Polyphenol addition to cooked ham modifies the abundance of fecal volatile metabolites involved in the formation of preneoplastic colon lesions

Claudia Giménez-Campillo
University of Murcia
Natalia Campillo
University of Murcia
Natalia Arroyo-Manzanares
University of Murcia
Carlos M. Martínez
Instituto Murciano de Investigación Biosanitaria
Carlos de Torre Minguela (carlos.de3@um.es)
University of Murcia
Pilar Viñas
University of Murcia

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Abstract

Epidemiological studies have shown that haem iron from processed meat is a key element involved in the colon carcinogenesis. The haem iron induces lipid peroxidation in the colon lumen during digestion, enabling the formation of cytotoxic molecules derived from these reactions. The cytotoxic molecules generated are highly dependent on dietary factors such as lipid sources, calcium levels or the presence of antioxidants during digestion. Here, we investigated whether nitrite substitution by polyphenols as a food additive could modulate the *in vivo* luminal lipid peroxidation in the colon and consequently, reduce the formation of mucin-depleted foci in a chemical-induced colon cancer rat model. The addition of polyphenols to the cooked ham increases its antioxidant capacity, reducing the lipid peroxidation measured before and during the digestion. We observed a reduction in cytotoxic aldehydes in fecal water from animals fed with polyphenols as well as a decrease in the formation of mucin-depleted foci. The antioxidant capacity derived from polyphenols modifies the luminal environment of the colon, allowing the identification of a specific molecular signature derived from the analysis of fecal volatile organic compounds. In this molecular signature is included the reduction in the abundance of (2E,4E)-2,4-hexadienal, a carcinogenic aldehyde derived from lipid peroxidation.

1. INTRODUCTION

A large number of epidemiological studies in humans have made it possible to establish a positive association between the consumption of processed meat and the risk of developing colon cancer [1–4]. Consequently, in 2015, the International Agency for Research on Cancer (IARC) classified the consumption of processed meat as carcinogenic to humans[5]. The Working Group pointed out the role of several components of processed meat involved in colon carcinogenesis, both endogenous, as haem iron, and exogenous molecules generated during their elaboration, as nitroso-compounds (NOCs) or heterocyclic aromatic amines.

In the last years, several animal models have been evaluated to clarify the molecular mechanisms that could link the haem iron and the red meat to colorectal cancer, analyzing the composition of feces from animals fed with haem or meat diet [6]. The animals fed with high concentration of haem as hemin or hemoglobin showed increased levels of luminal lipid peroxidation in the colon, measured as Thiobarbituric Acid Reactive Substances (TBARS) that correlated with increased fecal water cytotoxicity *in vitro*[7–10]. These results were highly dependent on dietary factors such as lipid sources and calcium levels, but demonstrated the *in vivo* carcinogenic role of aldehydes derived from luminal lipid peroxidation in the colon[11].

In addition, several studies have shown the *in vivo* promotion of mucin depleted-foci (MDF), preneoplastic lesions in the colon, through the promotion of luminal lipid peroxidation in the colon. This *in vivo* model employed an initiation step with an intraperitoneal injection of genotoxic chemical followed by a low-calcium, high-lipid diet supplemented with high levels of haem as processed meat[12–14]. The carcinogenic effect of processed meat in this animal model depends on luminal lipid peroxidation in the colon and can

be reduced by directly adding antioxidants to the diet, such as tocopherol[15], or by marinating processed meat with vegetable extracts[16].

The analysis of fecal water using high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC– HRMS) has enabled to track the formation of some toxic aldehydes derived from *in vivo* lipid peroxidation[17]. This methodology requires an initial step of derivatization that allows to quantify the abundance of multiple toxic aldehydes such as malondialdehyde (MDA) and (2E)-4-hydroxy-non-2-enal in the aqueous environment of the colon. However, this approach is not able to study the changes in other small molecules present in the colon.

Fecal volatile organic compounds (VOCs) are molecules derived from complex physiological processes present in the large intestine. In this anatomical location, the diet composition and microbiome play an important role in the generation of these compounds together with the colon epithelial cells. These VOCs are small molecules with a high vapor pressure at room temperature that can be easily analyzed by gas chromatography–mass spectrometry (GC-MS), allowing to identify metabolites associated with pathophysiological processes in colon [18,19].

In the present study, we have explored whether the incorporation of polyphenols in processed meat could modify the fecal volatile metabolites profile *in vivo* and, consequently, reduce the formation of genotoxic molecules such as the products derived from lipid peroxidation. Moreover, we have tested the effect of this processed meat with a higher antioxidant capacity on the formation of preneoplastic lesions using a chemical-induced colon carcinogenesis model where luminal lipid peroxidation in the colon is promoted.

2. RESULTS AND DISCUSSION

2.1. Cooked ham analysis

In accordance with the animal model selected for this study[13], cooked hams were air exposed at 4°C in the dark for 5 days before the elaboration of animal diets. In this step, we explored the role developed by nitrite and polyphenols on the formation of toxic molecules during the oxidation of meat. The addition of polyphenols significantly decreased the levels of hexanal, a marker of lipid peroxidation[20], compared with cooked hams containing nitrite (Table 1). In agreement with these results, we obtained a ten-fold higher level of antioxidant activity (radical scavenging capacity) in cooked hams produced with polyphenols compared to those produced with nitrite (Table 1).

Table 1
Cooked ham analysis after their air exposure for five days at 4°C in the dark.

	Nitrite	Polyphenols
Lipid peroxidation (hexanal (mg/kg ham))	6.6 ± 1.4	2.6 ± 0.3***
Radical scavenging capacity (Trolox eq µmol/100 g ham)	37 ± 3	424 ± 23***
Nitrosyl-heme (N_2O (mg/kg ham))	42 ± 19	N.D. ***
N.D.: Not detectable (limit of detection, LD 0.29 mg/kg)		
n = 5 independent samples of each kind of cooked hams obtained in 5 different weeks.		
*** Indicates significant difference with nitrite cooked ham ($p < 0.001$, Mann-Whitney test).		

The addition of nitrites in the elaboration of cooked hams has been proposed as a factor involved in the reduction of protein and lipid peroxidation *in vitro*[21]. Our data highlight a significant improvement in protection against lipid peroxidation due to the substitution of nitrite by polyphenols in the elaboration of cooked hams. Besides that, the nitrosyl-haem content detected in polyphenol-cooked hams was very low, under detection limit, compared with nitrite-cooked hams (Table 1), suggesting also a decreased risk in the formation of nitrosylated molecules.

2.2. Histopathological changes in colon epithelium from rats fed with cooked ham diet

The animals fed with cooked ham diets gained similar weight than control animals fed with meat-free diet (Fig. 1A). Pathological examination of colon epithelium from animals exposed to a haem-enriched diet shows a hyperproliferation of intestinal epithelium in response to haem-induced damage[11,22,23]. In the present study, the histological analysis of the colon epithelium of rats fed with cooked ham diets showed significant thickening compared to animals fed with meat-free diet, as we observed a significant growth in crypt length in animals treated with cooked ham diets, which was greater in polyphenol-meat diet group (Fig. 1B). This colon epithelium thickening seems to be not homogeneous because we observed a significant thickening in the proximal colon segment of animals fed with polyphenol-meat diet (Fig. 1C). In contrast to the other colon segments for both groups of animals fed with cooked hams, where no differences could be observed.

Additionally, we explored the nature of these changes in colon epithelium of animals fed with meat diet and observed diet-depending changes in the number of goblet cells present in the proximal and middle colon segments. The quantification of goblet cells showed a significant increase of these cells in proximal and middle colon segments of animals fed with polyphenol-meat diet (Fig. 1D-E). These results suggest a protective effect of polyphenols against the damage induced by processed meat in the colon epithelium. The presence of a higher number of goblet cells in animals fed with polyphenols favor the

maintenance of mucous layer avoiding chronic inflammatory processes which may be involved in colon carcinogenesis [24].

In addition to these histopathological changes observed, we also observed a significant reduction in the number of MDF detected in animals fed with polyphenol-meat diet (Fig. 2A-B). This reduction in the number of MDF was also significant considering proximal and middle colon segments (Fig. 2C). Taken together, these results suggest a protective role of polyphenols against colon tumorigenesis promoted by processed meat diet in a carcinogen-induced rats model.

2.3. Genotoxic molecules in fecal water from rats fed with cooked ham diets

The formation of genotoxic molecules induced by nitrosylated and non-nitrosylated haem iron during the gastrointestinal digestion of red meat is associated with colon carcinogenesis promotion[11,25]. In particular, the generation of genotoxic molecules derived from lipid peroxidation contributes to the formation of preneoplastic lesions, MDF, in the carcinogen-induced rat model used in this study[11,13,26]. Moreover, the addition of antioxidants to diet containing meat with nitrites as preservative has shown a beneficial effect inducing a reduction in meat-induced carcinogenesis as well as the generation of genotoxic molecules in the same animal model[15,27].

The measurement of fecal aldehydes by TBARS assay is a marker of intestinal lipid peroxidation[8] and according with published data, the addition of cooked ham in animal diets induced the formation of a high amount of fecal aldehydes that can be measured as MDA equivalents (MDA eq) in the fecal water. The substitution of nitrate by polyphenols in the cooked ham diets produced a significant reduction in the *in vivo* formation of TBARS (Table 2). These data support a link between the antioxidant capacity of polyphenols and the protective effect observed in the carcinogen-induced rat model, associated with a limitation of luminal lipid peroxidation in the colon. In this study, we incorporate the polyphenols during the elaboration of cooked hams instead of simply marinating the meat with a vegetal extract with polyphenols, which also reduces the formation of toxic fecal aldehydes.

Table 2
Fecal water analysis from rats fed with meat-free diet or cooked ham diets for 90 days after AOM i.p. injection

	Nitrite	**Polyphenols**
TBARS in FW (MDA eq µmol/24h)	65 ± 19	48 ± 16^{+}
AOM, azoxymethane; FW, fecal water; MDA, malondialdehyde; TBARS, Thiobarbituric Acid Reactive Substances		
Nitrite-meat diet and polyphenol-meat diet (n = 12)		
+ Indicates significant difference with nitrite-meat diet ($p < 0.05$, Mann-Whitney test).		

2.4. Analysis of fecal VOCs from rats fed with different cooked ham diets

A total of 104 VOCs were detected in each of the 24 fecal samples analyzed (12 corresponding to stools from rats fed with nitrite-meat diet and 12 corresponding to stools from rats fed with polyphenols-meat diet). Among them, eighteen VOCs were fully identified, including carbon chains with single bonds (hexane, octane, methylcyclohexane), with double bonds (1-butene, 1-pentene), ketones (acetone, 2-heptanone, 3-methyl-2-butanone, 2-pentanone), aldehydes (hexanal, (2E,4E)-2,4-hexadienal, 3-methylbutanal), alcohols (1-hexanol, 2-heptanol, 2-hexen-1-ol, 1-butanol), esters (propyl acetate) and nitrogen compounds (4-ethyl-1-methyl-4,5-dihydro-1H-pyrazole).

Univariate analysis performed with 104 detected VOCs showed significant differences in 22 of them, highlighting the decrease of two aldehydes derived from lipid peroxidation in the samples from rats fed with polyphenol-meat diet (Table 3). In accordance with the results obtained from cooked ham analysis, we observed a significant reduction of hexanal abundance, a marker of lipid peroxidation, in the samples from rats fed with polyphenol-meat diet. Moreover, we also identified a reduction in the abundance of (2E,4E)-2,4-hexadienal, a naturally formed product from autoxidative degradation of polyunsaturated fatty acids with a well stablished carcinogenetic activity[28]. These results support the *in vivo* antioxidant activity developed by polyphenols included in the cooked hams and their protective effect derived from the reduction in the formation of genotoxic molecules as (2E,4E)-2,4-hexadienal.

Table 3
Fecal VOCs with a significant difference in abundance between rats fed with a nitrite-containing and polyphenol-containing meat diet.

RT (min)	ID	Compound	*m/z*	Fold change	p-value
10.79	18	Hexane	56.9	1.45	0.0086
11.74	24	Unidentified	42.0	1.29	0.0304
11.98	27	Unidentified	42.9	1.41	0.0351
14.67	40	Unidentified	55.9	1.34	0.0226
14.67	41	Unidentified	40.9	1.44	0.0226
16.18	49	Unidentified	56	1.87	0.0464
16.26	51	Octane	42.9	1.69	0.0043
16.55	53	Unidentified	43.0	1.44	0.0464
16.56	54	1-Pentene	40.9	1.43	0.0464
17.14	56	Hexanal	55.9	1.41	0.0061
18.46	64	1-Hexanol	56.0	1.82	0.0043
18.94	67	2-Hexen-1-ol	43.0	1.52	0.0086
18.97	68	Unidentified	55.9	1.38	0.0029
19.89	74	(2E,4E)-2,4-Hexadienal	40.9	1.65	0.0262
19.91	75	Unidentified	56.0	1.48	0.0262
19.96	76	Unidentified	56.0	1.43	0.0029
20.01	77	3-Methyl-2-butanone	43.0	1.39	0.0262
20.20	79	Unidentified	43.0	1.45	0.0304
20.34	80	Unidentified	41.0	1.50	0.0061
21.23	89	Methylcyclohexane	41.0	1.45	0.0086
21.55	93	1-Butanol	41.0	1.54	0.0013
22.43	99	Unidentified	40.9	1.49	0.0141

RT, retention time; ID, identification number provided by MS-DIAL; Fold change nitrite-meat diet/polyphenol-meat diet; p value determined by Mann-Whitney test.

In order to obtain a comprehensive view of the impact of polyphenols as food additive in the colonic luminal environment, we performed an unsupervised analysis of all fecal VOCs detected in the animal study. Through principal component analysis (PCA), we observed that the data set obtained from the

analysis of fecal VOCs allows to partially discriminate the samples belonging to the two groups studied (Figure S1), which suggests the presence of a molecular signature that could characterize the polyphenol-meat diet group.

We applied a supervised analysis building an orthogonal partial least squares discriminant analysis (OPLS-DA) model using Unit Variance (UV) scale to determine the dominant factors present in fecal VOCs data set that allow a full discrimination between the samples obtained from rats fed with polyphenol-meat diet and rats with fed nitrite-meat diet. Figure 3A shows the full discrimination of the groups studied (Q2 value of 0.636), which allows to demonstrate the validity of the model. Furthermore, the cross-validation percentage is 100%, it does not misclassify any sample.

The calculation of the variable influence on the projection (VIP) obtained for each fecal VOC included in the OPLS-DA model showed that 44 of them have a significant weight in the discriminant model (VIP value > 1) and therefore, this set of 44 fecal VOCs could be considered as a molecular signature of the effect of polyphenols in the luminal environment of the colon (Table S1). When constructing the OPLS-DA model, the chemometric model takes into account the collinear variables, not each variable separately as in univariant statistical tests. Therefore, in addition to 20 fecal VOCs showing significant differences in univariate analysis, a further 24 fecal VOCs were considered by the mathematician model.

It should also be noted that among the markers with a VIP value greater than 1, there are 14 of the identified compounds (only 4 of the identified compounds have no weight for the model) and 30 that could not be identified. In addition, all the aldehydes identified considered as products of lipid peroxidation (hexanal, (2E,4E)-2,4-hexadienal) are included in the set of 44 fecal VOCs with significant weight in the discriminant model and the analysis of the relative abundance of fecal VOCs showed a higher overall abundance of fecal VOCs in the group of animals fed a nitrite-containing meat diet (Fig. 3B).

The loading plot of the OPLS-DA model shows the weight of each fecal VOC in the discrimination between the group of rats fed with nitrite-meat diet and the group of rats fed with polyphenol-meat diet (Fig. 3C). Despite the large number of fecal VOCs studied, it is clearly observed how only a reduced number of fecal VOCs contributes to the discrimination of the group of animals fed with a polyphenolic-meat diet, while the majority are involved in the discrimination of nitrite-meat diet including all the fecal VOCS with a VIP value > 1.

Overall, the results of multivariable analysis obtained from OPLS-DA model have shown a molecular signature that demonstrates the influence of polyphenols in the luminal environment of the colon reducing the occurrence of numerous VOCs including genotoxic aldehydes as (2E,4E)-2,4-hexadienal. Consequently, the reduction in the number of fecal VOCs would be associated with the physiological effects observed in the chemical-induced colon cancer rat model: proliferation of globet cells in the colonic epithelium and the reduction in the formation of preneoplastic lesions (mucin-depleted foci).

3. CONCLUSION

The substitution of nitrate by polyphenols in the cooked ham diets has shown a beneficial effect by reducing the formation of genotoxic fecal aldehydes and preneoplastic lesions in a carcinogen-induced rat model. In addition, the analysis of fecal VOCs profiles obtained from this animal model has shown a characteristic signature associated with the presence of polyphenols that allows to differentiate from group fed with nitrite-meat diet and suggest a role as modifier of colon luminal environment. In summary, these results suggest an antioxidant and protective effect developed by polyphenols in the large intestine that affects the abundance of toxic fecal metabolites.

4. MATERIAL AND METHODS

4.1 Reagents

Key reagents and their sources were as follows: azoxymethane (AOM), 1,1,3,3-tetramethoxypropane, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-thiobarbituric acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), chlorobenzene, hexanal, 1-octanol and formaldehyde were supplied by Sigma Aldrich (St. Louis, MO, USA) and dimethyl sulfoxide, methanol and sulfuric acid by AppliChem GmbH (Darmstadt, Germany). All solvents used in this work were HPLC grade. Chlorobenzene was used as internal standard (IS) in the HS-GC–MS analyses. This was prepared by diluting 2 µL of chlorobenzene in 25 mL of dimethyl sulfoxide to obtain a concentration of 80 µL/L. Polyphenol-rich extract was obtained from PROSUR SAU (Murcia, Spain), commercialized as NATPRE T-10 HT S (20% of total polyphenols).

4.2 Animal study design

Twenty-eight rats (F344/DuCrl), between 4 and 5 weeks of age, were obtained from Charles River Laboratories (St Germain l'Arbresle, France). The animals were housed in cages by pairs under controlled light conditions (12 h light-dark cycle) and controlled temperature/humidity conditions in accordance with the animal experimentation guidelines. The animals were maintained in the Animal Facilities at the University of Murcia (authorized facility No. ES 300305440012) and both the experimental design and methods performed followed ARRIVE guidelines for animal research. All animal experiments were approved by Ethical Committee for Animal Experimentation of the University of Murcia (authorization code: A13211206) in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. After seven days of acclimatization, twenty-four rats received a single i.p. injection of AOM (20 mg/kg) diluted in 0.9% m/v sodium chloride aqueous solution and were randomly allocated to two groups (nitrite-meat diet n = 12 and polyphenol-meat diet n = 12). Four animals were fed with meat-free diet as controls. The remaining animals were fed with a specific diet based upon the group of study (nitrite-meat diet or polyphenol-meat diet). Body weights were monitored every week during the study (90 days after AOM injection). Between days 78 and 80, each rat was put in a metabolic cage and stools and urine were collected and frozen at -20°C. At day 90, animals were killed by CO_2 asphyxiation in a random

order and colons were removed and fixed in 4% buffered formaldehyde (Panreac Química, Barcelona, Spain).

4.3 Animal diets

The experimental diets were elaborated using modified AIN76 powered diet according to Santarelli et al[13]. Both meat-free diet and base diet used for cooked ham diets were made by Research Diets Inc. (New Brunswick, NJ) and their composition was balanced for protein, iron and fat. The composition of meat-free diet was (g/100 g): casein, 46.7; sucrose, 27.8; corn starch, 5.9; cellulose, 5.0; lard, 5.2; safflower oil, 5.00; mineral mix S10001, 3.5; vitamin mix V10001, 1.0; DL-methionine, 0.6; choline bitartrate, 0.20. The composition of base diet without ham was (g/100 g): casein, 5.0; sucrose, 27.8; corn starch, 5.0; cellulose, 5.0; safflower oil, 5.00; mineral mix S10001 calcium free, 3.5; dicalcium phosphate, 0.21; vitamin mix V10001, 1.0; DL-methionine, 0.3; choline bitartrate, 0.20.

Cooked hams were elaborated from pig shoulder meat at the PROSUR Meat Laboratory. Nitrite ham was cured with 1.8 g of salt, 0.012 g sodium nitrite (120 ppm $NaNO_2$) and 0.03 g sodium ascorbate per 100 g of meat, meanwhile polyphenol ham was cured with 1.4 g of salt and 1 g polyphenol rich extract (NATPRE T-10 HT S) per 100 g of meat. Hams were then cooked until reach 68°C core temperature (maximum oven temperature was 73–75°C). Cooked hams of 1.3 kg were stored at -20 ºC in vacuum sealed plastic bags with low-oxygen permeability until their use. Before mix with powered diet base to elaborate cooked ham diets, slices of cooked hams were air exposed at 4°C in the dark for 5 days for their oxidation according to Santarelli et al[13]. The cooked ham diets were elaborated by mixing 53 g of powered base diet with 187 g of ham.

4.4 Hexanal analysis of cooked hams

Hexanal was analyzed by headspace with gas chromatography and mass spectrometry (HS-GC-MS) consisting of a 7890A GC-System gas chromatograph from Agilent Technologies (Agilent Technologies, Palo Alto, California, USA), equipped with a temperature-controlled vaporizer (PTV) model CIS4-C506 and an automatic injector (Headspace model Multipurpose Sampler MPS), both from Gerstel (Mülheim an der Ruhr, Germany) following a chromatographic protocol described by Mandić et al[29]. The GC system was coupled to a mass spectrometer (5975C inert MSD-triple axis detector from Agilent Technologies). Briefly, cooked ham samples (1 g) placed in 10 mL vials containing 10 µL of 80 µL/L chlorobenzene (IS) solution were heated in the HS sampler at 80°C for 30 min, and then injected (12 mL/min for 10 s) into the GC system with a split ratio (1:25) while the inlet was maintained at 250°C. Hexanal in samples was separated by a DB-624 column (60 m, 0.25 mm, 1.4 µm), applying a temperature programme consisting of an initial temperature of 40°C for 5 min, which was increased by 10°C/min to 150°C, held for 2 min, and next was increased at 25°C/min to 240°C and then maintained at 240°C for 10 min. The mass spectrometer was operated using electron-impact (EI) mode (70 V) and the temperature of the ion source was 230°C. Analyses were carried out using selected ion monitorization (SIM) mode. Standard solutions in the 0.030–0.158 µg/mL concentration range were used for calibration purposes. The Mass Hunter

Workstation Data Acquisition software (Agilent Technologies, Palo Alto, California, USA) was applied for data processing.

4.5 Nitrosyl-haem content analysis of cooked hams

Nitrosyl-haem content of cooked hams was analyzed according to Hernández et al[30]. Briefly, 15 g of cooked ham was homogenized with 5 g of ice and 12 mL of water at 7000–9000 rpm using a homogenizer (IKA, t25 digital Ultraturrax), then the mixture was centrifuged at 1125 x g for 5 min at 10°C. An aliquot of 8 mL of supernatant was transferred into a 20 mL vial and then, 8 µL of 1-octanol and 4 mL of 20% v/v sulfuric acid were added. The vial was immediately sealed and gently shaken for 30 s. The samples were incubated for the headspace analyses at 30°C for 45 min, then injected into the 7890A GC system equipped with an Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, California, USA).

4.6 Radical scavenging capacity of cooked ham

The radical scavenging capacity of cooked hams was determined by DPPH assay according to Cheng et al[31]. Briefly, 5 g of cooked ham was homogenized with 10 mL of methanol using a homogenizer (IKA, t25 digital Ultraturrax) at 3000–4000 rpm for 2 min and then, the mixture was centrifuged at 1620 x g during 15 min. The supernatant was used to determine radical scavenging capacity and the absorbance at 515 nm was monitored using a UV-visible spectrometer plate reader (Multiscan GO, Thermo Scientific, USA).

4.7 Tissue collection, processing and histopathological analysis of colon tissue

The whole colon was extracted and fixed in 4% buffered formaldehyde (Panreac Quimica, Barcelona, Spain) for 24 h. The whole organ was divided in 3–4 sections which were placed into histologic cassettes. The samples were then processed and paraffin embedded. To determine the number of MDF, three micrometers-thick sections of the samples were stained with Alcian blue pH 2.5 histochemical procedure by using a commercial kit (Vector Laboratories, Burlingame, California) following the commercial recommendations. Briefly, after deparafination and rehydration, sections were treated with acetic acid for 3 min, and incubated with Alcian Blue solution for 30 min at 37 ºC. The sections were then discolored with acetic acid and counterstained with Fast Red solution. Positive stain was identified as a light-blue cytoplasmic stain. MDF were identified according with the criteria described previously by Caderni et al. (2003)[32]: little or absence of crypt mucin in more than 3 crypts, distortion of the lumen of the crypts and/or elevation of the lesion in comparison with normal mucosa. Numbers of MDF per colon and their multiplicity (crypts per MDF) were counted under a light microscope at high magnification x400.

The media length of the colonic villous was determined by measuring villous length in x100 magnified images from longitudinal sections of the specimens stained with a standard hematoxylin and eosin (H&E) stain procedure in 20 random fields. The number of goblet cells were determined by counting all goblet cells in 5 random fields (x100) from longitudinal sections of the specimens stained with Alcian Blue. The result was expressed by the median of goblet cells per field. All the slides and morphometric

determinations were performed by using a standard Zeiss Axio Scope AX10 light microscope (Carl Zeiss) with a high resolution digital camera (AxioCam 506 color) with a commercial digital analytic software (Zeiss Zen, Ver. 3.0).

4.8 TBARS analysis in fecal water

Fecal water was prepared according to Pierre et al[8] by reconstituting freeze-dried stools by adding 1 mL of distilled water to 0.5 g of freeze-dried stools. Samples were incubated at 37°C for one hour, mixed thoroughly during the incubation time and then centrifuged for 15 min at 13200xg. The supernatant (fecal water) was analyzed immediately. TBARS were measured in fecal water according to Ohkawa et al[33]. The amount of TBARS was determined as MDA equivalents by measure of the absorbance of the butanol extract at 532 nm using a UV-visible spectrometer plate reader (Multiscan GO, Thermo Scientific, United States) against a standard (1,1,3,3-tetramethoxypropane at 0, 0.5, 1, 1.5, 2 µM).

4.9 Analysis of fecal volatile organic compounds (VOCs)

Fecal VOCs were analyzed by HS-GC-MS on a 7890A GC-System gas chromatograph from Agilent Technologies (Agilent Technologies, Palo Alto, California, USA), equipped with a temperature-controlled vaporizer (PTV) model CIS4-C506 and an automatic injector (Headspace model Multipurpose Sampler MPS), both from Gerstel (Mülheim an der Ruhr, Germany). The GC system was coupled to a mass spectrometer (5975C inert MSD-triple axis detector from Agilent Technologies). Fecal samples (1 g) placed in 10 mL vials containing 10 µL of 80 µL/L chlorobenzene (IS) solution were heated in the HS sampler at 80°C for 30 min, and then injected (12 mL/min for 10 s) with a split ratio (1:10) into the GC system through the inlet maintained at 250°C. VOCs in samples were separated in a DB-624 column (60 m, 0.25 mm, 1.4 µm), by applying a temperature programme at an initial temperature of 40°C for 5 min, which was increased by 10°C/min to 150°C for 2 min, then the temperature was increased 25°C/min to 240°C and then maintained at 240°C for 10 min. The mass spectrometer was operated using electron-impact (EI) mode (70 eV) and the temperature of the ion source was 230°C. Analyses were carried out using scan mode from 29 to 150 *m/z*. The Mass Hunter Workstation Data Acquisition software (Agilent Technologies, Palo Alto, California, USA) was applied for data processing.

4.10 Data processing and statistical analysis

For fecal VOCs processing, MS-DIAL software (rev. 4.80 Windows) was used to detect and identify the markers present in the samples. The mass range was set to 29–160 *m/z* based on the acquisition method. In order to perform markers detection, 1000 amplitudes were set as the minimum height, using a level of 3 scans and an average peak width of 20 scans. The data points were smoothed with a linear weighted average. A peak resolution of 0.5 was set for peak deconvolution, as lower values could lead to signal noise being misinterpreted as peaks and higher values could reduce the number of resolved chromatographic peaks. To identify the metabolites, the software uses the retention indexes calculated by the Kovats method, which requires the injection of a mixture of alkanes. The injected mixture of alkanes containing C8 to C40 was provided by Sigma Aldrich (St. Louis, MO, USA) and was analysed at a concentration of 2 µg mL^{-1} under the same conditions as the fecal samples. A tab-delimited file of the

number of carbon atoms of the alkanes detected and their retention time was then created and inserted into the identification programme. The deconvoluted spectra were compared with a GC-MS metabolomic MSP spectral library from RIKEN. For the identification purposes, tolerances of 0.5 min, 20, and 0.5 Da were set for retention time, retention index and *m/z*, respectively. A match criterion of more than 70% was taken into account. Finally, the alignment was carried out using a tolerance of 70% for the spectral similarity and a tolerance of 0.075 min for the retention time.

All features obtained by MS-DIAL were used to build chemometric models using SIMCA version 14.1 software (Umetrics, Sartorius Stedim Biotech AS, Umea, Sweden). The chemometric models (PCA and OPLS-DA) were built on the set of all samples using UV scaling. The success of the model was assessed using the following parameters: Q2(cum) (cumulative ability to predict) and percentage of cross-validation.

In order to evaluate histopathological changes and the formation of genotoxic molecules, normal distribution was evaluated by Shapiro-Wilk test. Mann-Whitney or Kruskal-Wallis tests were applied for two-group or more than two-group comparisons, respectively. Data processing, statistical analysis and graphics were performed using, Prism software (Graph-Pad Software, Inc) and R Studio version 1.4.1717 software. P-value is indicated as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; $p > 0.05$ not significant (*ns*).

Abbreviations

AOM, azoxymethane; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FW, fecal water; H&E, hematoxylin and eosin; HS-GC-MS, Headspace with gas chromatography and mass spectrometry; i.p., intraperitoneal; MDA, malondialdehyde; MDF, Mucin depleted-foci; VOCs, volatile organic compounds; TBARS, Thiobarbituric Acid Reactive Substances; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

Declarations

Author Contributions

CGC: Validation, Investigation, Data Curation, Writing – original draft preparation, Visualization. NC: Writing – review and editing, Supervision, Visualization; N.A.M: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Writing—review and editing; C.M.M.: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing—review and editing; C.d.T.-M.: Conceptualization; Investigation; Methodology; Supervision; Validation; Writing—original draft. P.V.: Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Supervision; Validation; Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The protocol and experimental procedures were approved by the Ethical Committee of Experimental Animal Care of the University of Murcia (authorization code: A13211206).

DATA AVAILABILITY

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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CONFLICT OF INTEREST

C.d.T.-M. is member of R&D Department of PROSUR SAU, a Spanish international food manufacturer. PROSUR SAU provided one of its products (NATPRE T-10 HT S) to be tested. All cooked hams studied in the project were elaborated at PROSUR SAU.

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Figures

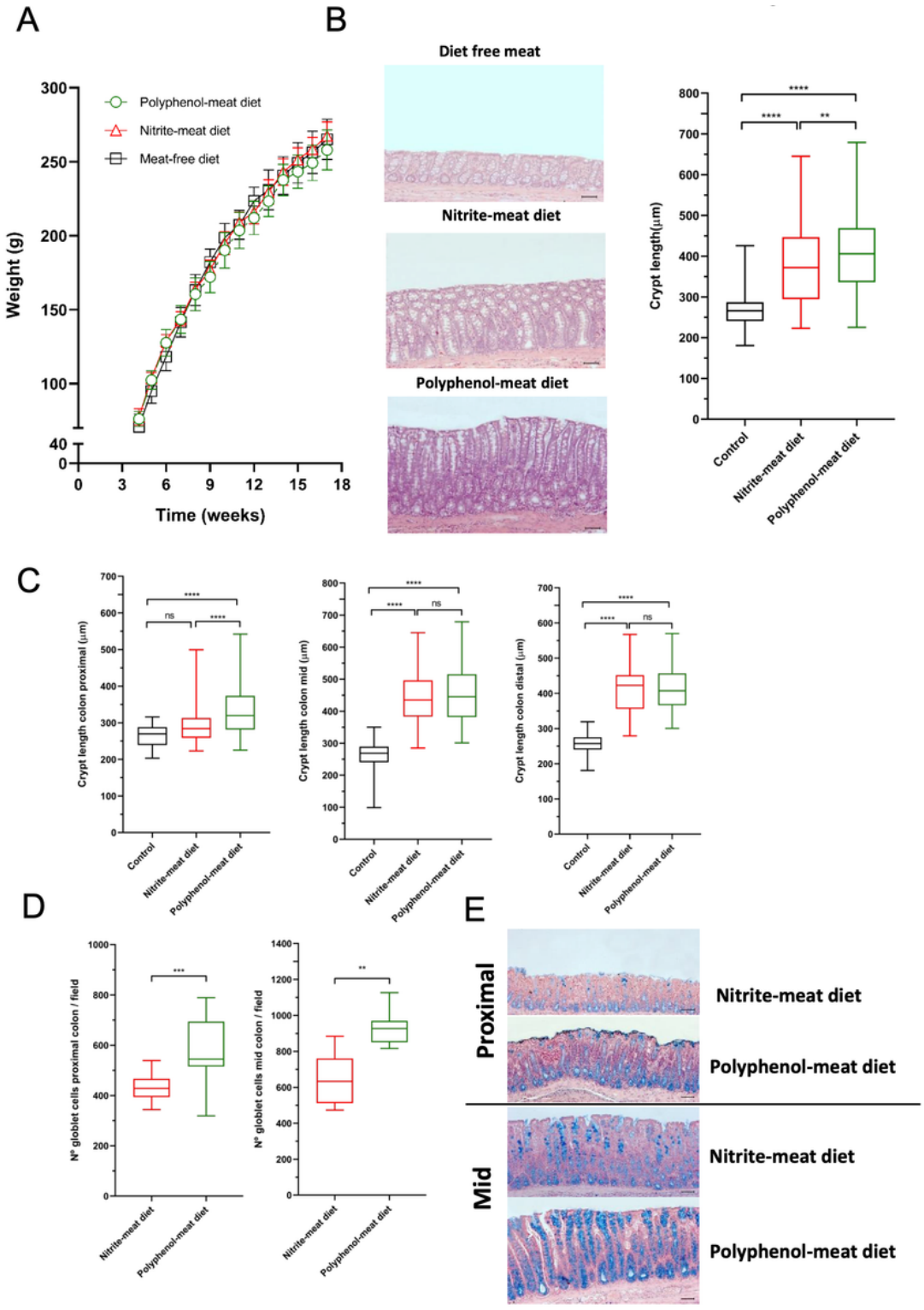

Figure 1

Effect of cooked ham diets on colon epithelium of rats fed for 90 days after i.p. injection of AOM. A) Changes in body weight. B) Representative images of colon tissue sections stained with H&E and global crypt length assessment in colon tissue. C) Crypt length assessment in proximal, middle and distal colon segments. D) Quantification of goblet cells from colon segments (proximal and middle). E) Representative images of tissue sections from proximal and middle colon stained with H&E and Alcian

blue pH 2.5. Scale bar represents 100 mm. Data presented in A are expressed as the mean ± SD. Diet meat free (n = 4), nitrite-meat diet and polyphenol-meat diet (n = 12). Data presented as boxplots, median (center line) while the box contains the 25th to 75th percentiles of dataset. The black whiskers mark the 5th and 95th percentiles. Mann-Whitney and Kruskal-Wallis test **p<0.01; ***p<0.001; ****p<0.0001; not significant (*ns*).

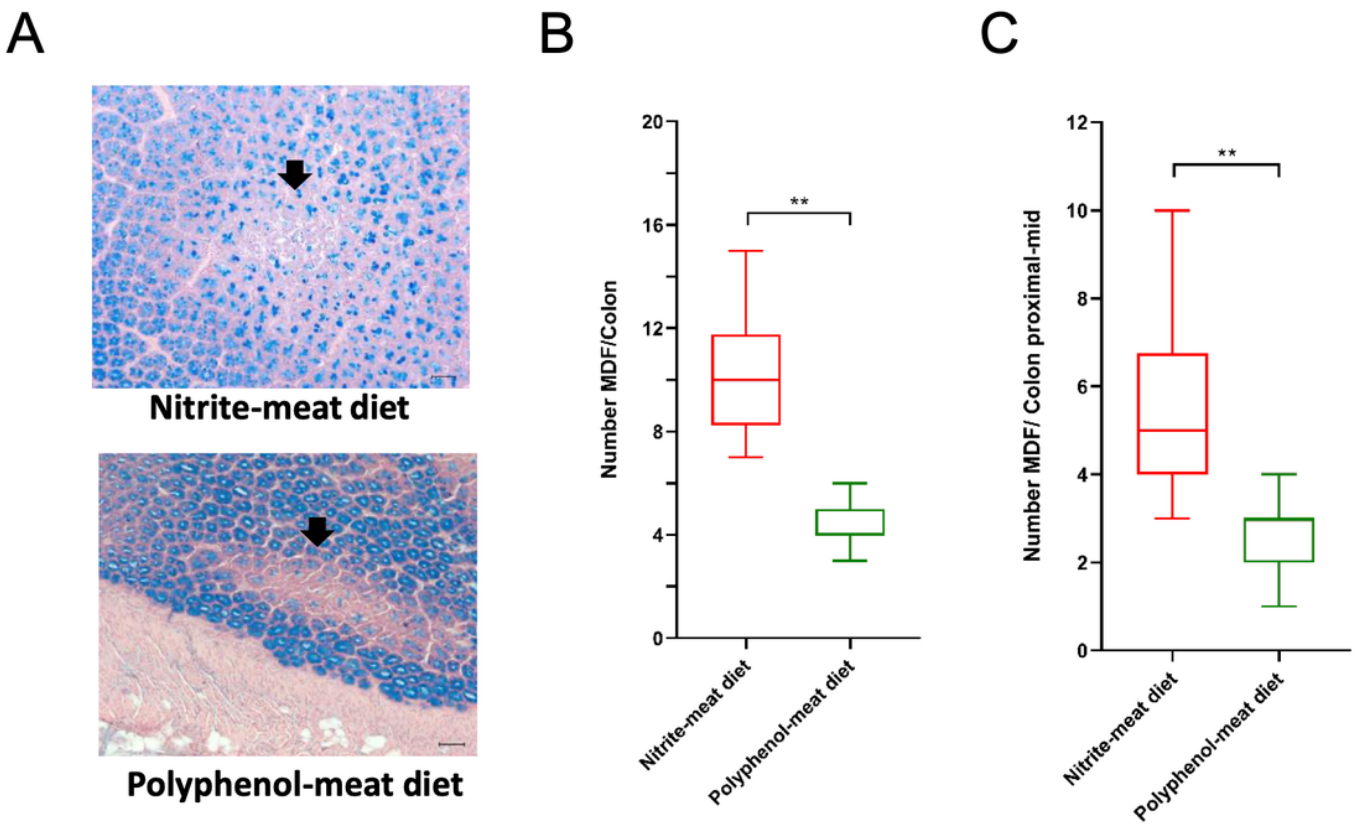

Figure 2

Reduction of Mucin-depleted foci (MDF) in colon epithelium from rats fed with polyphenol-meat diet. A) Representative images of MDF in colon tissue sections stained with Alcian blue pH 2.5. and H&E. B) Quantification of MDF lesions per colon. C) Quantification of MDF lesions per colon in the proximal and middle segments. Scale bar represents 100 mm. Data are presented as boxplots, median (center line) while the box contains the 25th to 75th percentiles of dataset. Nitrite-meat diet and polyphenol-meat diet (n = 12) Kruskal-Wallis test **p<0.01.

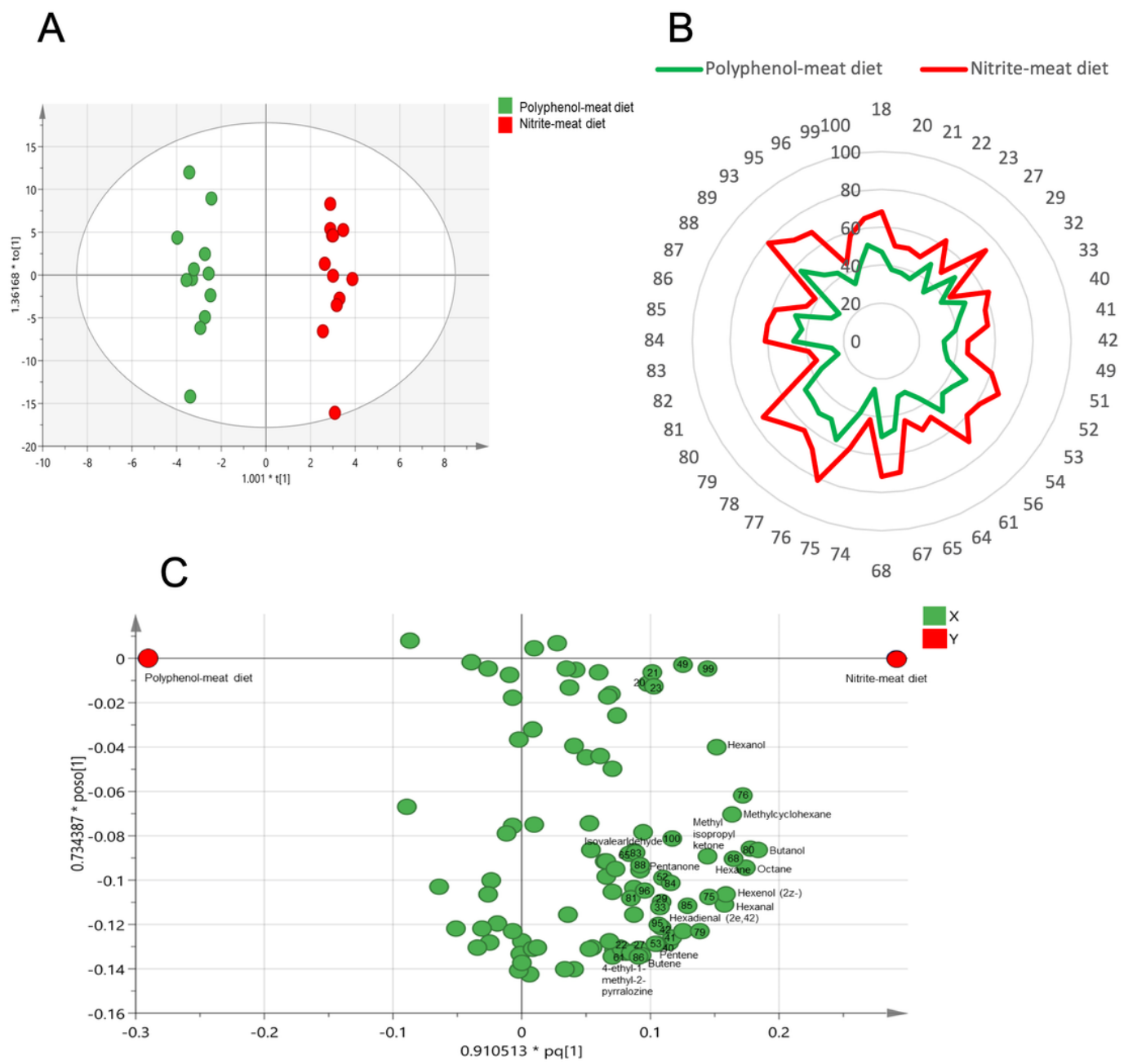

Figure 3

OPLS-DA score, loading and mean relative abundance plots. A) PCA plot obtained of OPLS-DA binary model using UV scaling to discriminate between fecal samples from rats fed with polyphenol-meat diet (blue circles) and fecal samples from rats fed with nitrite-meat diet (red circles). B) Radar chart representing the mean relative abundance of 44 fecal VOCs with a VIP>1 in the 24 samples analyzed. Polyphenol-meat diet (green plot) and nitrite-meat diet (red plot). C) Loading scatterplot of OPLS-DA binary model using UV scaling to discriminate between fecal samples from rats fed with polyphenol-meat diet and fecal samples from rats fed with nitrite-meat diet.

Supplementary Files

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