Behaviour of citrus pectin and modified citrus pectin in an azoxymethane/dextran sodium sulfate (AOM/DSS)-induced rat colorectal carcinogenesis model

Highlights:

1. Effect of citrus pectins was evaluated in an AOM/DSS cancer rat model.
2. A reduction in weight and blood glucose levels was observed in pectin fed rats.
3. A high mortality was observed in pectin and modified pectin fed individuals.
4. pH decrease and high increase in Proteobacteria were observed in these individuals.
5. Pectin and modified citrus pectin did not reduce the tumorigenesis in the used model.

Abstract

We have assessed the impact of citrus pectin and modified citrus pectin on colorectal cancer in rats (*Rattus norvegicus* F344) to which azoxymethane and DSS were supplied. The lowest intake of food and body weight were detected in animals fed with citrus pectin, together with an increase in the caecum weight, probably due to the viscosity, water retention capacity and bulking properties of pectin. Neither citrus pectin nor modified citrus pectin gave rise to a tumorigenesis prevention. Moreover, in both, more than 50% of rats with cancer died, probably ascribed to a severe dysbiosis state in the gut, as shown by the metabolism and metagenomics studies carried out. This was related to a decrease of pH in caecum lumen and increase in acetate and lactic acid levels together with the absence of propionic and butyric acids. A relevant increase in *Proteobacteria* (*Enterobacteriaceae*) were thought to be one of the reasons for enteric infection that could have provoked the death of rats and the lack of cancer prevention. However, a reduction of blood glucose and triacylglycerides level and an increase of *Bifidobacterium* and *Lactobacillaceae* were found in animals that intake pectin, as compared to universal and modified citrus pectin feeding.

Behaviour of citrus pectin and modified citrus pectin in an azoxymethane/dextran sodium sulfate (AOM/DSS)-induced rat colorectal carcinogenesis model

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Abstract

Large intestine cancer is one of the most relevant chronic diseases taking place at present. Despite therapies have evolved very positively, this pathology is still under deep investigation. One of the recent approaches is the prevention by natural compounds such as pectin. In this paper, we have assessed the impact of citrus pectin and modified citrus pectin on colorectal cancer in rats (*Rattus norvegicus* F344) to which azoxymethane and DSS were supplied. The lowest intake of food and body weight were detected in animals fed with citrus pectin, together with an increase in the caecum weight, probably due to the viscosity, water retention capacity and bulking properties of pectin. The most striking feature was that, neither citrus pectin nor modified citrus pectin gave rise to a tumorigenesis prevention. Moreover, in both, more than 50% of rats with cancer died, probably ascribed to a severe dysbiosis state in the gut, as shown by the metabolism and metagenomics studies carried out. This was related to a decrease of pH in caecum lumen and increase in acetate and lactic acid levels together with the absence of propionic and butyric acids. A relevant increase in *Proteobacteria* (*Enterobacteriaceae*) were thought to be one of the reasons for enteric infection that could have provoked the death of rats and the lack of cancer prevention. However, a reduction of blood glucose and triacylglycerides level and an increase of *Bifidobacterium* and *Lactobacillaceae* were found in animals that intake pectin, as compared to universal and modified citrus pectin feeding.

Key words: cancer, pH decrease, intestinal microbiota, gut, dysbiosis, lactic acid, acetic acid

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy in the world, being the second reason of cancer deaths in 2018 [1]. As it is known, CRC can imply severe health complications related to the illness itself and the side effects of surgery and/or therapy [2]. In a recent study on the incidence and mortality of CRC in 39 countries, it has been have shown that the occurrence of colon and rectal cancers is increasing in countries with medium to high development degrees, mainly in the case of young people [3]; therefore, it is necessary to increase the early detection methods and to continue with the investigations that can shed light on the prevention and treatment of this pathology.

CRC usually is developed during several years when a sequence of genetic modifications (towards polyps, adenoma and carcinoma) gives rise to tumours that are more common in the distal large intestine, including the descending colon and rectum, as compared to the proximal sections. Although some CRC forms can be of genetic origin, most CRC cases have a relationship with the lifestyle and diet. In this sense, a diet based on dietary fiber and the use of cancer-therapeutic or cancer-preventive natural compounds are considered efficient and affordable approaches [4].

A plethora of scientific articles has linked a high fiber consumption with a lower frequency of large intestine cancer. Particular interest has been sparked in the case of pectin, mainly derived from citrus, that is used as important technological food ingredient and also for its bioactivity [5]. Experimental studies have also showed a limited consistency on the effects of pectin on CRC with results of inhibition, no effect, or even tumour augmentation [6-9]. Several factors related to pectin such as the source, extraction and purification methods can affect the effectiveness of the assays since the extracted pectin could have rather dissimilar structural features. This fact seems to play an important role in terms of molecular weight (Mw), methyl esterification degree (DM), composition of galacturonic acid (GalA) and neutral sugars such as galactose and arabinose [8].

Pectin is a complex hetero-polysaccharide occurring in plant cell walls and its precise chemical structure is still under debate. The most recognized model combines the structural domains of

homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II). HG corresponds to 65% of pectin molecules, with a linear backbone composed of α-(1,4)-D-galacturonic acid, partially methyl-esterified in the C6, or acetylated in O-3 and/or O-2. RG-I corresponds to 20–35% of pectin molecules; this chain is composed of hundreds of repeating disaccharides [→4)α-D-galacturonic acid and (1–2)α-L-rhamnose(1→]n, and may present side chains of molecules of L-arabinose and D-galactose. RG-II represents 10% of pectin molecules and it is a well-preserved and extremely complex molecule, where the main backbone is HG with four heteropolymer side chains with more than 17 rare monosaccharides and 20 different types of bonds [10].

Due to its highly branched complex, pectin is poorly soluble in water, limiting its use. Thus, Modified Citrus Pectin (MCP) has been developed by chemical, enzymatic or heat treatment of citrus pectin to produce a mixture of low Mw polysaccharides that could have a stronger therapeutic role against cancer as compared to full citrus pectin [11,12].

In colon cancer cell lines, several studies have demonstrated the efficiency of different citrus pectin and MCP, and their fractions, and even different mechanisms of action have been postulated [8]. Ai et al. (2018) assayed, against Caco-2 cells, different fractions obtained by an enzymatic treatment and subsequent ultrafiltration. Among the samples tested, the highest activity was found in the fraction of RG-II, probably due to its peculiar branched structure and low Mw [13]. In the case of Ramos do Prado et al. (2019), the production of MCP fractions was by heat treatment and ultrafiltration [14]. In HCT116 and HT29 colon cancer cells, the highest antiproliferative effect was observed when HG oligomers were de-esterified and enriched in arabinogalactan I and poor in RG-I.

On the contrary, the limited *in vivo* information available on the effect of citrus pectin on CRC shows contradictory results using different animal models, different types of modified pectin and carcinogen doses. Scarce attention has been considered to effects on the microbiota and the relationship with the pectin structure. Moreover, in some cases, the effect of citrus pectin is considered together with other bioactive compounds [8,15]. Ohkami et al. (1995) stated that the intake of 20% of citrus pectin in rats

injected with azoxymethane (AOM) decreased the multiplicity of colon tumours and they hypothesised that a decrease of β-glucuronidase activity was the most important mechanism, although this effect was much higher in the case of apple pectin [16]. According to Jacobasch et al. (2008), who used a model of animals with a genetic predisposition for intestinal neoplasia (APCMin/$^{+}$ mice), pectins (with low and high DM) were ineffective for reduction of tumorigenesis in the small or large intestine and for suppressing COX-2 activity, an enzyme that plays a key role in the pathogenesis of tumour progression [7]. These results were in line with those earlier obtained by Jacobs and Lupton (1986) who stated in Sprawe-Dawley rats that the intake of pectin and other soluble fiber could increase proximal colon tumorigenesis [6]. However, Nangia-Makker et al. (2002) observed in BALB/c mice, with implanted tumours in the colon, that the daily oral administration of MCP reduced the growth of those implanted tumours and subsequent metastasis [17].

On the basis of this background, we have carried out an exhaustive study on the effect of commercial citrus pectin and Modified Citrus Pectin (MCP) in an animal model (*Rattus norvegicus* F344) developed for colorectal cancer using a combination of azoxymethane and DSS as carcinogenic compounds. Structural and physicochemical characteristics of both test substances have been considered in this animal model. Also, different tumorigenesis parameters (tumour size, number, area) have been analysed, together with metabolic data (short-chain fatty acids, glycemia, etc.), physiological (food intake, weight, number of hyperplastic Peyer's patches, caecum weight) and metagenomics of gut microbiota.

2. Materials and Methods

2.1. Manufacturing and samples

Commercial citrus pectin (trade name Ceampectin, ESS-4400) was kindly provided by CEAMSA® (Porriño, Pontevedra, Spain). Modified Citrus Pectin (MCP) was kindly provided by Econugenics®, Inc. (Santa Rosa, CA, USA).

2.2. Physicochemical characterisation of substrates

A high Mw citrus pectin as well as MCP were used in this study. Physico-chemical characterisation of each substrate and the feed mixtures was carried out in samples before assays. Product composition was determined regarding carbohydrates, DM, Mw, water retention capacity (Wr) and pH (**Table 1**). Monomeric composition of pectins was analysed after acid hydrolysis with 2 M trifluoroacetic acid (TFA) at 110 ºC during 4 h. The released monosaccharides were derivatised by the formation of trimethylsilyl oximes, following a previous method [18]. Then, samples were analysed by gas chromatography coupled to a flame ionisation detector (GC-FID) and equipped with a fused silica capillary column DB-5HT (5% phenyl methylpolysiloxane, 30 m x 0.25 mm x 0.1 µm, Agilent J&W Scientific, Folsom, CA, USA). Oven temperature program started in 150 ºC and increased to 165 ºC at 1 ºC/min and up to 300 ºC in a rate of 10 ºC/min. Injector and detector temperature were 280 and 350 ºC, respectively. Nitrogen was used as the carrier gas at 1 mL/min of flow rate. Samples were injected in split mode 1:5. Quantification was done through the internal standard method (*β*-phenyl-glucoside).

Estimation of the Mw was conducted by HPSEC-ELSD [19]. Samples were filtered (0.45 µm), analysed on a LC 1220 Infinity System (Agilent Technologies, Boebligen, Germany) and detected on an ELSD System 1260 Infinity (Agilent Technologies, Boebligen, Germany). Mobile phase used was 0.1 M $NH_4CH_3CO_2$, at a flow rate of 0.5 mL/min for 50 min at 30 ºC. Pullulans of Mw 805, 200, 10, 3 and 0.3 kDa were used as standards.

DM of the samples was analysed by Fourier transform infrared spectroscopy (FTIR) [20]. The DM was determined as the average of the ratio of the peak area at 1747 cm^{-1} (COO-R) over the sum of the peaks 1747 cm^{-1} (COO-R) and 1632 cm^{-1} (COO^-).

W_r was determined following the method of Chau & Huang (2003) [21]. Pectins were incubated with distilled water (1:10, w/v) for 24 h with continuous agitation. Then, samples were centrifuged at 1006

x *g* for 30 min. W_r was expressed as mL of water held by 1 g of pectin. In addition, pH of samples was measured using a pH-meter FE20 (Mettler Toledo GmBH, Schwerzenbach, Switzerland).

2.3. Animal and experimental design

In the inducted colorectal cancer model a total of 30 male Fischer 344 rats were maintained in the Animal Facilities at the University of Oviedo (authorised facility No. ES330440003591). All rat assays were approved by the Ethics Committee of the Principality of Asturias (authorisation code PROAE 36/2018).

Rats (5 weeks old) were divided into 3 cohorts of 10 individuals each and fed ad libitum in individual cages. Cohort 1 was fed with universal feed (F cohort, 2014 Teklad Global 14% Protein Rodent Maintenance Harlan diet feed), which contained 6.7% protein, 5.8% fat, 53,6% carbohydrates, 20 % fibre, 4.7% ashes (**Table 2**). Cohort 2 was fed with a mixture feed prepared from universal feed where cellulose (BW200) was substituted by citrus pectin (20%) (FP cohort) (Research Diets Inc, NJ, USA). In a similar way, cohort 3 was fed with a preparation where cellulose was substituted with the modified citrus pectin (FMP cohort) (20%) (Research Diets Inc, NJ, USA).

2.4. Colorectal cancer induction and monitoring

The colorectal cancer inducing was carried out according to previously described methodology [22]. Assay took place one week after the animals arrived at the facility when the diets started. After one week of eating the corresponding diet, CRC was induced in eight rats from each cohort. The two other rats were kept free of CRC induction as absolute control animals. CRC induction was carried out in those eight rats of each cohort using azoxymethane (AOM, Sigma-Aldrich, Madrid, Spain) dissolved in sterile saline solution (0.9% NaCl) at a concentration of 2 mg/mL. This AOM solution was injected intraperitoneally at a final concentration of 10 mg per kg body weight. This AOM

treatment was repeated seven days after the first injection (weeks 2 and 3). The absolute control animals received sterile saline in both injections.

In weeks 4 and 15, rats received drinking water during seven days' treatment, containing 3% and 2% dextran sodium sulfate (DSS, 40.000 g/mol, VWR), respectively. This ulcerative colitis step was repeated twice because it enhances the pro-carcinogenic effect caused by AOM administration.

Rats were sacrificed by pneumothorax 21 weeks after the first administration of AOM. Throughout the entire process, rats were monitored for body weight and stool consistency/rectal bleeding.

2.5. Weight measurements

Rats were weighed regularly during the 21 experimental weeks; at reception of the animals, (week 1), at each of the AOM administrations (week 2 and 3), and at weeks 6, 10, 13, 18 and 21.

2.6. Blood and tissue samples

Before being sacrificed (bilateral pneumothorax) at week 21, rats were anesthetised (isoflurane) for the extraction of blood (2 mL) from the heart, which was then centrifuged at 3,000 rpm for 15 min, in order to collect and freeze the plasma at -20º C.

Small intestines were fresh removed and the hyperplastic Peyer's patches were counted. Their number in the experimental animals was calculated and compared with respect to the two absolute control animals from each cohort (animals 9 and 10). Weight and length of small intestines was also measured in all cohort individuals. Caecums were weighed immediately after sacrifice using a precision scale and then frozen at −20 °C.

Finally, the colon was opened longitudinally and washed with PBS (phosphate buffer saline) before keeping it in 4% formaldehyde at 4 ºC. Fixed colons were meticulously examined with a caliper in order to count the number of polyps larger than 1 mm on the inner mucosa surface. The largest

detected polyps were 10 mm in diameter. The shape of the polyps was identified as pedunculated (a disc connected via a peduncle to the colon mucosa), plane irregular, plane circular and spherical. Finally, the total polyp-affected area was calculated.

2.6.1. Plasma glucose and triacylglycerides analysis

Plasma glucose levels were measured using a Accutrend Plus and the reactive strips 11447475 (Roche, Barcelona, Spain). Plasma triacylglycerides levels were measured using the same equipment, but with reactive strips 11538144 (Roche, Barcelona, Spain).

2.7. HPLC-UV quantification of SCFA in caecum samples

Prior to HPLC analysis, short chain fatty acids (SCFA) were extracted from rat caecum, according to the method of Joseph et al. (2019) [23]. Caecum samples (0.2 g) were added to distilled water (1.6 mL) in order to get a final ratio of 1:8 (w/v). Then, extraction was performed by mixing powerfully in vortex for 1 min. Finally, samples were centrifuged for 10 min at 10000 x *g* and supernatant was filtered using a 0.22 µm syringe filter (Symta, Madrid, Spain). Samples were injected on a HPLC system (Agilent Technologies, Germany) equipped with a UV-975 detector. Separation was done through a Rezex ROA Organic Acids column (300 cm x 7.8 mm) (Phenomenex, Macclesfield, UK) at a flow rate of 0.5 mL/min (isocratic elution) at 50 ºC. The mobile phase was 0.005 N H_2SO_4 and detection was performed at a wavelength of 210 nm. Identification and quantification of peaks were done through external standards solutions of SCFA (acetic, propionic, butyric, formic, lactic, valeric and isovaleric acid) in different concentrations (1-100 mM).

2.8. Genomic DNA extraction and 16S ribosomal RNA sequencing for metagenomics

Genomic DNA (gDNA) was extracted from 200 mg of frozen (-80 ºC) caecum feces using E.Z.N.A.® DNA Stool kit (Omega BioTek Ref. D4015-02, VWR, Madrid, Spain) and provided 200 µL of genomic DNA. These gDNA samples were then quantified using a BioPhotometer® (Eppendorf,

Madrid, Spain) and their concentrations diluted to 6 ng/µL. Diluted samples were used for performing polymerase chain reactions (PCR) amplification, following the protocol of the Ion 16™ Metagenomic kit (Thermo Fischer Scientific, Madrid, Spain).

PCR amplification products were utilised to create a library using the Ion Plus Fragment Library kit for AB Library Builder™ System (Cat. No. 4477597, Thermo Fischer Scientific), with sample indexing using the Ion Xpress™ Barcode adapters 1-96 kit (Cat. No. 4474517, Thermo Fischer Scientific).

Template preparation was performed using the ION OneTouch™ 2 System and the ION PGM™ Hi-Q™ OT2 kit (Cat. No. A27739, Thermo Fischer Scientific). Metagenomics sequencing was performed using ION PGM™ Hi-Q™ Sequencing kit (Cat. No. A25592, Thermo Fischer Scientific) on the ION PGM™ System. The chips used were the ION 314™ v2, 316™ v2 or 318™ v2 Chips (Cat. No. 4482261, 4483188, 4484355, Thermo Fischer) with various barcoded samples per chip [24].

2.9. Phylogenetic analysis

The consensus excel table for each metagenomics sequencing was downloaded from ION Reporter 5.6 software (Life Technologies Holdings Pte Ltd, Singapore). 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 conducted 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). 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 80.000. Total number of reads was always over 110.000. Counts were normalised by sum scaling [25].

2.10. Statistical methods

Data were expressed as the mean value ± S.E.M. Statistical analyses were conducted using ANOVA test when the quantitative data presented normality and the variances were assumed equal. Normality was analysed using Shapiro-Wilk. In the absence of normality, Kruskal-Wallis test was used. The graphical representation of all these data was generated using GraphPad Prism software (version 8, GraphPad Software, San Diego, CA, USA). In all cases, a p value < 0.05 was considered statistically significant (*p < 0.05; ** p < 0.005; *** p < 0.0005; **** p < 0.0001) [25].

3. Results

3.1. Effect of pectin diets on body weight

Body weight was affected in all cohorts due to the different feeding after the AOM and DSS treatment. In general, all cohorts gained weight during the experiment after the DSS treatment maintaining a continuous gain along the experimental weeks. Rats with inducted CRC achieved a slightly lower weight values as compared to the control rats (**Figures 1A** and **1B**). It is noteworthy that cohort fed with pectin (FP) showed the lowest intake and body weights in all cohorts, followed by the cohort fed with modified pectin (FMP), whereas the cohort fed with the universal diet (F) showed the highest values.

Nevertheless, it should be noted that nine animals died during the assay; five in FP cohort and four in FMP cohort. They did not survive the DSS treatment, which was used to enhance the final production and size of the colon tumours, and died just after its administration. Five of them died during the next 10 days after finishing the first DSS treatment (mainly in FP cohort), three died one day after finishing the second DSS treatment and the last animal died 2 days before sacrifice day (mainly in FMP cohort).

3.2. Effect of pectin diets on caecum weight

Statistically significant differences in the caecum weight values between the three different cohorts were observed (**Figure 2**). Highest weight was detected in the FP cohort (14.4 ± 1.4 g) as compared to FMP (9.4 ± 1.9) and F (3.8 ± 0.6) cohorts.

3.3. Effect of pectin diets on hyperplastic Peyer's patches

The hyperplastic Peyer's patches in the small intestine was quantified when the animals were sacrificed. Peyer's patches contain high amounts of lymphocytes and are located in the mucosa layer of the small intestine. These lymphoid nodules can become hyperplastic and are, therefore, easily visible in the small intestine as rounded, protruding, white 2-3 mm ovals [24].

In this work, differences in the Peyer's patch mean values were not statistically significant between the universal feed cohort and pectin and modified pectin cohorts (**Figure 3**).

3.4. Effect of pectin diets on number of polyps and tumour-affected area

After sacrifice, colonic mucosa of each animal was analysed for the number of polyps which diameter ranged from 1 to 10 mm. Statistically differences were not observed in the number of polyps between rats from the different cohorts (**Figure 4A**). Moreover, the area of each polyp present in a given colon mucosa was calculated according to its shape and the total polyp area was computed for each animal. Highest tumour area was measured for F cohort (629.1 ± 270) with a reduction of 23.5% in FP cohort and 5% in FMP cohort, respectively; however, these reductions were not statistically significant (**Figure 4B**).

3.5. Effect of pectin diets on SCFAs production and blood glucose and triacylglyceride levels

Caecal production of SCFA is important since these compounds show interesting antitumor properties regarding CRC prevention. As it could be expected, acetate, which is the main product of

saccharolytic fermentation of polysaccharides, was the highest SCFA detected in all samples (**Figure 5A**). F cohort showed the lowest values of acetate (22.6 mM ± 5.5) as compared to FP (33.71 mM ± 7.4) and FMP (35.0 mM ± 6.3), respectively. Slight levels of lactic acid were also detected in all cohorts (0.4 mM ± 0.3; 1.9 mM ± 0.1 and 2.8 mM ± 1.4 for F, FP and FMP, respectively) (**Figure 5B**). Although lactate is not a SCFA, it is usually considered in the metabolism of bacteria as a product of saccharolytic fermentation. Regarding other SCFA, no quantifiably values were found in the samples with the chromatographic method used. In general, total organic acids observed (acetate and lactate) showed an increase in FP and FMP cohorts (in line with the lower pH observed in these groups; 6.33 ± 0.13 and 6.50 ± 0.15, respectively, vs F cohort 6.92 ± 0.19; **Figure 5C**), although these increases did not show statistically significant differences.

Regarding glucose levels determined in plasma (**Figure 5D**), citrus pectin presence in the FP cohort provided lower levels of glucose in the animals, which is in accordance with its relation of a better control of the caloric intake given its high resistance to intestinal digestion. Conversely, FMP, which is mainly composed of oligosaccharides (average Mw = 3.1 kDa) did not show any decrease in the glucose levels compared to the F cohorts. Nevertheless, all variations found in this analysis did not show any statistically significant differences.

Finally, plasma triacylglycerides levels showed a statistically significant reduction of this parameter in FP cohort (170.2 mg/dL ± 25.4) in comparison with F (324.7 mg/dL ± 27.3) and FMP (358.8 mg/dL ± 63.4) (**Figure 5E**).

3.6. Effect of pectin diets on intestinal microbiota

Average phyla compositions showed important differences between the three animal cohorts with and without disease (**Table 3**). At this level, one of the main differences observed was the high increase in *Bacteroidetes* in the FP cohort with respect to F and FMP cohorts in both CRC and healthy rats. No-CRC rats showed higher increases in this phyla compared to CRC rats. Additionally,

reduction in the *Firmicutes* levels was found in the FP cohort of CRC rats with respect to the F (20.7%) and FMP (19.9%) groups, where no-CRC animals showed decreases only in FP cohort, in a lesser extent, compared to F cohort (6.9%). Finally, the main difference observed in this level was the important increase in *Proteobacteria* in CRC rats, in FP (14.8%) and FMP (4.3%) groups compared to F group, whereas no-CRC animals showed a reduction of these bacteria, 3.1% and 2.3% reduction for FP and FMP cohorts compared to F, respectively.

At family level (**Figure 6, Table 4**), in the F cohort, the most abundant families were *Clostridiaceae* (14.91%), *Lachnospiraceae* (13.60%), *Bacteroidaceae* (12.78%), *Porphyromonadaceae* (11.86%), *Ruminococcaceae* (11.75%), and *Desulfovibrionaceae* (10.70%). In the case of FP cohort, the most abundant ones were *Prevotellaceae* (25.42 %), *Enterobacteriaceae* (13.04 %), *Lachnospiraceae* (12.12%), *Bacteroidaceae* (12.08%), and *Clostridiacea* (8.12%). Highest values found in FMP were for *Lachnospiraceae* (20.39%), *Bacteroidaceae* (13.74%), *Porphyromonadaceae* (10.49%), *Clostridiaceae* (8.28%), *Desulfovibrionaceae* (6.85%), *Enterobacteriaceae* (5.37%), *Lactobacillaceae* (5.25%) and *Ruminococcaceae* (5.17%).

At this level, different statistically significant increases can be observed compared to the F cohort. For example, *Lactobacillaceae* increased from 0.27% to 2.01% and 5.25% in FP and FMP cohorts, respectively. *Prevotellaceae* increased from 1.77% to 25.42% in FP group. *Enterobacteriaceae* showed high increase in FP individuals (13.04%) and FMP (5.37%) vs 0.35% in F group. *Suterellaceae* increased from 1.12% in F cohort to 4.05% in FP animals. *Lachnospiraceae* family showed an increase in FMP cohort (20.39%) in comparison with F (13.60%) and FP animals (12.12%).

Additionally, significant reductions were observed in *Porphyromonadaceae* (3.91% in FP, 11.86% in F and 10.49% in FMP). *Clostridiaceae* in FP (8.12%) and FMP (8.28%) vs 14.91% in F cohort and *Desulfovibrionaceae* (3.18%, 6.85% for PF and FMP, vs 10.70% for F cohort). *Ruminococcaceae* showed a value of 4.48% in FP, 5.17% in FMP and 11.75% in F cohort. (**Table 4**).

PCA analysis of gut microbiota composition at family level divided the animals in three clusters, indicating differences in the gut microbiota composition associated to these dietary interventions, where F and FMP animals are clustered closer than FP cohort (**Figure 7**).

Tables 5 and **1S** show the percent abundance of the genera and species with statistically significant differences between the three cohorts in the assay. The main differences are associated with a higher proportion of some genera (such as *Lactobacillus*) in the pectin administration diets (FP, FMP), some of them involved in SCFAs biosynthesis (*Bifidobacterium, Paraprevotella, Bacteroides, Eubacterium, Parasutterella, Blautia*), and a reduction in the populations of other genera in these cohorts (*Prevotella, Clostridium, Blautia*), including a significant reduction in some pro-inflammatory genera (*Ruminococcus* and *Bilophila*).

4. Discussion

Potential antitumor effects of commercial citrus pectin (CP) and modified citrus pectin (MCP) were studied in an animal model where CRC was generated using AOM/DSS. Chemical composition of both test substrates demonstrated to be similar regarding the monomeric composition (**Table 1**). The higher Mw and methylation degree observed in pectin support the highly complex structure of this substrate with a high number of side chains, already observed in previous studies [26], whereas MCP was mainly composed of a galacturonic acid backbone and free mono- and oligosaccharides, showing a lack of methylation degree (0%). Pectin structure provides an important water retention capacity being almost 15-fold higher than that of MCP (10 mL/mg *vs* 0.7 mL/mg). In this sense, pectin, as well as other dietary fiber, is known to impact on satiety and satiation due to its properties of producing viscosity (satiety) and adding bulk to the food (satiation). Pectin has been shown to significantly delay gastric emptying time, hence increasing satiety [27-29], which can explain the lower intake of food observed in the FP cohort (**Figure 1C**) and, therefore, the lower body weight

observed in all the individuals (**Figure 1A**), although the important food intake reduction observed at week 6 was also associated to the secondary effects of the ulcerative colitis episode due to DSS administration. Conversely, MCP, with a lower Mw and DM than pectin and similar physicochemical properties to the universal feed regarding water retention capacity, showed higher intake values during the assay (FMP cohort), with almost similar responses to the universal feed individuals (F cohort) (**Figure 1C**). In addition, bacterial pectate lyase has shown to hydrolyse preferably low DM pectin structures, such as MCP, contributing, therefore, to their high intake and absorption [7]. Thus, higher body weight was observed in the FMP cohort as compared to pectin being almost as high as the F cohort was.

Regarding glucose content at the end of the study, high plasma levels were observed in F and FMP cohorts (> 200 mg/dL) (**Figure 5D**), whereas, pectin intake decreased glucose levels showing the lowest values (FP cohort) of all studied groups. The anti-diabetic and hypoglycemic effects of dietary fiber and pectin have been widely reported in previous *in vivo* and *in vitro* studies [30-32]. In this sense, the European Food Safety Authority (EFSA) has recognised a direct cause and effect relationship between the consumption of pectins and a reduction of postprandial glycemic responses in adults [27,33]. Studies with rats have demonstrated the effectiveness of pectin in reducing glucose levels in type 1 and type 2 diabetic rats [34,35]. Conversely, the low Mw carbohydrates composition and low viscosity in FMP produced higher glucose levels, since it has been reported that a reduction in the viscosity of pectins can reduce significantly the effect on postprandial hyperglycaemia [36]. A plausible explanation for this is that glucose intake is reduced with a high viscosity possibly due to a combination of delayed gastric emptying, reducing macronutrient absorption and preventing diffusion of glucose through the lumen to the epithelium [37,38]. In the same sense, plasma triacylglycerides showed a statistically significant reduction in the case of pectin cohort (FP) in comparison with the two other cohorts (**Figure 5E**), due to a similar positive effect.

At the end of the experiment, all surviving animals were sacrificed. It has to be noted that FP diet caused the death of five CRC rats and four rats did not survive in the FMP cohort, whereas CRC control cohort rats (F) did not show any mortality. One possible explanation to this is the fact that these two diets based on pectins, caused a dysbiosis at the intestinal microbiota level, with higher percentages of pro-inflammatory taxons, especially in the *Proteobacteria* phylum, which was not observed in the no-CRC rats (**Table 3**). This dysbiosis is more extreme in the FP cohort (**Figure 7**), where more animals' deaths took place, and also it took place during the first DSS challenge. However, dysbiosis in the FMP cohort is less accentuated and these animals' deaths took place closer to the last experimental weeks. DSS challenges are helpful for induction of a stronger CRC phenotype due to its ability to cause ulcerative colitis as pro-inflammatory trigger of CRC. This ulcerative colitis increases the intestinal permeability, enhancing the transfer of bacterial cells from lumen to intestinal submucosa tissue, inducing a pro-inflammatory status; and in FP and FMP animals this higher permeability is probably increasing the presence in intestinal submucosa of highly pro-inflammatory taxons (such as *E. coli*) (**Table 5, Table 1S, Figure 6**). Remarkably, those rats fed with either pectin or modified citrus pectin but that were also kept free of CRC induction did not exhibit any increase in pro-inflammatory taxons. In a mouse model, virulent *E. coli.* was accumulated after antibiotic treatment and can disseminate systematically when the intestinal epithelial barrier is breached by DSS, thereby inducing lethal inflammasome activation [39]. In a similar way, DSS-induced intestinal inflammation markedly increased the proliferation of *Citrobacter rodentium* in the intestine [40]. Thus, the reduced barrier function, as could be taking place in our study, would enable more interaction with the epithelium, resulting in an increased delivery of mutagenic and/or proinflammatory metabolites produced by *Enterobacteriaceae* [41,42].

To assess the effect of the pectin diets on CRC, histological parameters such as caecum weight, number of hyperplastic Peyer's patches, number of colon tumours, and total tumour area in the colon mucosa were measured.

Caecum weight was significantly increased in individuals from FP cohort, and, to a lesser extent, in the FMP animals (**Figure 2**). This effect could be ascribed to a higher stimulation of bacterial cell growth [43] in the case of pectin. However, the most plausible cause may be the physicochemical properties of pectin, such as the high viscosity, water retention capacity and bulking properties, which are higher in pectin in comparison with MCP [28].

Concerning hyperplastic Peyer's patches, no statistically significant differences were found between all three cohorts (**Figure 3**). Peyer's patches are abundant in lymphocytes and become hyperplastic when alterations in the digestive tract, which affect the animal's immune condition, take place, as may occur in response to some chemicals, pathogens or toxins [44,45]. This parameter has been used as a marker of the general pro-inflammatory condition of the small intestine mucosa in all individuals in response to the CRC induction treatment [43,46]. However, in our case, the absence of significant differences revealed that pectin does not affect the presence of these mucosal structures in the experimental model used.

Regarding the last histological parameters measured, number of colon tumours and the total tumour area in the colon mucosa (**Figure 4**), any significant difference between all cohorts were found.

The limited available *in vivo* information on the effect of citrus pectin on CRC and the contradictory results makes it difficult to elucidate the mechanism of action of these substrates, where most studies have been carried out in *in vitro* assays [8,47,48]. However, there are *in vivo* reports that do not support the chemopreventive effect of these pectins in line with this work. Jacobs et al. (1986) reported that different fibre such as oat bran, guar and citrus pectin could increase by 4.5 to 5 times the yield of proximal colonic adenocarcinomas, providing stimulus to cell proliferation in a 1,2-dimethylhydrazine (DMH) colonic cancer model in rats [6]. These authors attributed that a reduction in colonic luminal pH, similar to the observed in our work (≥ 0.3), while not providing any protection, may even enhance colon tumorigenesis. In addition, Jacobasch et al. (2008) found that citrus pectins

(with high and low methylation degree, 70% and 37%, respectively) did not inhibit tumorigenesis regardless their DM in $APC^{Min/+}$ mice [7]. Moreover, those pectins seemed even to accelerate CRC carcinogenesis since all polyps found in pectin-fed animals were large adenocarcinomas whereas only 80% in control diet mice were large adenocarcinomas. As basic requirements for colorectal anticarcinogenic effect can be a sufficient high fermentative butyrate production and an adequate butyrate absorption. These authors attributed this behaviour to an insufficient butyrate supply, since fermentation of pectin delivered only low amounts of butyrate. This might lead to a deficient energy metabolism and an ineffective function of butyrate as a promoter of normal cell differentiation and inducer of apoptosis in tumour cells, which could also explain the obtained results in the present study.

Thus, changes in the luminal pH may affect the uptake of luminal compounds by colonocytes and their action on these cells; increasing tumorigenesis as observed in our results [49]. Decreases in the pH could increase hydrogen sulphide concentrations (pKa = 7.04) [50], which easily penetrates the biological membrane amplifying its deleterious and pro-inflammatory effect on colonocytes respiration at excessive concentration [51]. Moreover, modification of the luminal pH *per se* may affect colonic epithelial cell physiology where lower colonic luminal pH in patients with ulcerative colitis has been observed as compared to healthy patients [52]. Low external pH has been shown to dramatically increase the expression of p-glycoproteins, related with multidrug resistance, in human colon carcinoma cell lines [53], rendering these cells more resistant to chemotherapeutic agents.

Interestingly, an evaluation of the abilities to prevent colorectal cancer of different dietary fibre in an AOM rat model showed that pectin from green cincau (*Premna oblongifolia* Merr.) was able to increase butyrate levels, however, no antiproliferative properties were observed [9]. Despite the SCFA stimulation, feeding with pectin led to an increase in proliferation within the colon and an increase in preneoplastic lesions, thus, appeared to be acting more like a pro-carcinogen. These authors maintained that it was possible that more pectin (> 5 %) needed to be consumed by rats to act

as a protective, which was not confirmed in our work (20 %), or that pectin may need to be delivered with other nutrients or fibre source to be protective in AOM/DSS models as observed in other studies [54,55].

Analyses of organic acids showed important differences in acetate levels, as well in lactic acid amounts (**Figures 5A and 5B**). Acetate has been previously reported as the main SCFA from pectin structures fermentation [56]. The high presence of this metabolite can be justified due to that acetate is generated by many bacterial groups that inhabit the colon, with approximately one-third of the product coming from reductive acetogenesis [57,58]. Absence of propionic and butyric acids in our study is in line with the no protective effect against tumorigenesis observed, since the presence of these metabolites have been widely correlated with the inhibition of growth of different CRC lines, induction of apoptosis of tumour cells and enhancement of anti-inflammatory properties, [4,8,59] whereas low levels of these metabolites can increase the risks of CRC and inflammatory gut diseases [7,60,64]. Moreover, in line with our results, elevated concentrations of luminal lactic acid have been reported in active colitis and CRC cases [62,63], a factor that could explain again that more animals died in our study during DSS challenges.

Analysis of microbiota of survival animals at phylum level showed significant differences between FP cohort versus F and FMP cohorts (**Table 3**). The *Firmicutes/Bacteroidetes* coefficient, which has been described as a parameter associated with obesity and type II diabetes [64,65], was reduced in FP (0.78) when compared to F and FMP cohorts (1.75 and 1.76, respectively), due to the increase in *Bacteroidetes* and diminution in *Firmicutes*, supporting the hypoglycemic effect of high Mw citrus pectin.

Higher *Bacteroidetes* population in FP was mainly produced due to the significant increase in *Prevotellaceae* family (**Figure 6, Table 4**). Species within genera *Bacteroides* and *Prevotella* are the primary pectin-degraders, possessing carbohydrate-active enzymes (CAZymes) within the

polysaccharide utilization loci [66-68]. However, the decrease in families, such as *Porphyromonadaceae,* observed in FP cohort might contribute to the absence of propionate production since these families contain numerous genera involved in propionate production [4]. The marked reduction in *Firmicutes* phylum was mainly produced by the decrease of the *Ruminococcaceae, Clostridiaceae* and *Eubacteriaceae* families, as it was observed in previous *in vitro* studies with pectin [26,69]. The reduction observed in *Faecalibacterium* genus, (especially *F. prausnitzii, Ruminococcaceae* family) could also contribute to the low anticarcinogenic properties observed in pectins cohorts, since its presence has been related with anti-inflammatory properties and it is described as a key bacteria species in promoting health [69,70].

Strikingly, a massive increase in *Proteobacteria* phylum was also observed in FP cohort due to the increase in *Enterobacteriaceae* family (13.04 %) (**Figure 6**). This family did not show any increase in a previous *in vitro* study with the same pectin [69]. Higher *Proteobacteria* populations and, particularly, *Enterobacteriaceae* family (including *E. coli*) are found in the gut microbiota of patients with IBD, which is a known risk factor for CRC [71]. In this sense, generally recognised pathogenic species, such as *E. coli, Salmonella* and *Serratia* increased in FP cohort compared to F cohort. This dysbiotic status has been correlated with various immune, metabolic and neurological disorders, in both intestinal and extra-intestinal sites [72]. As a consequence, susceptibility to enteric infection can be markedly increased. *Salmonella enterica* for example, poorly colonize the mouse intestine in the presence of commensal microbiota, however, it can proliferate and induce inflammation if the resident bacterial community is disrupted [73]. Thus, the presence of inflammation or an altered bacterial community facilitates the overgrowth of potentially harmful bacteria by decreasing the production of protective mucins and antimicrobial peptides.

In contrast, certain beneficial effect can also be identified when pectin is present such as the reduction of *Desulfovibrionaceae* family (*Proteobacteria* phylum), whose high levels have been associated with damages at the mucosal level caused by reduction of the mucin barrier [74].

High levels of *Prevotellaceae* family, as observed in our study in the FP cohort (25.42%) (**Table 4**), have also been associated in some studies with a healthier status [75,76]. In this study, it has been also observed a significant increase in *Bifidobacteriaceae* family (*Actinobacteria* phylum) in both FP and FMP cohorts, mainly due to the increase of *Bifidobacterium*; as well as in *Lactobacillaceae* family (*Lactobacillus* genus, *Firmicutes* phylum). Both families have been associated to several health benefits [72,77,78].

5. Conclusions

No previous studies have been carried out on the evaluation of the potential anticarcinogenic properties of citrus pectin and modified citrus pectin in *in vivo* models based on the use of azoxymethane/dextran sodium sulfate (AOM/DSS) to induce colorectal cancer in rats. Neither citrus pectin nor modified citrus pectin tested were able to inhibit tumorigenesis in this rat model. Strikingly, both pectins, particularly citrus pectin, seemed to induce a decrease of luminal pH of caecum and a huge dysbiosis degree in the CRC rats at the intestinal microbiota level, leading towards a potential proinflammatory status, even causing the death of five and four animals (of a total of eight) in pectin and modified pectin cohorts, respectively. Thus, a high increase in *Proteobacteria* (proinflammatory bacteria) and a reduction in *Faecalibacterium* genus were observed mainly in the former. These results were in line with the absence of butyric and propionic acids and the levels of lactic and acetic acid. On the other hand, citrus pectin demonstrated an important impact in the decrease of glucose and triacylglycerides in plasma, probably related to the lower feeding and body weight as compared to modified citrus pectin and universal feed cohorts. These results agree to the low *Bacteroidetes/Firmicutes* ratio. Citrus pectin and modified citrus pectin also demonstrated to stimulate the growth of other positive bacteria such as *Prevotellaceae, Bifidobacteriaceae and Lactobacillaceae* families. Summing up, the consumption of pectin such as citrus pectin and modified citrus pectin could not be beneficial in an inflammatory-tumour status due to an important worsening

of the pathology related to a severe unbalance of the intestinal microbiota. However, in a status of health, these pectins have relevant benefits not only in the gut but also at systemic level. Although the results obtained under the conditions assayed in this investigation seems to indicate the ineffectiveness of commercial citrus pectin and modified citrus pectin to exert a benefit in the prevention of CRC, more research is needed with other animal models in order to understand the intricate behaviour of this polysaccharide in this severe pathology.

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References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre and A. Jemal, *CA. Cancer J. Clin.*, 2018, **68**, 394–424.

[2] C. K. H. Wong, C. L. K. Lam, J. T. C. Poon, S. M. McGhee, W. L. Law, D. L. W. Kwong, J. Tsang and P. Chan, *J. Eval. Clin. Pract.*, 2012, **18**, 1203–1210.

[3] M. C. Wong, J. Huang, V. Lok, J. Wang, F. Fung, H. Ding and Z.-J. Zheng, *Clin. Gastroenterol. Hepatol.* DOI:10.1016/j.cgh.2020.02.026.

[4] P. Louis, G. L. Hold and H. J. Flint, *Nat. Rev. Microbiol.*, 2014, **12**, 661–672.

[5] E. G. Maxwell, N. J. Belshaw, K. W. Waldron and V. J. Morris, *Trends Food Sci. Technol.*, 2012, **24**, 64–73.

[6] L. R. Jacobs and J. R. Lupton, *Cancer Res.*, 1986, 1727–1734.

[7] G. Jacobasch, G. Dongowski, S. Florian, K. Müller-Schmehl, B. Raab and D. Schmiedl, *J. Agric. Food Chem.*, 2008, **56**, 1501–1510.

[8] W. Zhang, P. Xu and H. Zhang, *Trends Food Sci. Technol.*, 2015, **44**, 258–271.

[9] S. U. Nurdin, R. K. Le Leu, A. Aburto-medina, G. P. Young, J. C. R. Stangoulis, A. S. Ball and C. A. Abbott, *Int. J. Mol. Sci.*, 2018, **19**, 2593.

[10] F. R. B. Marenda, F. Mattioda, I. M. Demiate, A. de Francisco, C. L. de Oliveira Petkowicz, M. H. G. Canteri and R. D. de Mello Castanho Amboni, *J. Polym. Environ.*, 2019, **27**, 549–560.

[11] V. V. Glinsky and A. Raz, *Carbohydr. Res.*, 2009, **344**, 1788–1791.

[12] V. J. Morris, N. J. Belshaw, K. W. Waldron and E. G. Maxwell, *Bioact. Carbohydrates Diet. Fibre*, 2013, **1**, 21–37.

[13] L. Ai, Y. C. Chung, S. Y. Lin, K. C. Lee, P. F. H. Lai, Y. Xia, G. Wang and S. W. Cui, *Food Hydrocoll.*, 2018, **83**, 239–245.

[14] S. B. R. do Prado, T. M. Shiga, Y. Harazono, V. A. Hogan, A. Raz, N. C. Carpita and J. P. Fabi, *Carbohydr. Polym.*, 2019, **211**, 141–151.

[15] F. Odun-Ayo, J. Mellem and L. Reddy, *Food Sci. Technol.*, 2017, **37**, 478–482.

[16] H. Ohkami, K. Tazawa, I. Yamashita, T. Shimizu, K. Murai, K. Kobashi and M. Fujimaki, *Japanese J. Cancer Res.*, 1995, **86**, 523–523.

[17] P. Nangia-Makker, V. Hogan, Y. Honjo, S. Baccarini, L. Tait, R. Bresalier and A. Raz, *J. Natl. Cancer Inst.*, 2002, **94**, 1854–1862.

[18] K. M. Brobst and C. E. Lott Jr, *Cereal Chem.*, 1966, **43**, 35–43.

[19] N. Muñoz-Almagro, F. Rico-Rodriguez, M. Villamiel and A. Montilla, *Food Chem.*, 2018, **252**, 271–276.

[20] N. Muñoz-Almagro, A. Montilla, F. J. Moreno and M. Villamiel, *Ultrason. Sonochem.*, 2017, **38**, 807–819.

[21] C. F. Chau and Y. L. Huang, *J. Agric. Food Chem.*, 2003, **51**, 2615–2618.

[22] J. Fernández, F. J. Moreno, A. Olano, A. Clemente, C. J. Villar and F. Lombó, *Front. Microbiol.*, 2018, **9**, 1–14.

[23] N. Joseph, K. Vasodavan, N. A. Saipudin, B. N. M. Yusof, S. Kumar and S. A. Nordin, *J. Funct. Foods*, 2019, **57**, 103–111.

[24] J. Fernández, L. García, J. Monte, C. J. Villar and F. Lombó, *Genes (Basel).*, DOI:10.3390/genes9030133.

[25] J. Fernández, V. G. De Fuente, M. T. F. García, J. G. Sánchez and B. I. Redondo, *Lipids Health Dis.*, 2020, **19**, 1–19.

[26] A. Ferreira-Lazarte, V. Kachrimanidou, M. Villamiel, R. A. Rastall and F. J. Moreno, *Carbohydr. Polym.*, 2018, **199**, 482–491.

[27] A. Mortensen, F. Aguilar, R. Crebelli, A. Di Domenico, B. Dusemund, M. J. Frutos, P. Galtier, D. Gott, U. Gundert- Remy, C. Lambré, J. Leblanc, O. Lindtner, P. Moldeus, P. Mosesso, A. Oskarsson, D. Parent- Massin, I. Stankovic, I. Waalkens- Berendsen, M. Wright, M. Younes, P. Tobback, S. Ioannidou, S. Tasiopoulou and R. A. Woutersen, *EFSA J.*, 2017, **15**, 4866.

[28] S. Christiaens, S. Van Buggenhout, K. Houben, Z. Jamsazzadeh Kermani, K. R. N. Moelants, E. D. Ngouémazong, A. Van Loey and M. E. G. Hendrickx, *Crit. Rev. Food Sci. Nutr.*, 2016, **56**, 1021–1042.

[29] C. D. I. Lorenzo, C. M. Williams and J. E. Valenzuela, *Nutr. Rev.*, 1989, **47**, 268–270.

[30] J. Cui, X. Gu, Q. Zhang, Y. Ou and J. Wang, *Food Funct.*, 2015, **6**, 1635–1642.

[31] Q. Chen, L. Zhu, Y. Tang, Z. Zhao, T. Yi and H. Chen, *Ann. N. Y. Acad. Sci.*, 2017, 1–15.

[32] G. Grunberger, K.-L. C. Jen and J. D. Artiss, *Diabetes. Metab. Res. Rev.*, 2014, **32**, 13–23.

[33] European Food Safety Authority, *EFSA J.*, 2010, **8**, 1462.

[34] Y. Liu, M. Dong, Z. Yang and S. Pan, *Int. J. Biol. Macromol.*, 2016, **89**, 484–488.

[35] D. C. Silva, A. L. P. Freitas, C. D. S. Pessoa, R. C. M. Paula, J. X. Mesquita, L. K. A. M. Leal, G. A. C. Brito, D. O. Gonçalves and G. S. B. Viana, *J. Med. Food*, 2011, **14**, 1118–1126.

[36] S. L. Brenelli, S. D. S. Campos and M. J. A. Saad, *Brazilian J. Med. Biol. Res.*, 1997, **30**, 1437–1440.

[37] S. Ou, K. Kwok, Y. Li and L. Fu, *J. Agric. Food Chem.*, 2001, **49**, 1026–1029.

[38] M. O. Weickert and A. F. H. Pfeiffer, *J. Nutr.*, 2008, **138**, 439–442.

[39] J. S. Ayres, N. J. Trinidad, R. E. Vance and C. Biology, *Nat. Med.*, 2012, **18**, 799–806.

[40] N. Kamada, Y.-G. Kim, H. P. Sham, B. A. Vallance, J. L. Puente, E. C. Martens and G. Núñez, *Science (80).*, 2012, **336**, 1325–1329.

[41] T. Secher, A. Samba-louaka, E. Oswald and J. Nougayrède, *PLoS One*, 2013, **8**, 1–17.

[42] J. C. Arthur, E. Perez-chanona, M. Mühlbauer, S. Tomkovich, J. M. Uronis, T. Fan, B. J. Campbell, T. Abujamel, B. Dogan, A. B. Rogers, J. M. Rhodes, A. Stintzi, K. W. Simpson, J. Jonathan, T. O. Keku, A. A. Fodor and C. Jobin, *Science (80).*, 2013, **338**, 120–123.

[43] J. Fernández, E. Ledesma, J. Monte, E. Millán, P. Costa, V. G. de la Fuente, M. T. F. García, P. Martínez-Camblor, C. J. Villar and F. Lombó, *Sci. Rep.*, 2019, **9**, 1–17.

[44] S. Mishra, A. Tripathi, B. P. Chaudhari, P. D. Dwivedi, H. P. Pandey and M. Das, *Toxicol. Appl. Pharmacol.*, 2014, **279**, 186–197.

[45] M. Bailey, F. J. Plunkett, H. Rothkötter, K. Haverson and C. R. Stokes, *Proc. Nutr. Soc. (2001)*, 2001, **60**, 427–435.

[46] C. Jung and J. Hugot, *Int. J. Inflam.*, 2010, 1–12.

[47] L. Ai, Y. Chung, S. Lin, K. Lee, P. F. Lai, Y. Xia, G. Wang and S. W. Cui, *Food Hydrocoll.*, 2018, **83**, 239–245.

[48] H. Cheng, S. Li, Y. Fan, X. Gao, M. Hao, J. Wang, X. Zhang, G. Tai and Y. Zhou, *Med. Oncol.*, 2011, **28**, 175–181.

[49] F. Blachier, M. Beaumont, M. Andriamihaja, A. M. Davila, A. Lan, M. Grauso, L. Armand, R. Benamouzig and D. Tomé, *Am. J. Pathol.*, 2017, **187**, 476–486.

[50] F. Blachier, A. M. Davila, S. Mimoun, P. H. Benetti, C. Atanasiu, M. Andriamihaja, R. Benamouzig, F. Bouillaud and D. Tomé, *Amino Acids*, 2010, **39**, 335–347.

[51] S. Mimoun, M. Andriamihaja, C. Chaumontet, C. Atanasiu, R. Benamouzig, J. M. Blouin, D. Tomé, F. Bouillaud and F. Blachier, *Antioxid. Redox Signal.*, 2012, **17**, 1–10.

[52] S. Nugent, D. Kumar, D. Rampton and D. Evans, *Gut*, 2001, **48**, 571–577.

[53] L. Y. Wei and P. D. Roepe, *Biochemistry*, 1994, **33**, 7229–7238.

[54] Y. Cho, N. D. Turner, L. A. Davidson, R. S. Chapkin, R. J. Carroll and J. R. Lupton, *Exp. Biol. Med.*, 2012, **237**, 1387–1393.

[55] F. Odun-ayo, J. Mellem, T. Naicker and L. Reddy, *Anticancer Res.*, 2015, **4776**, 4765–4775.

[56] B. Gómez, B. Gullón, R. Yáñez, H. Schols and J. L. Alonso, *J. Funct. Foods*, 2016, **20**, 108–121.

[57] T. L. Miller and M. J. Wolin, 1996, **62**, 1589–1592.

[58] J. Fernández, S. Redondo-Blanco, I. Gutiérrez-del-Río, E. M. Miguélez, C. J. Villar and F. Lombó, *J. Funct. Foods*, 2016, **25**, 511–522.

[59] H. Zeng, *World J. Gastrointest. Oncol.*, 2014, **6**, 41–51.

[60] D. L. Topping and P. M. Clifton, *Physiol. Rev.*, 2001, **81**, 1031–1064.

[61] A. Wächtershäuser and J. Stein, *Eur. J. Nutr.*, 2000, **39**, 164–171.

[62] A. H. Uga, P. Loganathan, A. Habeeb and M. Oyeka, *Ann. Clin. Case Reports*, 2019, **4**, 3–4.

[63] H. Hove and P. B. Mortensen, *Dig. Dis. Sci.*, 1995, **40**, 1372–1380.

[64] C. De Filippo, D. Cavalieri, M. Di, M. Ramazzotti and J. Baptiste, *Proc. Natl. Acad. Sci.*, 2010, **107**, 14691–14696.

[65] R. E. Ley, P. J. Turnbaugh, S. Klein and J. I. Gordon, *Nature*, 2006, **444**, 1022–1023.

[66] N. Larsen, C. Bussolo de Souza, L. Krych, T. Barbosa Cahú, M. Wiese, W. Kot, K. M. Hansen, A. Blennow, K. Venema and L. Jespersen, *Front. Microbiol.*, 2019, **10**, 1–13.

[67] H. J. Flint, K. P. Scott, S. H. Duncan, P. Louis and E. Forano, *Gut Microbes*, 2012, **3**, 289–306.

[68] E. C. Martens, E. C. Lowe, H. Chiang, N. A. Pudlo, M. Wu, P. Nathan, D. W. Abbott, B. Henrissat, H. J. Gilbert, D. N. Bolam and J. I. Gordon, *Plos Biol.*, 2011, **9**, 1–16.

[69] A. Ferreira-Lazarte, F. J. Moreno, C. Cueva, I. Gil-Sánchez and M. Villamiel, *Carbohydr. Polym.*, 2019, **207**, 382–390.

[70] S. Miquel, R. Martín, O. Rossi, L. G. Bermúdez-Humarán, J. M. Chatel, H. Sokol, M. Thomas, J. M. Wells and P. Langella, *Curr. Opin. Microbiol.*, 2013, **16**, 255–261.

[71] I. Mukhopadhya, R. Hansen, E. M. El-omar and G. L. Hold, *Nat. Rev. Gastroenterol. Hepatol.*, , DOI:10.1038/nrgastro.2012.14.

[72] G. P. Donaldson, S. M. Lee and S. K. Mazmanian, *Nat. Rev. Microbiol.*, 2016, **14**, 20–32.

[73] K. Endt, B. Stecher, S. Chaffron, E. Slack, N. Tchitchek, A. Benecke, K. Endt, L. Van Maele, J. Sirard, A. J. Mueller, M. Heikenwalder, J. Andrew, R. Strugnell, C. Von Mering and W. Hardt, *PLOS Pathog.*, 2010, **6**, 1–18.

[74] H. Song, W. Wang, B. Shen, H. Jia, Z. Hou, P. Chen and Y. Sun, *Cancer Sci.*, 2018, 666–677.

[75] B. Flemer, M. Herlihy and M. O. Riordain, *Gut Microbes*, 2018, **0**, 1–5.

[76] C. Ferrario, R. Statello, L. Carnevali, L. Mancabelli, C. Milani, M. Mangifesta, S. Duranti, G. A. Lugli, B. Jimenez, S. Lodge, A. Viappiani, G. Alessandri, M. D. Asta, D. Del Rio, A. Sgoifo, D. Van Sinderen, M. Ventura and F. Turroni, *Front. Microbiol.*, 2017, **8**, 1–11.

[77] C. M. Guinane and P. D. Cotter, *Ther. Adv. Gastroenterol. Rev.*, 2013, 295–308.

[78] P. Louis and S. H. Duncan, *Nat. Rev. Gastroenterol. Hepatol.*, 2012, **9**, 577–589.

Figure

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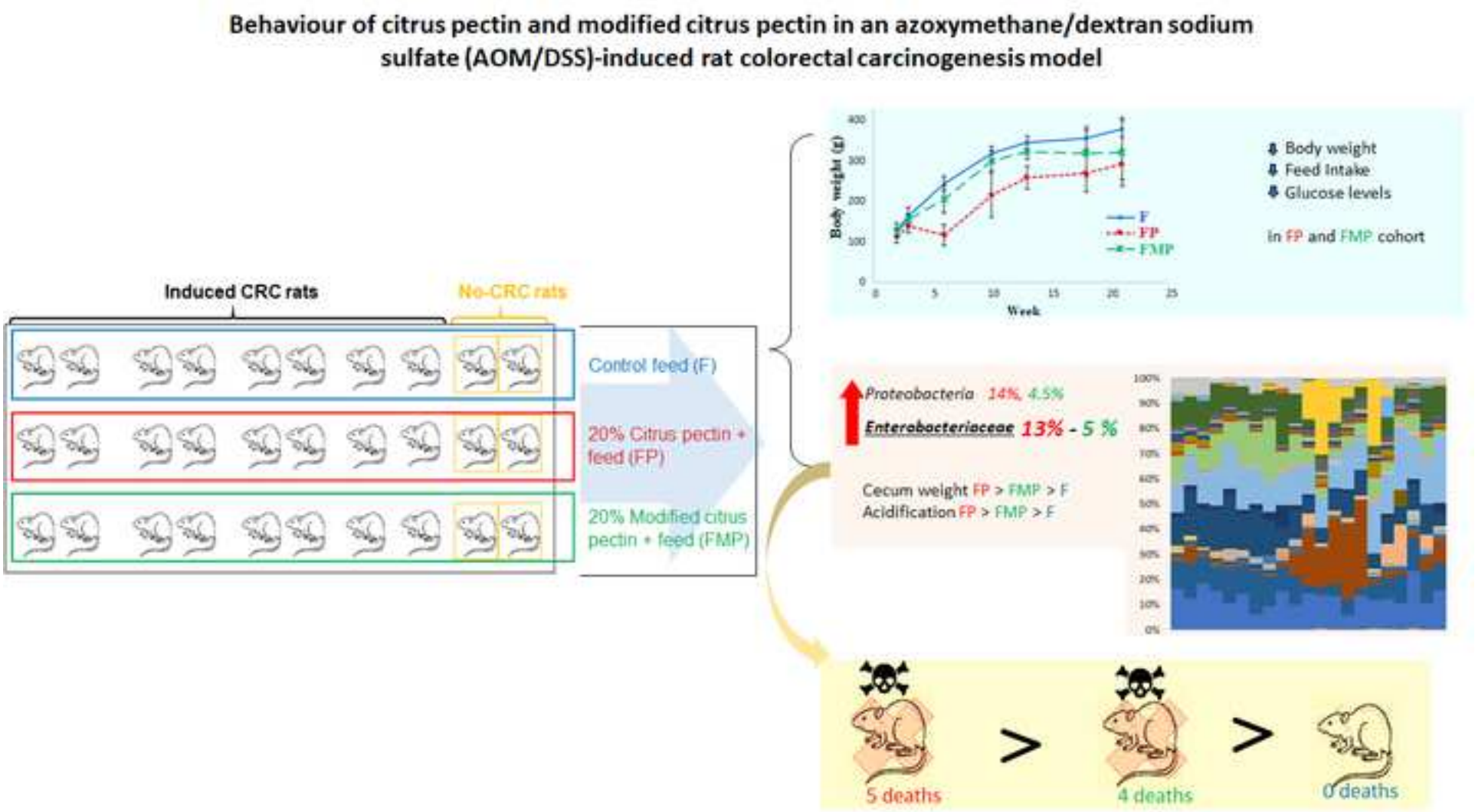

Table

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