα, reduced histological inflammation scores and modification of the overall gut microbiota composition and SCFA production. These results provide an experimental demonstration that pectin containing prebiotic ingredients can be metabolized, to a certain extent, by the disrupted microbiome in colitis models, as previously suggested through *in silico*[18] and *in vitro* investigations.[17] It should be noted that despite its content in prebiotic pectins, the apple pomace, as incorporated in the animals feed, is a complex mixture of ingredients;[16] hence, the possibility that other apple pomace components may be affecting the inflammatory response observed in the animals cannot be ruled out. Overall, our results warrant further investigation incorporating well-defined purified substrates for comparison, as well as

long-term evaluation of a comprehensive array of health and disease biomarkers associated with consumption of foodstuffs incorporating apple pomace. This will open the avenue towards the formulation of novel and safe prebiotic containing foods from pectin-rich agricultural by-products such as apple pomace.

Author contributions

ICT: investigation and formal analyses; CS: formal analyses; BC: investigation; MS: investigation and formal analyses; AM: conceptualization; LR: conceptualization, funding acquisition, and writing – original draft. All authors have read, edited and approved the final version of the manuscript.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

We thank the project RTI2018-095021-J-I00 funded by MCIN/AEI/10.13039/501100011033/, ERDF A way of making Europe and AYUD-2021-50910 from the autonomic Government of Principado de Asturias (FICYT), supported by FEDER. Carlos Sabater was the recipient of a Juan de la Cierva-Formación postdoctoral contract from MICINN, the Spanish Ministry of Science and Innovation (FJC2019-042125-I).

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