Article

A diet based on cured acorn ham with oleic acid content promotes anti-inflammatory gut microbiota shifts and prevents ulcerative colitis in an animal model

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ABSTRACT

Background

Diets based on meat products are not recommended in the case of ulcerative colitis (UC). However, some foods, as those containing high oleic acid and a low omega-6/omega-3 ratio show anti-inflammatory properties. The objective here is to test if some traditional cured meat products, as acorn-fed ham (high levels of oleic acid), may be useful for controlling inflammatory diseases as UC in animal models.

Methods

3 rat cohorts have been used: vegetable rat feed, control ham and acorn-fed ham (a traditional ham where high oleic acid concentration from acorns is storage in the muscle fat). UC was induced with DSS in drinking water *ad libitum* for one week. Short-chain fatty acids (SCFAs) and 16S rRNA from bacterial populations were analyzed in cecum samples. Colon samples were analyzed for histological parameters (inflammatory cell density, mucosa damages, myeloperoxidase).

Results

In the acorn-fed ham cohort, a protective effect was observed with respect to UC disease activity index, inflammatory cells density, colon mucosa alterations, myeloperoxidase levels, blood total antioxidant capacity and lower levels of pro-inflammatory cytokines, in comparison with feed cohort. Both ham diets caused a reduction in *Firmicutes* and an increase in *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* in comparison with rat feed diet. Also, acorn-fed ham diet induced changes in gut microbiota composition, with pronounced enrichments in anti-inflammatory bacterial genera such as *Alistipes, Bacteroides, Blautia, Butyricimonas* and *Parabacteroides*.

Conclusions

In the acorn-fed ham cohort, as a result of the dietary intake of oleic acid and low intake of omega-6 fatty acids, a strong preventive effect against UC symptoms was observed, indicating a valuable effect of this traditional Mediterranean cured meat product.

Keywords: oleic acid, acorn feed ham, gut microbiota, ulcerative colitis

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1. Introduction

Some traditional foods present in the Mediterranean diet contain nutraceutical compounds with anti-inflammatory bioactivities which may be useful under certain gastrointestinal conditions. Ulcerative colitis (UC) is the most common form of inflammatory bowel disease (IBD), followed by Crohn's disease (CD). In the European Union, UC affects 178,000 new individuals each year and about 2.1 million patients in total. Though the etiology of both UC and CD is still unknown, they share an inflammatory basis. In UC, for example, there are higher mucosal levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-17 or TNFα. UC and CD show a linkage in terms of genetic susceptibility as well, such as the NOD2 and IL23R genes, which are involved in immune response to microbes. Also, IBD patients show alterations in gut microbiota characteristics with respect to the canonical bacterial populations from healthy individuals (dysbiosis). This includes increased *Proteobacteria* (such as *E. coli*) and *Bacteroidetes* (such as *Prevotella* spp, as opposed to *Bacteroides* spp.) rates, and lower *Firmicutes* populations [1–8].

Several environmental factors have been proposed to modulate the onset of UC in children and adults. Lower levels of vitamin D are associated with higher UC incidence. Vaginal delivery and breastfeeding seem to be protective factors against UC, as well as rural lifestyle and exposure to pets. All these factors supposedly increase gut microbiota diversity. However, antibiotic therapy before 5 years of age has been linked to increased UC onset, as it is a factor that diminishes gut microbiota diversity. Smoking, sedentary lifestyle, air pollution, infections by *Salmonella* or *Campylobacter*, or colonization by *Mycobacterium avium* also show a positive correlation with UC development, probably because they trigger inflammatory responses in the gastrointestinal tract [9,10].

Diet is another important environmental factor linked with UC development and relapses. Dietary fiber from vegetables and fruits in a normal diet shows a protective effect. This is probably due to the gut production of SCFAs by microbiota fermentation of fiber, a type of metabolite with anti-inflammatory effects. The protective effect of dietary prebiotic fiber leads to a reduction of gut inflammatory biomarkers in UC patients, such as fecal calprotectin [11–14]. Processed meat foods (sausages, hamburgers, etc.) are risk factors for UC onset. Conversely, a normal diet high in omega-3 fatty acids (low omega-6/3 ratio) is associated with lower risk of UC [12,15].

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In general, Western diets (high saturated fat and high sugar content, high omega-6/3 ratio, low fiber) have been associated with IBD onset. In contrast, the Mediterranean diet (low saturated fat, low omega-6/3 ratio, high fiber) has been associated with an anti-inflammatory gut status, therefore preventing dysbiosis and IBD [8].

One of the anti-inflammatory actors in the Mediterranean diet is its low omega-6/3 ratio. This diet is high in protective omega-3 fatty acids from vegetables (α-linolenic) and fish (eicosapentaenoic (EPA), docosapentaenoic (DPA) and docosahexaenoic (DHA) acids), and low in omega-6 (linoleic, arachidonic or adrenic acids). The omega-6/3 ratio in traditional diets rich in vegetables and fish is considered to be 1, whereas in some European and North American countries this ratio is around 15. This high value induces a pro-inflammatory status associated with increased incidence of cancer, as well as cardiovascular and inflammatory diseases (such as UC). A low omega-6/3 ratio (such as 2:1) has been shown to attenuate inflammatory mediators production in UC animal models, downregulating pro-inflammatory cell populations such as Th1 (which produces IFN-γ), Th2 (which produces IL-4) and Th17 (which produces IL-17A), $CD4^+$ T-helper, and at the same time upregulating Treg cell populations titers (which have anti-inflammatory effects, by modulating T-helper cells) [16]. Another anti-inflammatory actor in the Mediterranean traditional diet is its high oleic acid content. This monounsaturated fatty acid is able to reduce gut pro-inflammatory cytokine levels in animal models for UC generated by the chemical inducer dextran sodium sulfate (DSS)[8,17,18].

During the course of this research, a rat animal model for UC was induced with DSS (in drinking water, administered *ad libitum* for one week) and the protective effect of a diet based on traditional acorn-fed Iberian ham was tested, in comparison with conventional cured ham and rat feed. Acorn-fed Iberian ham is a cured meat product with a low omega-6/3 ratio, traditionally from Southwestern Spain and Portugal. This low omega-6/3 ratio is due to the fact that, in these geographical areas, free-range Iberian pigs fed exclusively on acorns (from green oaks and cork trees) and grass during the months prior to their sacrifice. Acorns are seeds with a low omega-6/3 ratio and high oleic acid content (63%). Consequently, these healthy fatty acids are stored in Iberian pig muscle tissue (as ham) during the free-range feeding months of these pigs on acorns [19]. Continuous use of this traditional cured

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acorn-fed ham in the human diet is interesting as it provides a gut anti-inflammatory status regarding important gut disorders as UC.

2. Materials and methods

2.1. Animals and experimental design

A total of 30 male Fischer 344 rats were maintained in the Animal Facilities at the University of Oviedo (authorized facility No. ES330440003591). All rat experiments were approved by the Ethics Committee of the Principality of Asturias (authorization code PROAE 23/2016).

Rats (5 weeks old) were divided into 3 cohorts of 10 individuals each and fed *ad libitum*. Rats were maintained in individual cages at controlled temperature, humidity and light cycle. Cohort 1 was fed with universal feed (2014 Teklad Global 14% Protein Rodent Maintenance Harlan diet feed). Cohorts 2 and 3 were fed only with acorn-fed Iberian commercial ham and control ham (from pigs fed conventional porcine feed: corn, barley, soy, wheat, whey, palm oil, inorganic salts), respectively. Every day, 25 g of the corresponding ham was added to each rat cage, and the leftovers discharged the next day. The daily Ham diets consisted in cubic pieces of the respective ham (1 cm size), which were stored at 4 ºC before daily addition to rat cages. Feed and hams composition is referred on Tables 1 and 2.

2.2. UC induction and monitoring

One week after the arrival of the animals to the animal facility, the three respective diets started. After one week feeding on the corresponding diet, UC was induced in 8 rats from each cohort. Induction was carried out using autoclaved drinking water containing 3% DSS (40,000 g/Mol, Alpha Aesar) for 7 days, administered *ad libitum*.

The rats were monitored weekly for food and drinking water intake, weight loss and stool consistency/rectal bleeding using a modified protocol from a published work on UC disease activity index (DAI) [7].

2.3. Blood and tissue samples

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One week after finishing the administration of DSS, all fasted rats were anesthetized (isoflurane) and sacrificed (pneumothorax) for the extraction of blood (2 mL from heart, centrifuged at 3,000 rpm 15 min and then the plasma was frozen), the small intestine (fresh, for Peyer's patches quantification), the whole colon (fresh or kept in 4% formaldehyde at 4 ºC, depending on the test) and the cecum (frozen at -20 ºC). 2 rats from each cohort were left free of DSS as absolute controls (no UC).

2.4. Physical measures

The rats were weighed every week during the 3 experimental weeks: at the beginning of DSS administration (day 7), at the end of DSS administration (day 14) and just before sacrifice (day 21).

2.5. Histological studies

Colon length: the percentage of its reduction in the experimental samples was calculated with respect to the colons of the 2 control animals from each cohort.

Peyer's patches: hyperplastic Peyer's patches were counted along the small intestine. Their number in the experimental animals was calculated with respect to the small intestines' Peyer's patches of the 2 absolute control animals from each cohort (animals 9 and 10).

Macroscopic score assessment of ulcerative colitis: this parameter was measured by an external investigator, according to a published score [20].

Reparative changes in colon mucosa, colon epithelium alterations and inflammatory cell density in colon: the distal colon samples were opened along the longitudinal axis and fixed for 24 h in 4% phosphate-buffered formaldehyde at room temperature before being embedded in paraffin blocks, in accordance with routine procedure. Specimens were sectioned in 5 μm thick sections and were stained using hematoxylin and eosin. Microscopic diagnosis was performed on microphotographs obtained by an Olympus BX-53 microscope and a DP73 digital camera connected to a computer with CellSens software. The images were used to identify widespread epithelial erosions, the degree of loss of goblet cells and crypts, and the degree of inflammatory infiltrate (from mucosa to submucosa), as well as the presence of lymphoid follicles. The inflammatory cells were analyzed for type (lymphocytes, plasma cells and neutrophils), intensity (mild, moderate and severe degree) and the presence of reparative changes (with or without epithelial regeneration and mucin depletion).

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2.6. Myeloperoxidase assay in colon mucosa

A 0.5 cm longitudinal section from each colon was excised and this pro-inflammatory enzyme was quantified following a published protocol [21].

2.7. Total antioxidant capacity in blood plasma

Total antioxidant activity was measured in plasma samples using a commercial FRAP (ferric reducing activity of plasma) assay kit (Bioquochem SL, Ref. Kf-01-003). A standard curve of different Trolox (a vitamin E analogue) concentrations was used for comparison.

2.8. Pro- and anti-inflammatory cytokines analysis in blood plasma

IFN-γ, IL-1β, IL-6, IL-10, IL-17a, TGF-β1 and TNF-α tests were performed on blood plasma samples, using commercial Elisa kits (Abnova Ref. KA0273, KA1502, KA0278, KA0274, KA1001, KA0279, KA0280) and following the manufacturer's instructions.

2.9. GC-MS quantification of SCFAs in feces using deuterated standards

400 mg of frozen cecum feces were thawed and resuspended in 1,716 µl milli-Q H_20 in 5 ml glass vials, homogenized by vortexing. Then, deuterated SCFAs standards were added as internal controls: deuterated acetate, butyrate, propionate and valerate (Cambridge Isotope Laboratories, USA), to a final concentration of 0.4 mM each. Finally, 400 µl of 50% H_2SO_4 and 800 mg NaCl were added. This mixture was resuspended and 1 ml of ethyl acetate was added as an extraction solvent. Samples were stirred for 1 h at 300 rpm and 25 ºC, and centrifuged for 5 min at 3500 rpm. 500 µl of supernatants were transferred to a new vial. This extraction was repeated twice.

The GC-MS equipment was an Agilent 7890A (Agilent Technologies) equipped with an inert XL MSD with a triple-Axis detector. Acquisition was done using Chemstation software. The capillary chromatographic column was DB-FFAP (30m, 0.25 mm ID, 0.25 µm film thickness). Helium was used as the carrier gas at 1 mL/min. Injection was made in splitless mode with an injection volume of 1 µL and an injector temperature of 200 ºC. A glass liner with a glass wool plug at the lower end of the liner was used to avoid the contamination of the GC column with nonvolatile fecal material. A blank sample was inserted between experimental samples to check for memory effects.

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The column temperature, initially 50 ºC (1 min), was increased to 150 ºC at 5 ºC/min and, finally, to 230 ºC at 15 ºC/min (total time 20 min). The temperature of the ion source, the quadrupole and the interface were 230 ºC, 150 ºC and 220 ºC, respectively. Scanning ions were 45 and 76 m/z for deuterated propionic acid, 45 and 74 m/z for propionic acid, 43 and 73 m/z for isobutyric acid, 63 and 77 m/z for deuterated butyric acid, 60 and 73 m/z for butyric acid, 60 and 87 m/z for isovaleric acid, 63 and 77 m/z deuterated isovaleric acid, 60 and 73 m/z for valeric acid and 60, 73 and 87 m/z for hexanoic acid. Identification of the SCFAs was based on the retention time of standards and with the assistance of the Wiley 7 library.

2.10. GC-MS quantification of fatty acids in meat samples and blood plasma samples

Lipids from blood plasma samples and *biceps femoris* muscle were extracted and methylated using the procedure described by [22]. Fat extracts were methylated in the presence of sulfuric acid and analyzed by gas chromatography. Previously fatty acid methyl ester (FAME) samples were identified by gas chromatography, as described elsewhere [23]. GC-MS was performed using an HP-6890 (Hewlett Packard, Avondale, PA, USA) gas chromatograph, equipped with a flame ionization detector and capillary column (HP-Innowax, 30 m by 0.32 mm ID and 0.25 μm polyethylene glycol-film thickness). A temperature program of 170 ºC to 245°C was used. The injector and detector were maintained at 250 °C. The carrier gas (helium) flow rate was 2 mL/min. For the identification of each fatty acid, pure standards were used (Sigma). The concentration of individual fatty acids was calculated as a % of total fatty acids. The results were expressed as grams per 100 g of detected FAMEs.

2.11. gDNA extraction and 16S rRNA sequencing for metagenomics

gDNA was extracted from 200 mg of frozen (-80 ºC) cecum feces using E.Z.N.A.® DNA Stool Kit (Omega Bio-Tek Ref. D4015-02), producing 200 μl of genomic DNA. gDNA samples were quantified using a BioPhotometer® (Eppendorf) and their concentrations diluted to 6 ng/μl. These diluted samples were used for performing a PCR amplification following the protocol of Ion 16™ Metagenomics kit (Thermo Fischer Scientific).

PCR amplification products were used to create a library using the Ion Plus Fragment Library kit for AB Library Builder™ System (Cat. No.4477597), with sample indexing using the Ion Xpress™ Barcode

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Adapters 1-96 kit (Cat. No. 4474517). Template preparation was performed using the ION OneTouch™ 2 System and the ION PGM™ Hi-Q™ OT2 kit (Cat. No. A27739). Metagenomics sequencing was performed using ION PGM™ Hi-Q™ Sequencing kit (Cat. No. A25592) on the ION PGM™ System. The chips used were the ION 314™ v2, 316™ v2 or 318™ v2 Chips (Cat. No. 4482261, 4483188, 4484355) with various barcoded samples per chip.

2.12. Phylogenetic analysis

The consensus excel table for each metagenomics sequencing was downloaded from ION Reporter 5.6 software. This excel table includes the percentages for each taxonomic level and was used for comparing frequencies between rat individuals and cohorts.

Taxonomic adscription up to species level was performed using the QIIME 2 (v.2017.6.0) open-source bioinformatics pipeline. Analysis of the microbiome community was carried out using R software (v3.2.4): non-supervised multivariate analysis (PCA). For LDA analysis, tab-delimited files were generated in R and computed at family level using Galaxy. Graphical representation of Galaxy output included only discriminative features with logarithmic LDA score higher than 3. The reference library used was the Curated MicroSEQ(R) 16S Reference Library v2013.1; Curated Greengenes v13.5. The number of mapped reads (after the ignored ones due to less than 10 copies) per sample was always over 60.000. Total number of reads was always over 110.000. Counts were normalized by sum scaling.

2.13. Statistical methods

Data were expressed as the mean value ± S.E.M. Statistical analyses were conducted using Student's *t*-test when the quantitative data presented normality and the variances were assumed equal. When the variances were assumed different, the Welch's *t*-test was used. When the quantitative data were not normal, the non-parametric Mann-Whitney U test was used. In the case of qualitative data, the χ^2 test was used. The graphical representation of all these data was generated using GraphPad Prism software, version 7. In all cases, a p value < 0.05 was considered statistically significant (*: $p<0.05$; **: $p<0.005$; ***: $p<0.0005$; ****: $p<0.0001$).

3. Results

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3.1. Nutritional composition comparison of acorn-feed ham and control ham

Both types of cured ham were analyzed with respect to percentages of humidity, total protein, total fat, total chlorides and total ash. The major difference here was in the total fat content, which was slightly higher in acorn-fed ham (21.4%) than in control ham (17.3%) [Table 1]. In the case of rat feed, the fat content was much lower (4%) [Table 1].

With respect to the specific composition of fatty acids for acorn-fed ham, control ham and rat feed, the main differences between acorn-fed ham and control ham are in the oleic acid content, because the content of this monounsaturated fatty acid is much higher in the acorn-fed ham (51.92%) than in the control ham (39.53%), and more than double in the rat feed (20.58%) [Table 2]. Also, the levels of omega-6 fatty acids, considered pro-inflammatory compounds, are double in the control ham (20.84%) than the in acorn-fed ham (11.74%), and almost 6 times higher in rat feed (58.82%). This omega-6 content difference is responsible for a much lower omega-6/omega-3 ratio in acorn-fed ham (9.97) as compared to the control ham (17.56), since omega-3 content is similar in both hams [Table 2].

Finally, both types of ham were analyzed for their nitrate and nitrite content. The nitrate concentration in the acorn-fed ham was 12.5 times lower (15.04 ppm) than in the control ham (188.67 ppm) [Table 1]. This substantial difference is due to the fact that neither nitrates nor nitrites are added as food preservatives in manufacturing acorn-fed ham.

3.2. Effect of acorn-feed ham on body weight and disease activity index

In all three cohorts, the animals' body weight was affected by DSS treatment [Figure 1]. In the feed cohort, 4 of the UC animals did not recover body weight after finishing the DSS treatment [Figure 1A]. In fact, these same 5 animals were the ones that later, after sacrifice, showed a higher disease activity index in the colon mucosa (degrees 3 and 4) [Figure 1E].

In the control ham cohort, 3 animals died at the beginning of the DSS challenge and another 3 animals died at the end of this treatment [Figure 1B]. These 6 deaths were due to colon hemorrhages during UC onset. Only 2 rats in control ham cohort survived until sacrifice (C1 and C5 rats, disease activity index degrees 2 and 4), apart from the 2 absolute control rats (C9 and C10). Therefore, during the rest of the study, and especially when comparing biological samples, most of the statistical analyses were

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carried out between the acorn-fed ham cohort and the feed cohort, since introducing data from the only 2 surviving animals from the control ham cohort was not appropriate.

In the acorn-fed ham cohort, weight gain slowed slightly during DSS treatment, but this parameter was recovered after the treatment ended. This recovery happened in all 8 animals [Figure 1C].

Finally, the absolute control rats for all cohorts (feed, control ham and acorn-fed ham) maintained a continuous and normal weight gain along the experimental weeks and they showed an UC disease activity index of 0, as expected [Figure 1D].

3.3. Effect of acorn-feed ham on colon histological measurements

Statistically significant differences were observed between the acorn-fed ham cohort and the feed cohort with respect to the histological measurements assessed. The macroscopic damage score of UC in the acorn-fed ham cohort was 0.12 and 2.75 in the feed cohort [Figure 2A]. The colon epithelium alteration score was 2.50 in the acorn-fed ham cohort and 3.50 in the feed cohort [Figure 2B], and the inflammatory cell density in colon mucosa score was 1.50 in the acorn-fed ham cohort and 2.500 in the feed cohort [Figure 2C]. Histology studies on colon mucosa revealed that only acorn-fed ham animals had recovered a proper colon mucosa epithelium [Figure 3].

With respect to the myeloperoxidase assay (MPO), mean myeloperoxidase levels in the colon mucosa from the acorn-fed ham rats were much lower (0.13) than in the feed cohort (1.76), and this difference was statistically significant [Figure 2D].

The three other parameters associated with colon histological studies did not show statistically significant differences between the acorn-fed ham and the feed cohorts. These parameters were the reduction of colon length (which is associated with UC severity) [Figure S1A], the presence of reparative changes in colon mucosa (which indicates tissue recovery after colon mucosa ulceration) [Figure S1B] and the number of hyperplastic Peyer's patches in the small intestine [Figure S1C]. And finally, the Evans blue assay was also carried out with no statistically significant differences in colon permeability observed between the cohorts [Figure S1D].

3.4. Effect of acorn-feed ham on blood total antioxidant capacity and cytokine levels on DSS-treated animals

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The sacrificed acorn-fed ham cohort rats (one week after finishing the DSS treatment) showed a much higher total antioxidant capacity (FRAP) in the blood plasma (453.82 μM Trolox equivalent) than the feed cohort rats (315.41 μM Trolox equivalent), and this difference was statistically significant [Figure 2E].

In terms of cytokines, the main differences observed between the feed cohort rats and the acorn cohort rats were in the levels of IL-17, IFN-γ and TGF-β, though statistically significant differences were obtained only in the cases of the pro-inflammatory IL-17 (3.62 pg/mL mean value in the acorn-fed ham cohort and 15.92 pg/mL mean value in the feed cohort) [Figure 2F] and IFN-γ (173.81 pg/mL mean value in the acorn-fed ham cohort and 221.96 pg/mL mean value in the feed cohort) [Figure 2G].

3.5. Effect of acorn-feed ham on short-chain fatty acids concentrations in feces of DSS-treated animals

Various short-chain fatty acids (SCFAs) were measured by GC-MS in cecum feces collected after sacrifices. These SCFAs were propionic, butyric, isobutyric, valeric, isovaleric and hexanoic acids, which are known compounds involved in colon homeostasis and health. In these quantifications, deuterated standards were used for measurements (see materials and methods section). Statistically significant differences were observed for some of these SCFAs, with higher concentrations in the acorn-fed ham cohort in the cases of isobutyric acid (mean values of 2.06 mM in the acorn-fed ham cohort and 1.62 mM in the feed cohort) [Figure 4A], isovaleric acid (0.0098 mM in the acorn-fed ham cohort and 0.0023 mM in the feed cohort) [Figure 4B], and valeric acid (0.14 mM in acorn-fed the ham cohort and 0.063 mM in the feed cohort) [Figure 4C]. No statistically significant differences were observed with respect to the propionic acid levels between the cohorts [Figure 4D]. Finally, the butyric acid mean value was higher in the feed cohort (0.98 mM) than in the acorn-fed ham cohort (0.32 mM) [Figure 4E].

3.6. Effect of acorn-feed ham on fatty acids concentrations in blood plasma

Table 3 shows the percentages for each fatty acid in the blood plasmas of the acorn-fed ham cohort rats and feed cohort rats. In accordance with the type of food in each case, animals from the acorn-fed ham cohort showed higher plasma levels of the monounsaturated fatty acid oleic acid (31.61%). This

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value was double the oleic acid content in the feed cohort rats (16.21%), and the difference was statistically significant [Figure 4F].

In contrast, the plasma content of the omega-6 fatty acid linoleic acid was much higher in the feed cohort animals (20.05%) than in the acorn-fed ham cohort (10.56%) [Table 3], and this difference was also statistically significant [Figure 4I]. As this is the main omega-6 fatty acid present in these blood plasmas, this difference resulted in a higher total omega-6 plasma content in the feed cohort animals (33.64%) as compared to the acorn-fed ham cohort (21.79%). It also caused the omega-6/omega-3 ratio in the feed cohort animals to be considerably higher (36.67) than in the acorn-fed ham cohort (24.07) [Table 3]. Both these differences, the total omega-6 content and the omega-6/omega-3 ratio, were statistically significant [Figure 4G and 4H].

3.7. Effect of acorn-feed ham on intestinal microbiota

The main difference at the phylum level between the three sequenced cohorts in comparison to the absolute control rats (those ones lacking the DSS challenge) is that both ham diets showed a similar increase in *Bacteroidetes* (44% in both ham cohorts versus 13% in feed cohort) and *Proteobacteria* populations (21% in both ham cohorts versus less than 1% in feed cohort), and a similar decrease in *Firmicutes* (34% in both ham cohorts versus 84% in feed cohort) [Figure 5], with respect to feed cohort animals. The distribution of these phyla in all the rats treated with DSS was similar to their distribution in the absolute control animals of each cohort, with the exceptions of the F4 rat (and, to a lesser extent, the F3 rat) of the feed cohort, which showed a deep dysbiosis [Figure 5].

At the family level, in general, the composition found in both ham cohorts was similar, though different from that of the feed cohort animals [Figure 5D]. The acorn-fed ham cohort animals showed a relatively higher proportion of *Coriobacteriaceae* (*Actinobacteria*), *Bacteroidaceae* and *Rikenellaceae* (*Bacteroidetes*), *Desulfovibrionaceae* and *Sutterellaceae* (*Proteobacteria*), *Staphylococcaceae*, *Eubacteriaceae* and *Erysipelotrichaceae* (*Firmicutes*). The acorn-fed animals showed a lower proportion of *Prevotellaceae* (*Bacteroidetes*), *Ruminococcaceae*, *Lachnospiraceae* and *Lactobacillaceae* (*Firmicutes*) in comparison with the feed cohort animals [Figure 5D and S2]. The main exception to this similarity between both ham cohorts is the presence of 11% *Enterococcaceae* only found in the two animals of control ham cohort that survived the DSS challenge [Figure 5D and S2].

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353 The animals treated with DSS from each cohort showed a family distribution similar to that of their
354 counterparts without UC induction. The exception, again, was for rats F3 and F4 from the feed cohort
355 [Figure 5D]. It is also worth noting the significant presence (13% overall) of the family *Enterococcaceae*
356 in the two surviving animals from the control ham cohort after the UC challenge. The proportion of
357 this family in one of the control animals of the same cohort is around 1%, and in all other animals in
358 the experiment, this family is minimally present or totally absent.

359 PCA of gut microbiota composition divided the animals in two clusters, indicating differences in the
360 gut microbiota composition associated to both dietary interventions, feed and acorn-fed ham diets
361 (Figure 6A). Bacterial families with significant differences in their relative abundances between the
362 feed and acorn-fed ham cohorts are indicated in the LDA analysis (Figure 6B): in total, 32 families
363 explain in a significant way both types of diet.

364

365 **4. Discussion**

366 High doses of processed meat products are not recommended in a healthy diet, especially not for UC
367 patients. However, some traditional cured meat products, such as acorn-fed ham, contain very high
368 levels of the monounsaturated fatty acid, oleic acid, an anti-inflammatory fatty acid. Furthermore,
369 acorn-fed ham has a lower omega-6/omega-3 ratio than control ham and rat feed [Table 2]. The roles
370 of omega 3 and omega 6 fatty acids in DSS-induced UC are not simple and may be influenced by a
371 number of variables. They have been studied extensively, and although in some cases no difference
372 has been found in terms of the protective role of short-term omega 3 dietary fish oil supplementation
373 versus omega 6, in other animal models omega 3 exacerbated the induced UC initially due to a
374 reduction in adiponectin expression in subepithelial myofribroblasts [24,25]. However, most of the
375 published results point to the anti-inflammatory nature and protective role of the oleic acid and the
376 omega-3 fatty acids against UC. In the case of omega-3 these protective effects are due, in part, to the
377 production of the anti-inflammatory resolvins and reduced titers of TNFα and LTB_4 leukotrienes
378 [8,12,15,17,18,26,27]. Given the different levels of these fatty acids in the acorn-fed ham compared to
379 other meats, the goal of the study was to test if a diet based on a Mediterranean traditional meat

product with a high content of the anti-inflammatory oleic acid and a low omega-6/omega-3 ratio (due to a lower level of the pro-inflammatory polyunsaturated omega-6) would aid in diminishing the UC symptoms in a rat animal model for this disease. Recently, several bioactive peptides generated in cured ham and other fermented meats (*chorizo* sausages) have been associated to beneficial effects, such as antioxidant and cardioprotective ones [28]. The objective here was to demonstrate the benefits of maintaining acorn-fed ham as part of a traditional Mediterranean diet, regarding its protective effects against UC, as an example of a common inflammatory gut condition.

To assess the potential effects of these three diets on UC, once the animals were sacrificed, three histological parameters were studied: macroscopic damage score assessment, colon epithelium alteration and inflammatory cell density in colon mucosa [Figure 2A, 2B and 2C]. In all three cases, statistically significant differences were observed between the acorn-fed ham cohort and the feed cohort. All three parameters indicate the extent of the colon mucosa damage and the pro-inflammatory status and all three were clearly lower in the colons from the acorn-fed ham cohort rats. This indicated either that the acorn-fed ham diet helped prevent damage to the colon mucosa caused by DSS treatment or that the acorn-fed ham diet enhanced the recovery of the affected colon mucosa. A plausible explanation for this is the known anti-inflammatory effect of oleic acid, which is abundant only in the acorn-fed ham, as well as its low omega-6/omega-3 ratio [17,18]. These two parameters support the idea that maintain the traditional acorn-fed ham in a normal diet can provide important anti-inflammatory benefits to the gut health, without the need of drugs nor dietary supplements.

Further histological data regarding the myeloperoxidase levels in the colon mucosa also demonstrated this lower pro-inflammatory status in the acorn-fed ham rats. The myeloperoxidase test is usually carried out in UC studies because it serves as a quantitative method for identifying the presence of infiltrated granulocytes in the colon mucosa, a type of immune cell. The higher the myeloperoxidase value, the higher the pro-inflammatory status of the mucosa [29–31]. An analysis of these levels in the two cohorts revealed a statistically significant reduction in the myeloperoxidase levels in the acorn-fed ham rats [Figure 2D]. In the same way, a statistically significant higher UC disease activity index (DAI) was measured in the feed cohort (4) than in the acorn-fed ham cohort (0.87) [Figure 1E]. Along with the histological data, several blood plasma parameters were analyzed

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in both surviving cohorts. First, the total antioxidant capacity, measured with the FRAP method, was found to be higher in the acorn-fed ham rats, and this difference between the two cohorts was statistically significant [Figure 2E]. This is most likely due to the higher antioxidant composition (higher levels of monounsaturated and polyunsaturated fatty acids) of acorn-fed ham with respect to feed. Two pro-inflammatory cytokines were also less present in the acorn-fed ham cohort plasma with respect to the feed cohort plasma. These were IL-17 and IFN-γ [Figure 2F, 2G]. These immunological parameters present a biochemical explanation for the lesser damage observed in the colon mucosa of the acorn-fed ham cohort animals [Figure 2].

Similarly, considerable differences were observed in the fatty acid content of the blood plasmas [Table 3]. As expected from a diet rich in oleic acid, the acorn-fed ham cohort animals showed double the amount of oleic acid in their blood plasma [Figure 4F], lower omega-6 content and a lower omega-6/omega-3 ratio [Figure 4G and 4H]. All these parameters indicated a lower pro-inflammatory status in the acorn-fed ham animals, which was also clearly observed at the histological level, as described above.

Another parameter analyzed was the cecal content. Since it is in this organ that fermentation processes are carried out by its microbiota [32]. this analysis allows the identification of metabolic differences associated with the digestion of the three diets in the cecum of the animals [33–35].]. In the end, only three of the SCFAs analyzed were found in a higher concentration in the fecal cecum content of the acorn-fed ham cohort animals. These were isobutyric acid, isovaleric acid and valeric acid [Figure 4]. A canonical explanation for the absence of important quantities of butyric acid in the acorn-fed ham cohort animals' cecum (0.32 mM with respect to 0.98 mM in feed cohort rats) is the fact that ham diets do not supply fiber content [Table 1], the nutrient that is fermented by cecum microbiota to generate this SCFA [35,36].

With respect to gut microbiota changes, the case of the F3 and F4 rats is unique. Though all of the feed cohort animals survived after the DSS challenge, two of them, the F3 and especially the F4 rat, were in critical condition one week after the end of the treatment (DAI score 7 and 8 respectively) [Figure 1E]. These two rats lost between 21% and 25% of the body weight with respect to week 1 [Figure 1A]. Also, it is worth noting that the F3 and F4 rats lost this body weight in the week following the

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436 withdrawal of DSS from the drinking water [Figure 1A], i.e., during the expected period of recovery,
437 which indicated a bad prognosis.

438 The profile of the intestinal microbiota of these two animals (F3 and F4 rats) showed a dramatic
439 alteration at all taxonomic levels examined (especially in the F4 rat) in comparison with the other
440 animals from the feed cohort [Figure 5D]. At the phylum level, the F4 rat showed 52% *Bacteroidetes*,
441 38% *Firmicutes* and 9% *Proteobacteria* (*Firmicutes*/*Bacteroidetes* ratio of 0.7), while the other rats from the
442 feed cohort showed, on average, 13% *Bacteroidetes*, 86% *Firmicutes* and 0.5% *Proteobacteria*
443 (*Firmicutes*/*Bacteroidetes* ratio of 6.6). This indicated a deep gut dysbiosis in the F3 and F4 rats with
444 respect to the other animals in the feed cohort. In fact, this phylum distribution for the F3 and F4 rats
445 was very similar to the animals from the acorn-fed ham cohort (44% *Bacteroidetes*, 34% *Firmicutes* and
446 21% *Proteobacteria*, *Firmicutes*/*Bacteroidetes* ratio of 0.8). This similarity may indicate that the
447 *Firmicutes*/*Bacteroidetes* ratio is probably not sufficient to express the health status of the individual.
448 This is evidenced by the fact that, although both types showed the same *Firmicutes*/*Bacteroidetes* ratio,
449 the F4 rat was in critical condition but the acorn-fed ham cohort animals recovered and thrived.

450 Nevertheless, even though there were more similarities than differences in the relative proportions of
451 most families present in the F4 rat microbiota as compared to the average values of the acorn-fed ham
452 animals, significant differences were found at the genus and species levels. For example, the relative
453 proportion of the *Parabacteroides* genus was the same, 4.5%, in the F4 rat and in the acorn-fed ham
454 cohort animals. But while the distribution at the species level in the acorn-fed ham cohort animals
455 was 2.8% *Parabacteroides distasonis*, 0.7% *P. goldsteinii* and 0.01% *P. merdae*; in the F4 rat these values
456 were 0.7% *P. distasonis*, 0.5% *P. goldsteinii* and 3% *P. merdae*. It is perhaps this distinct distribution at
457 the species level that differentiates a sick animal (such as the F3 and F4 rats) from a healthy one (such
458 as those in acorn-fed ham cohort).

459 Similarly, the acorn-fed ham cohort animals showed a very different taxa distribution in their
460 intestinal microbiota from that of the feed cohort rats [Figure 5 and S2].

461 The significance of all these changes is difficult to determine. Likewise, it is difficult to establish the
462 most relevant taxa that were favored with the acorn-fed ham diet, which could be involved in
463 protecting against the DSS challenge. For example, *Bacteroides vulgatus* (*Bacteroidaceae* family) [Figure

464 5D and S2] has a relatively high presence in the gut microbiota of the acorn-fed ham cohort animals
465 (7.7%) compared with the feed cohort rats (0.4%). Additionally, several studies have found that it is
466 more commonly present at higher levels in healthy human controls than in UC or IBD patients and it
467 can provide different types of protection against UC [36–41] However, sialidase activity from *B.*
468 *vulgatus* mediates the release of sialic acid from intestinal tissue, driving intestinal inflammation and
469 microbial dysbiosis in mice after DSS administration [40].

470 *P. distasonis* (*Bacteroidetes* phylum, *Porphyromonadaceae* family, Figure 5D) has a greater presence in the
471 acorn-fed ham cohort rats (4%) than in the feed cohort rats (0.05%). As in the case of *B. vulgatus*,
472 opposing roles have been assigned to this species in the development of UC: as a reducer of intestinal
473 inflammation in mice treated with DSS by inducing the anti-inflammatory cytokine IL-10 [41], but
474 also as an enhancer of the inflammatory condition in mutant mice affected in the anti-inflammatory
475 intestinal peptidoglycan recognition proteins (Pglyrps) [42]. Perhaps the protective role of *P. distasonis*
476 requires the presence of these Pglyrps proteins in the intestinal mucosa, that is, homologues to these
477 proteins must be present in the wild type animals (*R. norvegicus*) used in this acorn-fed ham cohort
478 and could help *P. distasonis* achieve an anti-inflammatory effect, since animals from the acorn-fed ham
479 cohort with high numbers of this species have a better health status.

480 As was indicated in the results section, the acorn-fed ham cohort animals showed a great reduction
481 in phylum *Firmicutes* with respect to the feed cohort animals [Figure 5A, 5C]. Two families in this
482 phylum showed the largest reductions in the acorn-fed ham cohort: *Lachnospiraceae* (from 47% to 10%)
483 and *Ruminococcaceae* (from 12% to 2%) [Figure 5D and S2]. Both families include numerous species
484 with the ability to synthesize anti-inflammatory SCFAs (such as butyrate or propionate) from various
485 polysaccharidic prebiotic fibers [43,44]. These fibers are present in the feed diet (22.1%) but totally
486 absent in the two types of ham diets, even though other components in the ham matrix are able to
487 generate diverse SCFAs via gut microbiota, such as isobutyrate, isovalerate, valerate and propionate,
488 which are readily detected in these animals. Therefore, the absence (or reduced populations) of these
489 two families in the acorn-fed ham animals should facilitate the development of UC in these rats.
490 However, this was not the case and other factors were probably involved in the lower DAI for UC
491 seen in these animals [Figure 1E]. Within *Lachnospiraceae*, the populations of the mucolytic bacteria

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492 *Ruminococcus gnavus* were also reduced in the acorn-fed ham cohort animals (from 17.5% in feed
493 cohort rats to 1.5%). This bacterium has been reported to be more prevalent and more abundant in
494 CD and IBD patients [45–47] and may play an important role in inducing chronic intestinal
495 inflammation [48].

496 Nonetheless, even with this decrease in *Lachnospiraceae* populations, some genera increased, such as
497 *Blautia* (0.01% in the feed cohort rats to 4% in the acorn-fed ham animals). UC individuals and CD
498 patients have shown a lower abundance of *Blautia* species than their healthy counterparts (Wang et
499 al., 2017). *Blautia* species could maintain gut homeostasis in terms of its ability to produce the anti-
500 inflammatory SCFA propionate [44].

501 With this gut microbiota panorama, the loss of butyrate-synthesizing bacteria that led to a decrease
502 of butyrate in the cecal content [Figure 4E] could be compensated in the acorn-fed ham cohort rats
503 with an increase in microorganisms able to produce isobutyrate, isovalerate and valerate. An increase
504 in these three SCFAs has been observed by GC-MS of the cecal content of the acorn-fed ham cohort
505 animals [Figure 4A, 4B and 4C].

506 The higher proportion of *Bacteroidetes* phylum species found in the acorn-fed ham cohort [Figure 5]
507 could explain the maintenance in SCFAs production. Some of these genera can produce butyrate, such
508 as *Butyricimonas* (0.04% in the feed cohort to 1.2% in the acorn-fed ham animals), [51], but most
509 members of this phylum are mainly propionate producers [44]. *Alistipes* genus (*Rikenellaceae* family)
510 [Figure 5D and S2], for example, was undetectable in the feed cohort animals, but accounted for 1.6%
511 total bacteria in the acorn-fed ham cohort. Several studies have linked the presence of *Alistipes* genus
512 with a healthy state [52]. Accordingly, a decrease in this genus has been associated with inflammatory
513 processes [53]. More direct proof of its protective role in the development of UC was observed in the
514 attenuation of DSS-induced UC in mice after gavage with an *Alistipes* strain In addition to its ability
515 to synthesize SCFAs, succinate is also a significant end product of *Alistipes* metabolism, and this may
516 stimulate SCFAs production by other commensal microorganisms in the gut through the succinate
517 pathway [54]. For example, *Phascolarctobacterium* (*Acidaminococcaceae* family, *Firmicutes*) [Figure 5D
518 and S2] is a succinate-utilizing propionate-producer bacterium with an undetectable presence in the
519 feed cohort rats, but accounting for 0.7% in the acorn-fed ham animals [55].

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Another notable difference between the gut microbiota of the feed and acorn-fed ham cohorts, not related to SCFAs production, is the presence of the bacteria *Bilophila wadsworthia* (*Desulfovibrionaceae* family, [Figure 5D and S2]). Although this *Proteobacteria* occurs in the intestinal microbiota of healthy humans [56], it has been found to be frequently associated with inflamed appendices in children and adults and it can be considered an opportunistic pathogen [57]. Mouse models have also found evidence that IBD can be caused by *B. wadsworthia*. The susceptibility to *Helicobacter hepaticus*-induced colitis differed considerably between IL10$^{-/-}$ mutant mice originating from two different institutions, and this was associated with significant differences in *B. wadsworthia* gut populations [58]. A milk-derived, high saturated fat diet can markedly promote the flourish of *B. wadsworthia* in the gut, and these intestinal blooms of *B. wadsworthia* can lead to significantly increased UC in IL10$^{-/-}$ mutant mice, but not in wild type animals [59,60].

In the present study, the presence of *B. wadsworthia* in the feed cohort rats was undetectable, with the only exception being the dysbiotic F4 rat (1.3%), which was in poor condition. On the contrary, all animals from both ham cohorts, treated with DSS or not treated, showed between 10% to 26% *B. wadsworthia* populations (an average value of 16.5%). These data are in accordance with a previous work which showed that short-term consumption of a diet based only on animal products changed microbial community structure and increased the abundance of *B. wadsworthia* in the human gut [61]. More recently, oral administration of *B. wadsworthia* to specific-pathogen free mice resulted in the reduction of body weight and fat mass, apparent hepatosplenomegaly and elevated serum inflammatory factors, including serum amyloid A protein and IL-6, indicating a systemic inflammatory response [62].

On the contrary, in this study acorn-fed ham animals showing high *B. wadsworthia* populations had a lower DAI than feed cohort rats. In these acorn-fed ham animals, weight gain and recovery were better after the DSS challenge [Figure 1C]. Also, their pro-inflammatory cytokines plasma levels (such as TNF-α and IL-6) were not statistically different, indicating that more factors than only the presence of *B. wadsworthia* are required for the development of UC in this animal model.

A total of 32 bacterial families explain the main differences in microbiota composition between feed and acorn-fed ham cohorts. *Veillonellaceae, Neisseriaceae Peptococcaceae, Rhodospirillaceae* and other

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548 families [Figure 6B] better describe the effect of acorn-fed diet on gut microbiota composition, whereas
549 *Corynebacteriaceae*, *Marinifilaceae*, *Enterococcaceae* and other ones better define the microbiota
550 associated to feed diet [Figure 6B].

551 Apart from the microbiota, a noteworthy development during the experiment was the fact that 6 out
552 of 8 animals in the UC-induced rats from the control ham cohort died due to UC hemorrhages and
553 colon damages during DSS challenge [Figure 1B]. It is also worth noting that the considerable
554 difference in the growth rate of the absolute control animals (those lacking the DSS challenge) in the
555 three cohorts. On average, at the end of the three weeks experiment the acorn-fed ham cohort rats had
556 a 44% weight gain, while control ham cohort rats showed just a 13% weight gain, even less than the
557 feed cohort animals (32% weight gain) [Figure 1D].

558 The best explanation for these results is the presence of a significant amount of nitrates (189 ppm)
559 used as preservatives in the control ham [Table 1]. Nitrates and nitrites are common additives used
560 in meat and other food products. Due to their binding to muscle myoglobin, these chemicals are used
561 to provide the attractive deep red color of the meat in accordance with consumer habits and demand
562 [63]. But more importantly, they provide protection against food contamination and spoilage caused
563 by anaerobes (as *Clostridium* species), which make them essential substances with respect to consumer
564 health, preventing, for example, botulism outbreaks [64]. However, nitrates are not added to acorn-
565 fed ham during its manufacturing, and this traditional ham only contains 15 ppm (12.5 times less
566 nitrates than control ham) [Table 1]. The reduction of nitrates into the more toxic nitrites by intestinal
567 microbiota species is a well-known cause of poisoning in cattle, where nitrites induce methemoglobin
568 oxidation in erythrocytes, giving rise to methemoglobinemia [65]. High dietary nitrate concentrations
569 have been reported as putative agents leading towards thyroid disease, different cancer types (ovary,
570 bladder, colorectal, etc.), birth neurological defects and liver injury [66,67]. Also, it is known that
571 chronic nitrite ingestion acts as a toxic agent for the kidneys, intestines, liver, lungs and other organs,
572 and even as a pro-carcinogenic compound [68]. In only one ulcerative colitis mouse model study,
573 dietary nitrite has shown preventive and therapeutic effects on this disease (symptoms amelioration)
574 [69]. In other studies, nitrate ingestion is associated with cardioprotective effects. Also, patients
575 suffering active UC episodes showed increased levels of serum nitrate, in comparison with inactive

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UC individuals. Nitrate in these patients is an end-product of NO formation in the intestinal tissue, a pro-inflammatory mediator generated by macrophages, neutrophils and other cell types [70]. This pathophysiology may suggest that, in our rat model for UC, two sources of nitrates may be present simultaneously, causing the observed animal deaths. On the one hand, there is the ingested nitrate (from the control ham diet), which renders nitrite after its reduction by intestinal microbiota. And, on the other hand, there are significant concentrations of endogenous nitrate, derived from NO formation at intestinal mucosa (triggered by autoimmune cellular mechanisms), which will render nitrite in a similar way in this organ due to intestinal microbiota activity. It seems that in the control ham cohort, the extra doses of diet nitrate (and therefore nitrite formation) generated increased inflammatory stress (and in this case methemoglobinemia), causing these observed deaths.

In this work, indirect proof of nitrite formation from nitrates (nitrate reductase activity) can be deduced by observing the proportions of gut microorganisms able to carry out this chemical reduction. Previous studies from other authors have identified genomes associated with intestinal microbiota phylogenetic groups already known for the presence of nitrate reductase activity [71]. These studies showed that this enzymatic activity was predicted to be present in the majority of genomes belonging to the *Actinobacteria* phylum, as well as in two classes from *Proteobacteria* phylum (*Betaproteobacteria* and *Gammaproteobacteria*), with highest frequency within the *Enterobacteriaceae* family from *Gammaproteobacteria*. The percentage of these taxonomic groups was three times higher in the control ham cohort rats (13.7%) than in the acorn-fed ham cohort animals (4.7%) [Figure S3]. This difference could be due to the greater amount of nitrate present in control ham [Table 1], which would favor the growth of these nitrate-reducing gut bacteria and the formation of toxic amounts of nitrite in these control ham cohort rats. When these control ham cohort animals were exposed to the DSS challenge, most of them could not overcome the extra intestinal pro-inflammatory damage and died.

However, two out of the eight animals (C1 and C5 rats) survived in this control ham cohort [Figure 1B]. When their gut microbiota was compared with that of the control ham cohort rats without DSS challenge (C9 and C10 rats), the most striking difference was the relative amount of the *Enterococcus* genus, with a value of 12.6% in C1 and C5 rats, while in the animals lacking DSS challenge (C9 and

C10) it represented only 0.6% of total bacteria [Figure 5D]. The high presence of *Enterococcus* genus in the control ham cohort animals, in contrast with the other two cohorts where this genus represents less than 0.05%, could be due to the ability of these bacteria to grow (obtain energy) through nitrite reduction [72] after feeding with control ham. *Enterococcus* species are known for their metabolic reduction of nitrite to NO via the denitrification pathway (*nirK* and *nirS* genes) [72]. The proliferation of this nitrite-reducing genus could explain the survival of the C1 and C5 rats during the DSS challenge due to the probiotic properties of *Enterococcus* species and the demonstrated ability of these strains to suppress the development of DSS-induced experimental colitis [73–76], as has been evidenced with *Enterococcus faecalis* and *E. durans* This animal model has shown that a high dietary intake of meat products containing high nitrate content may be a reason for chronic (and in this case lethal) toxicity by nitrite formation, due to the intestinal reduction of nitrates. However, dietary supplementation with some *Enterococcus* species could be used to counteract sporadic nitrite intoxications in humans.

There is also a striking number (5.8%) of the *Peptostreptococcaceae* family in one of the surviving rats from control ham cohort (rat C5, Figure 5D). This family is present in values lower than 1% in all other animals. However, its role here is difficult to determine due to the contradictory character assigned to this family in the literature, either as an actor in different pathologies [77,78] or as a keeper of the gut homeostasis [79,80].

In conclusion, the acorn-fed ham diet changed the rats gut microbiota due to the different carbohydrate/protein content of the food ingested. The lower carbohydrate and higher protein content in the acorn-fed ham diet led to a decrease in saccharolytic *Firmicutes* species and to an increase in proteolytic *Bacteroidetes* and *Proteobacteria* [Figure 5]. This dysbiosis caused less butyrate-producing strains, but more isobutyrate, isovalerate and valerate producers, such that total SCFAs amounts in both cohorts were similar, including similar propionate producers [Figure 4]. Several other beneficial properties from the increased strains in the acorn-fed ham cohort contributed to maintain an appropriate gut homeostasis and to facilitate the recovery of these animals after the DSS challenge. These beneficial properties may include the fact that some intestinal microbiota species may flourish under inflammatory conditions in this organ, independent of their metabolic role, or

lack thereof, in the onset of that condition. In a similar way, some of the taxons which have been detected in higher amounts in the healthier animals (that is, those from acorn-fed ham cohort) may secrete or possess metabolites or proteins able to ameliorate inflammation conditions [81], apart from the proven anti-inflammatory effect of oleic acid.

As a second conclusion, the healthy fatty acid composition of the acorn-fed ham, with very high levels of the anti-inflammatory oleic acid and a low omega-6/omega-3 ratio, may serve as a prevention strategy for UC onset or progression, as it has been demonstrated in this animal model. Furthermore, along with oleic acid, other acorn-fed ham components may also provide direct or indirect intestinal microbiota modulation, enhancing the protective role of this nutraceutical. Therefore, in humans, a normal diet containing acorn-fed ham, like in this animal model, increases oleic acid body concentration (such as in plasma), and the higher levels of this healthy fatty acid could promote an anti-inflammatory effect at the gut level, helping to reduce UC symptoms. Future clinical studies in humans would be necessary to confirm the findings of this UC animal model

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906 **Figure Legends**

907 **Figure 1. Effect of acorn-fed ham on body weight and disease activity index (DAI). A**, percentage
908 of body weight reduction in the feed cohort; **B**, in the control ham cohort; **C**, in the acorn-fed ham
909 cohort; and **D**, in the absolute control rats (those lacking the DSS challenge). Data were taken every
910 week during the UC experiment. DSS treatment (UC status) took place between days 7 and 14 of the
911 experiment, and those days are the ones represented on graphics. **E**, disease activity index (DAI) for
912 the feed (4 ± 0.84), control ham (3 ± 1) and acorn-fed ham (0.87 ± 0.29) cohorts. DAI is the sum of two
913 parameters: body weight loss (0, more than 5% body weight gain; 1, less than 5% body weight gain
914 and less than 5% body weight loss; 2, from 5 to 10% body weight loss; 3, from 10 to 20%% body weight
915 loss; 4, more than 20% body weight loss); stool consistency (0, normal feces; 1, loose stool; 2, watery
916 diarrhea; 3, slimy diarrhea with little blood; 4, severe watery diarrhea with blood).

917

918 **Figure 2. Effect of acorn-fed ham on colon histological measurements, blood plasma total**
919 **antioxidant capacity and cytokines levels.** Circles and squares indicate the corresponding value or
920 score for each rat. **A**, macroscopic damage score assessment of UC: 1, no ulceration and local
921 hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation in only one site; 4, two
922 or more ulceration and inflammation sites; 5, ulceration bigger than 2 cm; value 6 to 11, one score
923 point per each 1 cm of extra ulceration. The macroscopic score assessment of UC was much lower
924 (0.12) in the acorn-fed ham cohort than in the feed cohort (2.75), and this difference was statistically
925 significant. **B**, colon epithelium alteration score: 0, no alteration; 1, focal loss of caliciform cells; 2,
926 extensive loss of caliciform cells; 3, loss of crypts lower than in 50% mucosa surface; 4, loss of crypts
927 in more than 50% mucosa surface and/or polypoid regeneration. The mean score for epithelium
928 alteration in the acorn-fed ham cohort (2.50) was lower than in the feed cohort (3.50) and this
929 difference was statistically significant. **C**, inflammatory cells density in colon mucosa score: 0, no
930 inflammation; 1, mild inflammation; 2, moderate inflammation; 3, severe inflammation. The mean
931 score for colon mucosa inflammatory cells density in the acorn-fed ham cohort (1.50) was lower than
932 in the feed cohort (2.00) and this difference was statistically significant. **D**, myeloperoxidase assay
933 (MPO). The mean value for this immune enzyme was much lower (0.13 MPO units) in the acorn-fed

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ham cohort than in feed cohort (1.76 MPO units), and this difference was statistically significant. **E**, FRAP total antioxidant capacity. The mean value for antioxidant capacity in the acorn-fed ham cohort (453.825 μM equivalent) was much higher than in the feed cohort (315.413 μM equivalent) and this difference was statistically significant. **F**, Mean plasma levels of the pro-inflammatory IL-17 cytokine, which were much lower in the acorn-fed ham cohort rats (3.625 pg/mL) than in the feed cohort rats (15.925 pg/mL), and this difference was statistically significant. **G**, mean plasma levels of interferon-γ (IFN-γ) were lower in the acorn-fed ham cohort rats (173.813 pg/mL) than in the feed cohort rats (221.963 pg/mL) and this difference was statistically significant. There are not statistical significant differences with the control ham cohort due to the lack of statistical power in this cohort (only two animals survived).

Figure 3. Histology studies on colon mucosa stained with hematoxylin and eosin. Magnification 10 x. **A**, Feed cohort, showing moderate inflammation and no re-epithelialization of colon mucosa. **B**, Control ham cohort, showing moderate inflammation and no re-epithelialization of colon mucosa. **C**, Acorn-fed ham cohort, showing mild inflammation and good re-epithelialization of colon mucosa (black arrow).

Figure 4. Effect of acorn-fed ham on short-chain fatty acids concentrations in feces and lipids in plasma. A, isobutyric acid mM concentration in cecum feces. The mean value for isobutyric acid concentration in cecums from the acorn-fed ham cohort (2.063 mM) was higher than in the feed cohort (1.621 mM) and this difference was statistically significant. **B**, isovaleric acid mM concentration in cecum feces. The mean value for isovaleric acid concentration in cecums from the acorn-fed ham cohort animals (0.009853 mM) was higher than in the feed cohort (0.002351 mM) and this difference was statistically significant. **C**, valeric acid mM concentration in cecum feces. The mean value for valeric acid concentration in cecums from the acorn-fed ham cohort animals (0.1439 mM) was higher than in the feed cohort (0.06325 mM) and this difference was statistically significant. **D**, propionic acid mM concentration in cecum feces. The mean value for propionic acid concentration in cecums from the acorn-fed ham cohort animals (0.8546 mM) was higher than in the feed cohort (0.7851 mM), but this difference was not statistically significant. **E**, butyric acid mM concentration in cecum feces. The mean value for butyric acid concentration in cecums from the acorn-fed ham cohort animals (0.3273

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mM) was higher than in the feed cohort (0.9898 mM) and this difference was statistically significant. There are not statistical significant differences with the control ham cohort due to the lack of statistical power in this cohort (only two animals survived). **F,** plasma levels of oleic acid in both rat cohorts, showed as percentage with respect to total plasma fatty acids. The mean value for plasma oleic acid in the acorn-fed ham cohort (31.61%) was higher than in the feed cohort (16.21%) and this difference was statistically significant. **G**, plasma levels of omega-6 fatty acids in both rat cohorts. The mean value for omega-6 fatty acids in the acorn-fed ham cohort (21.79%) was lower than in the feed cohort (33.65%), and also lower than in the control ham cohort and these differences were statistically significant. **H**, plasma omega-6/omega-3 ratio in both rat cohorts. The mean value for the omega-6/omega-3 ratio in the acorn-fed ham cohort (24.07) was lower than in the feed cohort (36.68) and this difference was statistically significant.**I**, plasma linoleic acid in both rat cohorts. The mean value for the linoleic acid in the acorn-fed ham cohort (10.56) was lower than in the feed cohort (20.05) and this difference was statistically significant.

Figure 5. Intestinal microbiota composition (Phyla, Families). Phyla composition (*Verrucomicrobia, Tenericutes, Proteobacteria, Firmicutes, Deferribacteres, Bacteroidetes, Actinobacteria*) for all the surviving animals in this study. **A**, feed cohort animals; **B**, control ham cohort animals; **C**, acorn-fed ham cohort animals. **D**, Families composition for all the surviving animals in this study. F: feed, C: control ham; A: acorn-fed ham

Figure 6. PCA and LDA analyses of gut microbiota composition. A: Gut microbiota PCA cluster analysis, showing that animals belonging to each of the two compared diet cohorts (feed and acorn-fed ham) show very distinctive characteristics. B: LDA analysis showing the families that better discriminate between feed and acorn-fed ham cohorts.

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989 **Tables**

990 **Table 1. Nutritional composition of acorn-feed ham, control ham and rat feed.** Although both hams
991 have a similar protein and ash content, acorn-feed ham fat content is higher (21.4%). Rat feed content
992 is minimal (4%). A major difference is found in the nitrates content, a common preservative added in
993 meat products, which is 15.06 ppm in the case of acorn-feed ham, but 188.67 ppm in the case of control
994 ham. One batch per food was measured.

	HUMIDITY %	PROTEIN %	FAT %	FIBER %	CHLORIDES %	ASH %	NITRATES ppm	NITRITES ppm
ACORN-FEED HAM	38.3	31.1	21.4	0	4.50	5	15.04	0.47
CONTROL HAM	45.1	30.9	17.3	0	5.70	6	188.67	0.54
FEED	6.9	14.3	4	22.1	0.3	4.7	0	0

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998 **Table 2. Fatty acids composition of acorn-fed ham, control ham and rat feed.** The percentages for
999 each fatty acid are shown. The data include saturated fatty acids (such as myristic acid or palmitic
1000 acids), monounsaturated ones (such as oleic acid or palmitoleic acid), omega-6 (such as linoleic acid
1001 or arachidonic acid) and omega-3 (such as α-linolenic or DHA). The relative percentages for saturated,
1002 monounsaturated and polyunsaturated fatty acids are also shown. The omega-6/omega-3 ratio is an
1003 important parameter with respect to healthy properties or a given food. This ratio is shown at the
1004 bottom of the table for the three types of food. One batch per food was measured.
1005

FATTY ACIDS		ACORN HAM %	CONTROL HAM %	FEED %
C14:0	Myristic	1.37	1.28	-
C16:0	Palmitic	20.81	21.52	14.72
C16:1	Palmitoleic	3.25	2.05	-
C17:0	Margaric	0.22	0.44	-
C17:1	Heptadecenoic	0.26	0.35	-
C18:0	Stearic	7.96	11.76	2.94
C18:1	Oleic	51.92	39.53	20.58
C18:2n6	Linoleic	10.26	19.59	58.82
C18:3n3	α-linolenic	0.75	0.87	2.94
C20:0	Arachidic	0.12	0.19	-
C20:1n9	Eicosenoic	1.16	0.86	-
C20:4n6	Arachidonic	1.30	1.03	-
C20:5n3	Eicosapentaenoic	0.08	0.08	-
C22:4n6	Adrenic	0.18	0.22	-
C22:5n3	DPA	0.17	0.11	-

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C22:6n3	DHA	0.18	0.12	-
		100%	100%	100%
SATURATED FA		30.49	35.19	17.66
MONOUNSATURATED FA		56.59	42.78	20.58
POLYUNSATURATED FA		14.09	22.89	61.76
ω-3		1.18	1.19	2.94
ω-6		11.74	20.84	58.82
ω-6/ω-3		9.97	17.56	20.40

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1008 **Table 3. Mean fatty acid levels in the blood plasma of rats belonging to acorn-fed ham and feed**
1009 **cohorts.** The percentages for each fatty acid are shown. The data include saturated fatty acids (such
1010 as myristic acid or palmitic acids), monounsaturated ones (such as oleic acid or palmitoleic acid),
1011 omega-6 (such as linoleic acid or arachidonic acid) and omega-3 (such as α-linolenic or DHA). The
1012 relative percentages for saturated, monounsaturated and polyunsaturated fatty acids are also shown.
1013 The omega-6/omega-3 ratio is an important parameter with respect to health issues of the animal. This
1014 ratio is shown at the bottom of the table for the two types of blood plasma.

FATTY ACIDS		PLASMA LEVELS IN ACORN-FEED HAM COHORT RATS %	PLASMA LEVELS IN FEED COHORT RATS %
C14:0	Myristic	0.67	0.881
C16:0	Palmitic	21.99	25.598
C16:1n7	Palmitoleic	1.51	3.420
C17:0	Margaric	0.59	0.775
C18:0	Stearic	13.40	11.693
C18:1	Oleic	31.61	16.215
C18:1n-7	11-Octadecenoic	5.15	3.928
C18:2n6	Linoleic	10.56	20.052
C18:3n3	α-linolenic	0.73	1.131
C20:0	Arachidic	0.19	0.12
C20:1n9	Eicosenoic	1.02	0.812
C20:4n6	Arachidonic	10.03	12.274
C22:4n6	Adrenic	1.20	1.320
C22:5n3	DPA	0.45	0.512
C22:6n3	DHA	1.10	1.389

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	100%	100%
ω-3	2.28	3.032
ω-6	21.79	33.646
ω-6/ω-3	24.07	36.678

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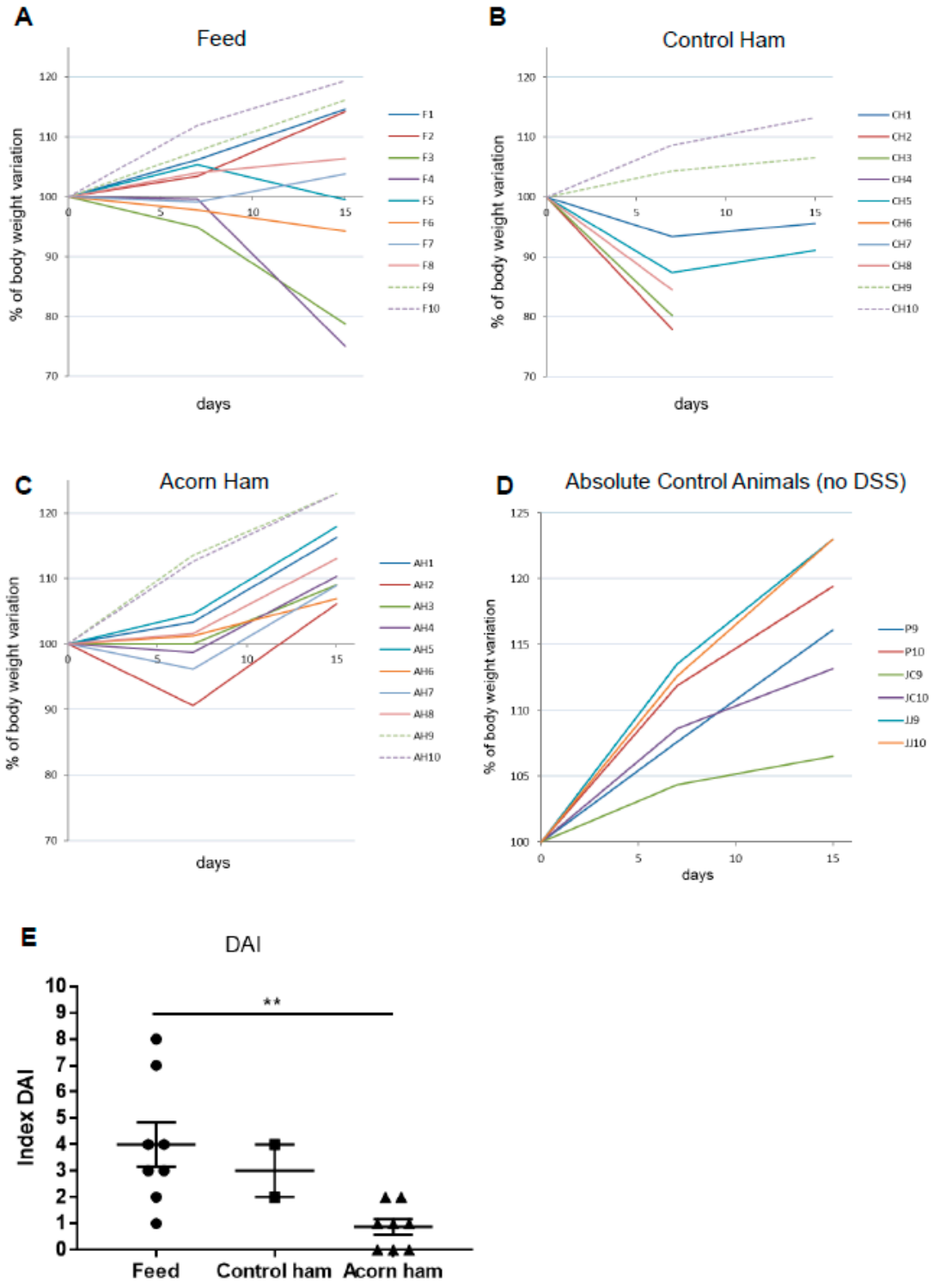

Figure 1

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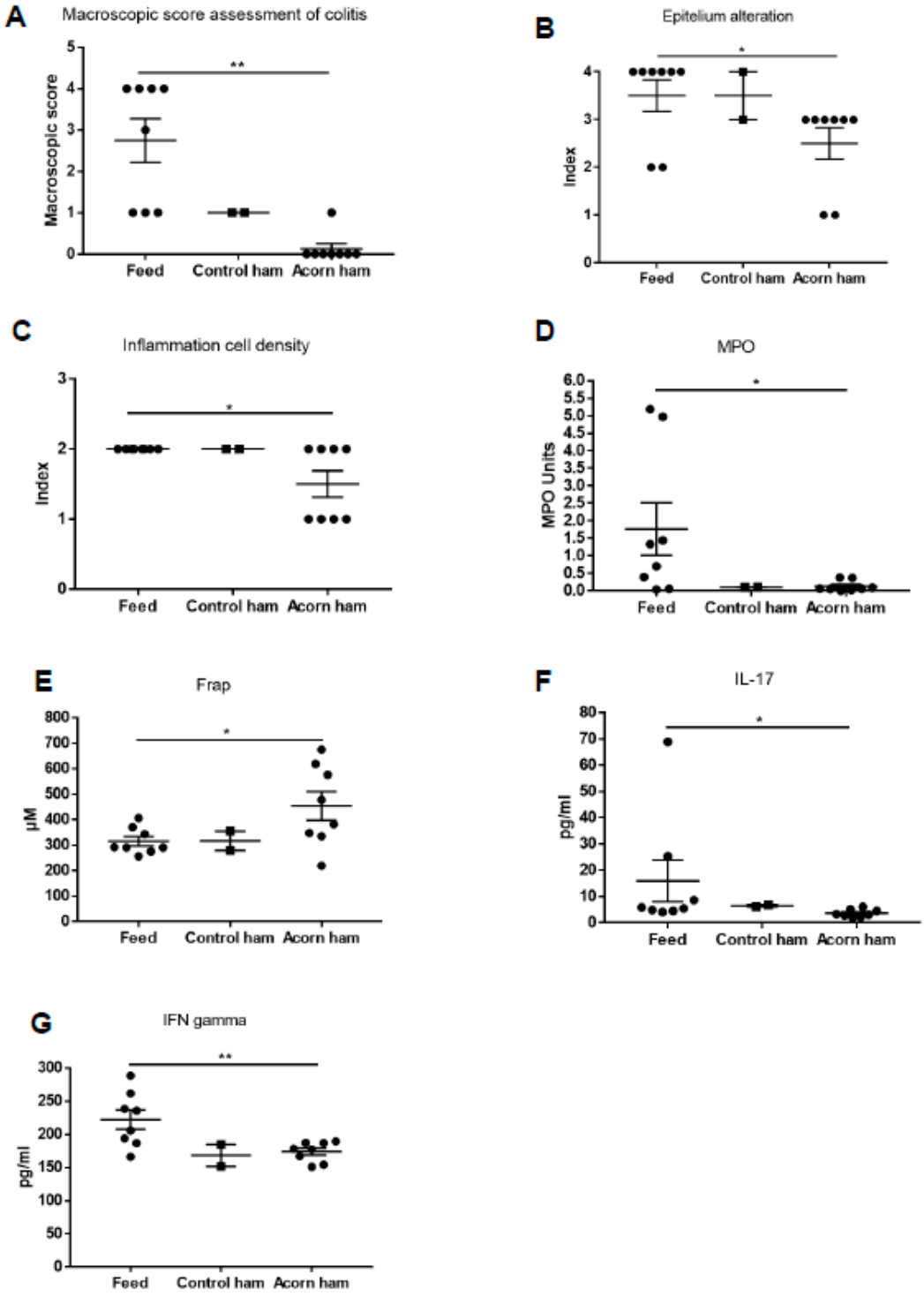

Figure 2

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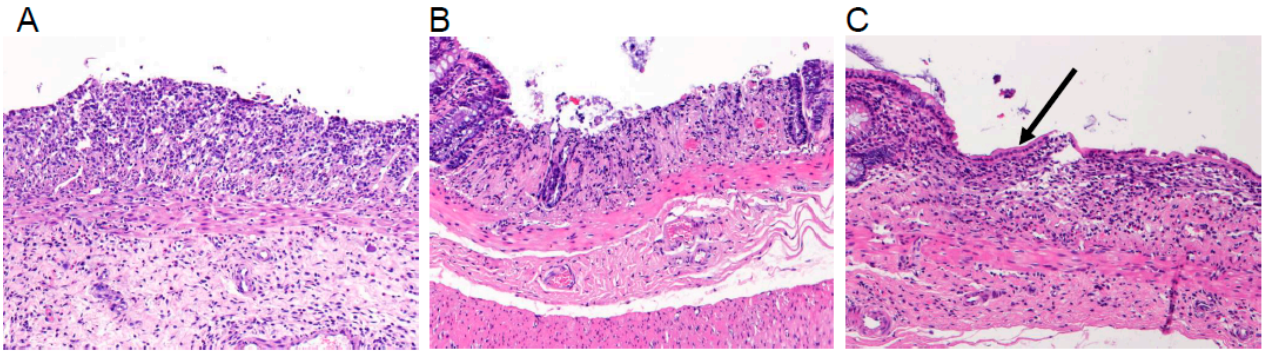

Figure 3

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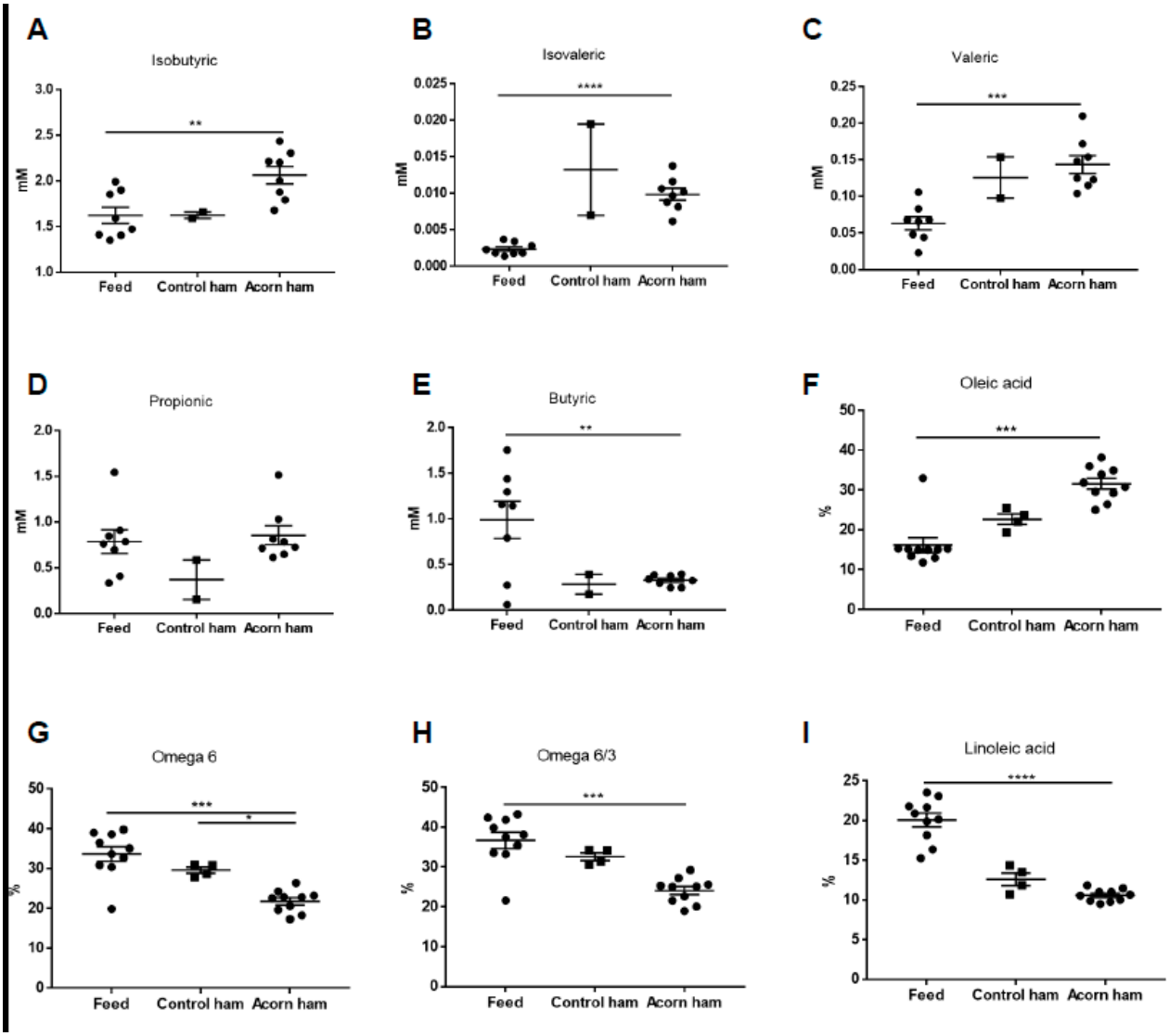

Figure 4

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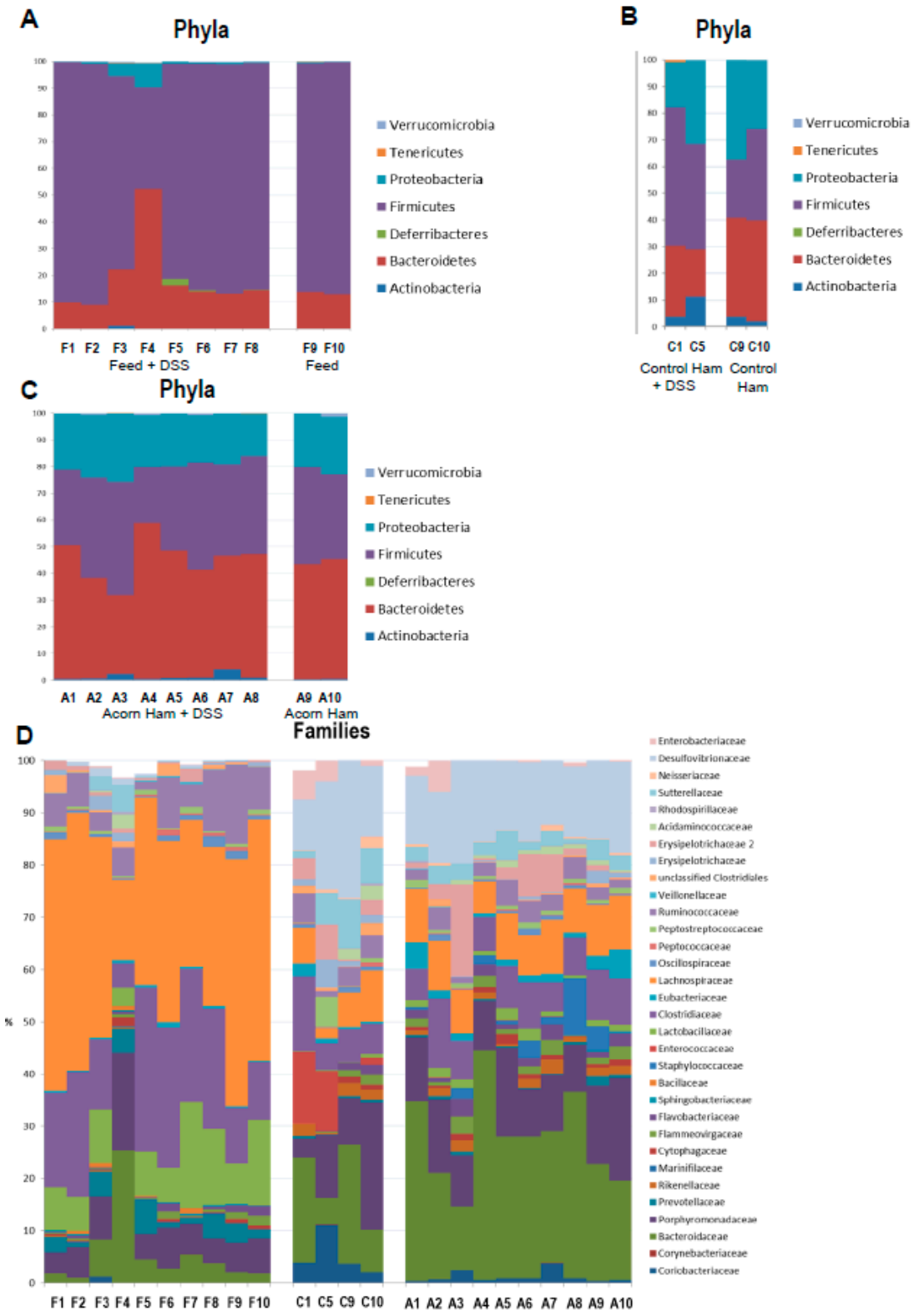

Figure 5

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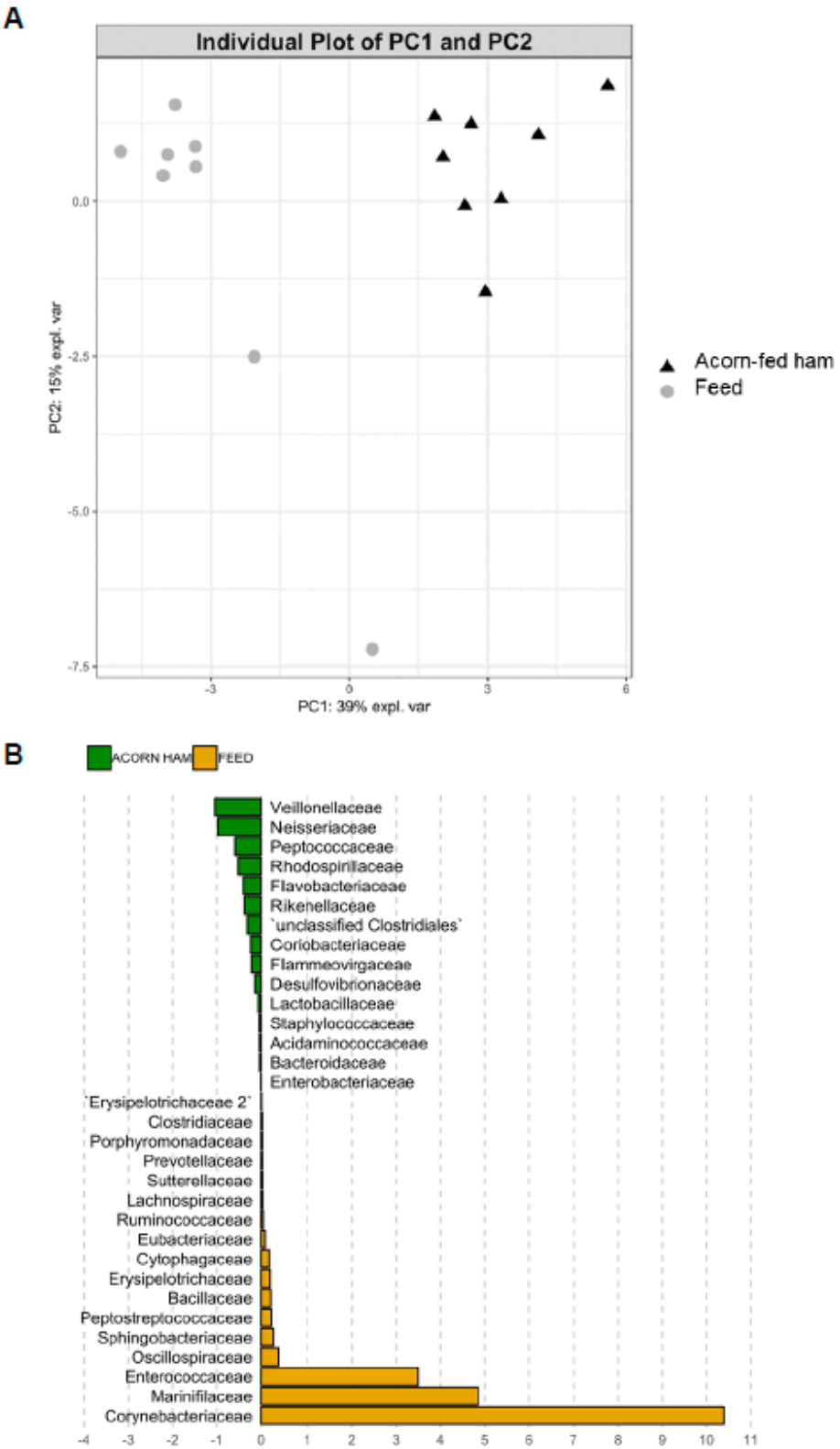

Figure 6