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# *Meat processing and colon carcinogenesis: cooked, nitrite-treated, and oxidized high-heme cured meat promotes mucin-depleted foci in rats*

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## Abstract

**Processed meat intake is associated with colorectal cancer risk, but no experimental study supports the epidemiologic evidence. To study the effect of meat processing on carcinogenesis promotion, we first did a 14-day study with 16 models of cured meat. Studied factors, in a 2 × 2 × 2 × 2 design, were muscle color (a proxy for heme level), processing temperature, added nitrite, and packaging. Fischer 344 rats were fed these 16 diets, and we evaluated fecal and urinary fat oxidation and cytotoxicity, three biomarkers of heme-induced carcinogenesis promotion. A principal component analysis allowed for selection of four cured meats for inclusion into a promotion study. These selected diets were given for 100 days to rats pretreated with 1,2-dimethylhydrazine. Colons were scored for preneoplastic lesions: aberrant crypt foci (ACF) and mucin-depleted foci (MDF). Cured meat diets significantly increased the number of ACF/colon compared with a no-meat control diet (P = 0.002). Only the cooked nitrite-treated and oxidized high heme meat significantly increased the fecal level of apparent total N-nitroso compounds (ATNC) and the number of MDF per colon compared with the no-meat control diet (P < 0.05). This nitrite-treated and oxidized cured meat specifically increased the MDF number compared with similar non nitrite-treated meat (P = 0.03) and with similar non oxidized meat (P = 0.004). Thus, a model cured meat, similar to ham stored aerobically, increased the number of preneoplastic lesions, which suggests colon carcinogenesis promotion. Nitrite treatment and oxidation increased this promoting effect, which was linked with increased fecal ATNC level. This study could lead to process modifications to make non promoting processed meat.**

**Author Keywords** processed meat ; cured meat ; ham ; viande saumurée ; jambon ; chercuterie ; épaule cuite ; colorectal cancer ; preneoplastic lesions ; MDF ; ACF ; lipid oxidation ; peroxydes ; cytotoxicity ; toxicology ; toxicité ; cancer promotion ; rats ; azoxymethane ; dimethylhydrazine

## Introduction

Colorectal cancer is one of the main causes of death in affluent countries. Environmental factors are involved in this cancer, particularly diet. Modifications in dietary habits could reduce this cancer burden up to 70% (1 ). In its 2007 report, the World Cancer Research Fund panel judges that “the evidence that red meat and processed meat are a cause of colorectal cancer is convincing” (2 ). The panel thus recommends: “Limit intake of red meat and avoid processed meat”, which is a challenge for the meat processing industry (3 ). Epidemiological studies indeed suggest that red meat and processed meat intake increases the risk of colorectal cancer. Three recent meta-analyzes show that consumption of red or processed meat is associated with the risk of colorectal cancer (4 –6 ). The average relative risk associated with consumption of red meat is modest but significant in the three studies (relative risk = 1.17, 1.35 and 1.28, respectively), as is the risk associated with consumption of processed meat (relative risk = 1.49, 1.32 and 1.20, respectively). We have estimated, from these meta-analyzes, that one gram of processed meat increases the risk of colorectal cancer eleven times, six times or twice more than one gram of fresh red meat respectively for the three meta-analyzes (7 ). Thus processed meat seems more closely associated with the risk of colorectal cancer than fresh red meat.

Several mechanisms have been assumed to explain the relationship between the risk of colorectal cancer and red meat intake. Red meat enhances the formation of putative carcinogenic N-nitroso compounds in human feces (8 –10 ). But N-nitroso compounds brought into the rat intestine by a bacon-based diet did not initiate nor promote preneoplastic lesions in rat colon (11 ). Meat cooked at a high temperature contains mutagenic heterocyclic aromatic amines that induce colon, mammary and prostate tumors in rodents and monkeys ( 12 ). But these aromatic amines might not play an important role in colorectal cancer incidence, since (i) chicken intake is a major contributor of aromatic amines intake, but it is not associated with the risk (13 ), and (ii) doses of aromatic amines that induce cancer in animals are 1000 to 100,000 times higher than the doses found in human food (14 ). Red meat also contains heme, the iron-bearing prosthetic group of myoglobin. Dietary hemin (heme stabilized by a chlorine atom, also called Ferriprotoporphyrin IX chloride) increases colonic epithelial proliferation and induces cytotoxicity of fecal water in rats (15 ). Dietary hemin, hemoglobin, and heme in meat promote dose-dependently the formation of preneoplastic lesions in the colon, aberrant crypt foci (ACF) and mucin depleted foci (MDF) (16 –18 ).

In addition, dietary heme increases amounts of lipid hydroperoxides in fecal water and cytotoxicity of fecal water (16 , 18 ). Since fecal water hydroperoxides and cytotoxicity were associated with heme-induced carcinogenesis, we have proposed to use these biomarkers in short term experiments to screen meat-induced promotion of colon cancer (17 ).

Processed meats cited in epidemiological studies include sausages, meat burgers, ham, bacon, and salami (7 ). Most frequent processes include salting, curing (adding sodium nitrite), smoking, cooking and drying. Through processing, the heme molecule is nitrosylated by sodium nitrite and the nitrosyl heme can be released from myoglobin during cooking (19 , 20 ). We assumed that nitrosylated heme is more toxic than native heme, which would explain why the consumption of processed meat is more closely associated with the risk of colorectal cancer than the intake of fresh red meat (7 ).

No experimental study has ever been conducted to clarify the effect of meat processing on colorectal carcinogenesis, using preneoplastic or tumor endpoints. However, we recently showed that freeze-dried cooked ham promotes colon carcinogenesis in carcinogen-injected rats (21 ). Here, we first investigated in a 14-day study the impact of meat processing on early biomarkers associated with promotion of heme-induced colorectal carcinogenesis in rats (17 ). We then measured in a 100-day study the promoting effect of four processed meats selected from the 14-day study. Carcinogenesis endpoints were 1,2-dimethylhydrazine-induced preneoplasic lesions (ACF and MDF) in rats. The results show that a model cured meat, similar to ham, can increase the number of preneoplastic lesions in the colon of a rodent model of carcinogenesis.

## Materials and Methods

### General Design

Two sequential studies were performed: a 14-day study investigated the effect of 16 cured meat models on early fecal and urinary biomarkers in rats, and a 100-day study measured the promoting effect of four cured meat models, selected among the 16 models, on preneoplastic lesions in carcinogen-initiated rats.

#### *Fourteen-day study: animals and design*

Ninety female Fischer 344 rats were purchased at four weeks of age from Charles River (Saint Germain l'Arbresle, France). Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. Rats were kept in an animal colony with temperature of 22°C and 12:12h light-dark cycle. They were allowed free access to tap water and the standard AIN76-diet (22 ). Rats were housed individually into metabolic cages. After 2 days of acclimatization, rats were randomly allocated to 17 groups (five rats per experimental group, but ten rats in the control group) and fed experimental diets for 14 days. Body weights were monitored on days 2, 7 and 14. Food and water intakes were measured at days 6–7 and 12–13. Feces were collected during the last two days and were frozen at −20°C. Urines were collected on day 13 and processed immediately.

Processed meats were analyzed for nitrosyl heme, pH, hexanal and pro-oxidant activity. The pro-oxidant activity of meat samples was also determined in an oil-water emulsion. Fecal water samples were analyzed for heme, thiobarbituric acid reactive substances (TBARS), and cytotoxic activity on three cell lines. Urine samples were analyzed for 1,4-dihydroxynonane mercapturic acid (DHN-MA). A Principal Component Analysis of data was then done to help selecting four processed meats that would be included in the 100-day carcinogenicity study.

#### *Fourteen-day study: Diets*

Pork processed meats and experimental diets were made in a specialized workshop by IFIP-Institut du Porc (Paris, France). Freeze-drying increases the formation of lipid oxidation products in meat (23 ). Thus processed meat was added as such (moist) to an AIN 76-base powder (UPAE, INRA, Jouy, France), so that each diet contained 55 g of processed meat dry matter per 100 g (dry weight, Table 1 ). Each diet contained one of 16 models of cured meat of pig, in a 2×2×2×2 design where four factors were crossed: (i) high heme (dark meat) content vs. low (light meat), (ii) cooking temperature 70°C (cooked meat) vs. 50°C (raw meat), (iii) added nitrite (with nitrite) vs. none, and (iv) exposure to air (oxidized) vs. anaerobic packaging (anaerobic). The sixteen experimental processed meats and diets were thus named as follows: [light cooked meat with nitrite, anaerobic], [dark cooked meat with nitrite, anaerobic], [light raw meat with nitrite, anaerobic], [dark raw meat with nitrite, anaerobic], [light cooked meat, anaerobic], [dark cooked meat, anaerobic], [light raw meat, anaerobic], [dark raw meat, anaerobic], [light cooked meat with nitrite, oxidized], [dark cooked meat with nitrite, oxidized], [light raw meat with nitrite, oxidized], [dark raw meat with nitrite, oxidized], [light cooked meat, oxidized], [dark cooked meat, oxidized], [light raw meat, oxidized], [dark raw meat, oxidized].

Dark meat was obtained from supraspinatus and infraspinatus pig muscles, which contains 15 to 17 mg heme/100 g, while light meat came from longissimus dorsi which contains 0.36 to 2 mg heme/100 g (24 , 25 ). Cooked meat was heated at 70°C for one hour in vacuum-sealed plastic bags in a water bath while "raw meat" was heated at 50°C: Raising temperature to 70°C denatures myoglobin which detaches from the heme (19 ). Nitrite-treated meat was cured with salt (NaCl) containing 0.6 g sodium nitrite/100 g salt, while

non-nitrite-treated meat was cured with ordinary salt without sodium nitrite. Cooked cured meat contained 2 g salt/100 g of meat whereas raw cured meat contained 2.5 g salt/100 g of meat. Anaerobic meat was packaged under vacuum immediately after processing, in plastic bags with low oxygen permeability (40 ml O2/$m^2$ /24h at 23°C and 75% relative humidity; VF90, Soussana, Orly, France). Packaging was efficient to prevent oxidation, since "anaerobic" meat contained 0.5 mg MDA equivalent per 100 g of anaerobic meat, compared with 1.8 mg MDA equivalent per 100 g of oxidized meat (Wilcoxon's test p = 0.04, full data not shown). Pieces of cured meat (25 g, length=4 cm, width=2.5 cm and thickness=1 cm) were kept at 4°C in the dark without any packaging for five days after cooking to obtain oxidized cooked cured meat. The 16 experimental diets were compared to a control diet containing 10 g fat/100 g of diet. The control diet, made by UPAE (INRA, Jouy), had the same protein, fat and iron contents than meat diets (Table 1 ). According to Pierre et al (17 ), all diets were low-calcium (0.27 g calcium phosphate/100 g diet) and n-6 fatty acids were provided by 5 g/100 g safflower oil (MP Biomedicals, Illkirch, France). All diets were vacuum-packaged to avoid further lipid oxidation and were stored at −20°C. They were given to rats every day around 5:00 p.m. during 14 days.

***One-hundred-day study: Animals and Design***

Fifty rats (same strain, gender and age as above) were housed in stainless steel, wire bottomed-cage of two rats. After five days of acclimatization to the animal colony and to the AIN 76 diet, each rat received a single i.p. injection of 1,2-dimethylhydrazine (Sigma Chemical, St Quentin, France; 180 mg/kg i.p.) in NaCl (9g/l). We chose to initiate all rats with the carcinogen, since the study was designed to show dietary promotion. Seven days later, they were randomly allocated to five groups (10 rats per group) and fed the experimental diets described below. Body weights were monitored every week during first four weeks, and then every two weeks. Food and water intakes were measured at days 20, 60 and 80. Feces were collected between days 90 and 95, and frozen at −20°C. Urines were collected between days 84 and 88 and frozen at −20°C (each rat was put in a separate metabolic cage to collect the urine). Animals were killed on days 98 and 99. Colons were removed and fixed in 10% buffered formalin (Sigma Chemical) between two sheets of filter paper with a blinding code. Aberrant crypt foci (ACF) and mucin depleted foci (MDF) were then scored. Fecal water samples (preparation described below) were analyzed for heme, TBARS and cytotoxicity. Urine samples were analyzed for DHN-MA.

***One-hundred-day study: Diets***

Four processed meats were selected from the data of the 14-day study: [dark cooked meat, oxidized]; [dark cooked meat with nitrite, oxidized]; [dark cooked meat with nitrite, anaerobic] and [dark raw meat, anaerobic]. Processed meats and diets were made by IFIP (Maison Alfort, France). Modified AIN-76 powdered diet was prepared and formulated by UPAE (INRA, Jouy, France). Each diet was a low-calcium diet (2.7 g calcium phosphate/kg) with 5 g of safflower oil/100 g of dry matter (Table 1 , lower panel). Diets were balanced to contain identical proportion of fat (15 g lipids/100 g) and protein (40 g proteins/100 g). They were compared with a control diet containing 15 g of lipids/100 g and 40 g of casein/100 g. The diets were divided into daily portions that were stored separately at −20°C in air-tight plastic bags sealed under vacuum to avoid lipid oxidation. They were given to the rats around 5:00 p.m. every day during 100 days.

**Characterization of Processed Meats**

***Meat composition***

Processed meats were analyzed for nitrosyl heme (26 ) and pH by an ISO-17025 accredited laboratory (LAREAL, Vannes, France).

***Pro-oxidant activity of meat samples***

Protein-stabilized oil-in-water emulsions prepared at acid pH were taken as a model food. The propensity of the ground meat products, added to the emulsions, to increase their rate of oxidation, was used to evaluate their pro-oxidant activity. Development of oxidation was followed by measurement of oxygen consumption by the ground meat products-emulsions mixtures kept in closed vials at 37 °C in the dark.

Oil-in-water emulsions were prepared with safflower oil (30 g oil; SICTIA, Marseille, France) and 10 $g.L^{-1}$ Bovin Serum Albumin (ref 103703; ICN Biochemicals-Inc, Aurora, OH, USA) in sodium phosphate buffer 0.1 M; pH = 6.0; $NaN_3$ 0.2g/l as previously described ( 27 , 28 ). The mean surface diameter ($d_{3,2}$ ) of droplets was around 1 μm. Droplet size distribution was determined with a Mastersizer 3600 (Malvern Instruments, Worchester, UK) and it was stable when the emulsions were stored at 37 °C.

Meat samples that were kept at −80°C until use were thawed at 4°C overnight and minced with a house meat-grinder. Four g of ground samples (all samples except the dry sausage) were homogenized for 2 min with Polytron homogeniser in 40 mL of 0,1 M, pH = 6.0 phosphate buffer, $NaN_3$ 0.2 g/l and the homogenates distributed (1 mL) in 22.4 mL headspace vials. The ground dry sausage was directly weighted (100 mg) and dispersed in 1 mL of the phosphate buffer. Three mL of freshly prepared emulsions were then added to the vials that were sealed with Teflon/silicon septa and aluminum crimp caps and rotated in the dark at 37 ± 1 °C. One vial was then taken from the chamber at regular time intervals for measurement of oxygen consumption that was measured by gas chromatography (29 ). The results were expressed in millimoles of consumed $O_2$ (mmol $O_2$ ) and the time needed to consume the oxygen initially present in the vials was evaluated from the time plots. The kinetics was performed at least in duplicate.

### *Hexanal concentration in the meat products*

Hexanal was selected as a specific and reliable marker of secondary products of lipid oxidation in meat products. It was analyzed by gas chromatography of the volatile compounds sampled in the headspace of the samples dispersed in phosphate buffer equilibrated at 37° C, with a solid phase micro extraction fiber (29 , 30 ). Fiber coated with polydimethylsiloxane (PDMS; 100 μm film thickness, Supelco, Bellefonte, PA) or carboxen/PDMS (75 μm film thickness, Supelco, Bellefonte, PA) was used according to the required sensitivity, that depended on the sample and its oxidation level. The quantification was achieved through standard addition method as follows: five hundred mg of ground samples prepared, as described above, were weighted in headspace vials and 3 mL of 0.1 M, pH = 6.0 phosphate buffer containing known amounts of hexanal, added in each vial. For every meat product six concentrations of hexanal were added to obtain hexanal amounts varying from 0 to 30 μg with PDMS fiber and 0 to 4.4 μg for carboxen/PDMS fiber. The tightly sealed vials were equilibrated for 30 min at 37°C under magnetic stirring. The solid phase micro extraction fiber was then exposed in the headspace for 5 min at 37 ± 1 °C. Gas chromatography analysis was then performed as described in Villière et al. (30 ). Peaks areas of hexanal were integrated and the volatile concentrations in the samples (pg/g) calculated from linear regressions that included the area measured with the sample with no addition of known amount of hexanal (six concentrations per sample). Final values were means of two determinations

## Fecal and Urinary Measures

### *Preparation of Fecal Water*

Fecal pellets were collected under each cage of two rats for 24 h, thus leading to five samples per group. To prepare fecal water, 1 ml of water was added to 0.3 g of dried feces. Samples were then incubated at 37˚C for one hour, stirred thoroughly every 20 min and then centrifugated at 20 000 × g for 15 min. The supernatant (fecal water) was collected and kept at −20˚C until use.

### *Heme and TBARS in Fecal Water*

Fecal water was analyzed because the soluble fraction of colon content would interact more strongly with the mucosa than the insoluble fraction (31 ). Heme concentration of fecal water was measured by fluorescence according to Van den Berg et al. (32 ) as described in Pierre et al. (18 ). We supposed that processed meat would induce lipid oxidation in fecal water as already shown with red meat, and lipid oxidation products present in fecal water are cytotoxic against colon cells (33 ). We thus measured TBARS in fecal water as a global measure of fecal lipid oxidation products. TBARS were measured in fecal water according to Ohkawa et al. (34 ), as previously described (17 ), and results are given as malondialdehyde (MDA) equivalent.

### *Cytotoxicity Assay of Fecal Water*

Cytotoxicity of fecal water was quantified for three cell lines according to Bonneson et al. (35 ) as previously described (17 ). The three lines were: cancerous mouse colonic epithelial cell line, CMT93 (ECAC); colon epithelial cell lines derived from C57BL/6J mice (Apc +/+) and Min mice (Apc +/−). This triple cellular model can contribute to understand the biological effects of fecal water on normal (Apc +/+), premalignant cells (Apc Min/+) and cancerous cells (CMT93).

CMT 93 cells were seeded in 96-well microtiter plates at 37°C ($1.6 \times 10^4$ cells per well in 200 μL of DMEM culture medium). At confluence, cells were treated for 24 h with fecal water sample diluted 10-fold by the culture medium and filtered (0.22μm). Cells were then washed with phosphate-buffered saline (PBS). Cytotoxicity of fecal water was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide test (MTT, 0.45mg/ml in PBS). One hundred microliters of MTT was added to each well. After incubation in the dark (3h 37°C), 100 μL of a 10% SDS-0.1 mol/L NaOH mixture was added and the absorbance of the reaction product (purple formazan) measured at 570 nm with a plate reader (35 ).

Apc +/+ and Apc Min/+ cells harbor a temperature-sensitive mutation of the simian virus 40 large tumor antigen gene (tsA58), under the control of interferon γ. These cells are ‘immortalized’, that is, they express active SV40 at the permissive temperature (33°C). Cells were cultured at permissive temperature of 33°C in Dulbecco-modified essential medium (DMEM) supplemented with 10% (v/v) fetal calf sera, 1% (v/v) penicillin/streptomycin, and 10 U/ml interferon g. The experiments were performed at non-permissive temperature of 37°C, and without interferon γ, to inhibit the SV40 transgene and limit proliferation. Apc +/+ and Apc Min/+ were seeded into a 96-well culture plates at the seeding density of $10^4$ cells in DMEM culture medium. Cells were grown at 33°C with interferon γ for 72h until subconfluence. They were then transferred at 37°C without interferon γ for 24h.

### *Urinary DHN-MA*

The 24-hour urine was collected under each individual metabolic rat cage. Urinary DHN-MA indicates the in vivo- and in diet-formation of 4-hydroxy-nonenal. DHN-MA assay was performed by competitive enzyme immunoassay as previously described (36 ), using DHN-MA-linked acetylcholinesterase enzyme (37 ). Each urine sample was assayed in duplicate.

### *ATNC analysis*

Fecal samples were stored at −20°C before they were transported on dry ice to Pollock and Pool Ltd, Reading, UK, for ATNC determination (38 ). Fecal samples were prepared for analysis by macerating feces with 10 times their weight of water and centrifuging. Supernatants were used for ATNC analysis. A total of 50 μl of the supernatant to be analyzed was injected directly into a refluxing mixture of propyl acetate and hydrogen bromide (added as 35%HBr in acetic acid). A further portion of each sample was pre-treated with sulfamic acid to destroy nitrite. After reaction for 5 min, 50 μl was injected into the refluxing mixture. N-Nitrosodipropylamine (160 ng) was injected into the system after the analysis of each sample as an internal standard to allow quantification of the —NNO group. The nitric oxide released as a result of denitrosation of the sample was passed into a thermal energy analyser (Thermal Electron Company, Waltham, MA, USA) in a stream of nitrogen gas where the amount of ATNC in the sample was quantified. The method detects ATNC though nitrolic acid, nitrosyl-hemoglobin and thionitrite are also denitrosated under these conditions. Results are expressed as the concentration of the common unit of structure, NNO, as mg/kg.

**ACF and MDF assays**

Rats were killed by CO2 asphyxiation in a random order at day 99 or 100. Colons were coded, fixed in formalin and scored for ACF incidence by Bird's procedure (39 ). Briefly, numbers of ACF per colon and of crypts per ACF were counted under light microscope at x 40 magnification in duplicate by two readers, blinded for the origin of the colon. Colons were stained for 6 min in a 0.05% filtered solution of methylene blue and ACF scoring criteria were: larger than normal crypts, microscopically elevated, a thick epithelial lining that stains darker than normal crypts, with a large pericryptal zone.

Colons were then stained with the high-iron diamine alcian blue procedure (40 ). Two investigators, blinded for the rat treatment, evaluated the number of MDF per colon and the number of crypts per MDF. MDF scoring criteria were: focus with at least three crypts with no or very little apparent mucin, and two of the following: crypts with distorted lumen, elevated lesion above the mucosa level (40 ).

**Statistical Analysis**

Results were analyzed using Systat 10 software for Windows and all data reported as mean ± SD. Values were considered firstly using one-way analysis of variance (ANOVA). If a significant difference was found between groups ($p<0.05$), comparison of each experimental group with the control group was made using Dunnett's test.

To analyze ACF and MDF data, we used a two-ways ANOVA (groups and readers): The interaction group x reader was never significant, thus data from the two readers were pooled. When total ANOVA was significant ($p<0.05$), pairwise differences between groups were analyzed using Fisher's least-significant-difference test.

Data from the 14-day study were analyzed by Principal Component Analysis with SIMCA-P 8.0 software. The aim of this analysis is to compress (or simplify) high-dimensional data by finding a linear combination of the original variables. The variance is maximized and new uncorrelated variables are created: the principal components. The number of principal components to retain in the model was kept as low as possible.

## Results

**1-Fourteen-day Study**

***General observation***

The final body weight of rats was 141±10 g, without significant difference between groups ($p>0.1$). [Light cooked meat with nitrite, anaerobic] and [dark raw meat with nitrite, anaerobic]-fed rats ate significantly more food than control-fed rats (13.0±1.9, 11.6±0.7 and 9.4 ±0.3 g/day, respectively, $p<0.05$). Rats given an oxidized diet drank significantly more water than control-fed rats ($p<0.05$), likely because of meat drying by air exposure.

**Fecal cytotoxicity and lipid peroxidation biomarkers (TBARS and DHN-MA)**

Fecal waters from processed meat-fed rats contained twice to five times more lipid oxidation products than control rats (71±156 vs. 34 ±19 μM MDA equivalent, all values significantly different from control, $p<0.05$, Table 2 ). Dark meat as compared to light meat, cooking and aerobic storage significantly increased TBARS value in fecal water, while the addition of nitrite reduced this value ($p<0.01$). The intake of three processed meat out of four was associated with increased cytotoxicity of fecal water on the three cell lines. Median cytotoxicity was the highest in fecal water from rats given [dark raw meat, anaerobic] and [light raw meat with nitrite, oxidized] diets. In contrast, compared with values found with control diet, [dark raw meat with nitrite, anaerobic], [dark raw meat with nitrite, oxidized], [light raw meat, anaerobic] and [light raw meat, oxidized] diets did not enhance cytotoxicity of fecal water on the three cell lines (Table 2 ). DHN-MA urinary excretion was 12- to 37-times higher in processed meat-fed rats than in control rats (Table 2 ). Four-factor ANOVA

demonstrated that (i) the four factors modified significantly TBARS in fecal water, (ii) cooking temperature and added nitrite modified cytotoxicity on Apc +/+ cell line, (iii) no factor had a significant effect on cytotoxicity on Apc +/− cell line, (iv) only cooking temperature modified cytotoxicity on CMT93 cell line and urinary DHN-MA.

**Processed meat characterization**

Mean pH value was 6.0±0.2. Among the 16 processed meats, pH was higher in dark meats than in light meats (pH 6–6.4, median 6.15 vs. 5.7–6, median 5.85, respectively, Table 3 ) and it was higher in cooked meats than in raw meats (pH 5.9–6.4, median 6.13 vs. 5.7–6.1, median 5.88, respectively, Table 3 ). Heme and NO from nitrite can form nitrosyl heme (7 ), and we speculated that nitrosyl heme is the promoting factor in processed meat. Low concentrations of nitrosyl heme (between 2 and 6 mg/kg) were found in meat without added nitrite (Table 3 ). The highest concentrations of nitrosyl heme were found in [dark cooked meat with nitrite, oxidized] and [dark cooked meat with nitrite, anaerobic] (51 and 40 mg/kg, respectively), and less than half theses values were found in [light cooked meat with nitrite, oxidized] and [light cooked meat with nitrite, anaerobic] (21 and 17 mg/kg). However, the effect of meat color on nitrosyl heme concentration did not reach significance ($p>0.05$). Pro-oxidant activity of the processed meats was measured by the time needed to consume oxygen in a closed vial in the presence of an oxidizable emulsion: high pro-oxidant activity leads to fast oxygen disappearance. All processed meat sampled exhibited pro-oxidant activity: the time needed to consume all oxygen in the vials was 22 to 90 h in vials with processed meat, but more than 500 h in standard emulsion vials. The pro-oxidant activity of dark meat was higher than this of light meat, of raw meat higher than this of cooked meat, and of meat with nitrite higher than meat without nitrite (hours to consume all oxygen: D<L, R<C, N<Z; all $p<0.01$, Table 3 ). In contrast, no effect of aerobic or anaerobic storage of the meats on pro-oxidant activity was observed ( Table 3 ). Table 3 showed that [light cooked meat, oxidized] and [light cooked meat, anaerobic] exhibited the lowest pro-oxidant activity while this activity was the highest for [dark raw meat with nitrite, anaerobic] and [dark cooked meat with nitrite, oxidized]. Meat peroxidation was measured by hexanal concentration. Concentration of hexanal in processed meat ranged from 1.3 μg/g in [dark cooked meat, oxidized] sample to 18 μg/g in [dark raw meat with nitrite, oxidized] sample.

A four-factor ANOVA has been achieved to see if the studied processes could significantly influence pH, nitrosyl heme, pro-oxidant activity or hexanal in processed meat. This ANOVA demonstrated that (i) color of meat (or heme level) and cooking temperature modified significantly pH, (ii) added nitrite modified nitrosyl heme concentration, (iii) the four factors except oxidation had a significant effect on pro-oxidant activity and, (iv) added nitrite modified hexanal concentration ($p < 0.01$), but no other factor.

**Choice of processed meats for the 100-day study: Statistical analysis with Principal Component Analysis**

The above 14-day study evaluated the effect of four factors (muscle darkness i.e., a proxy for heme content, cooking temperature, added nitrite and anaerobic packaging) upon three biomarkers linked to promotion of carcinogenesis by heme: TBARS in fecal water, cytotoxicity of fecal water and urinary DHN-MA. A Principal Component Analysis was performed to simplify the data set so that we could choose few contrasting diets for inclusion in a long term study. This tool transforms variables into a smaller number of uncorrelated variables called principal components explaining the greater variance. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. The Principal Component Analysis allows defining some subset observation to explain difference between experimental groups.

The variables included in the Principal Component Analysis were: body weight, intake of diet and water, volume of urine and weight of feces, fecal water cytotoxicity and TBARS, and urinary DHN-MA.

The three first Principal Component Analysis axes (3 principal components) explained 92% of the total variability of the data set (52%, 31% and 9% for axis 1, 2 and 3, respectively). The projection of the group onto principal components (score plots) 1 vs 2 and 2 vs 3 are given on Figure 1(A and B) . In these projections groups can be localized and it is obvious that the principal component analysis allows to separate groups according to the three first principal components.

The projection of data onto principal components (loadings plot) 1 vs 2 and 2 vs 3 is given on Figure 1(C and D) . In this projection, it is possible to explain that the dietary groups are separated thanks to variances in TBARs and cytotoxicity of fecal water (first and second axes) and difference between cytotoxicity of fecal water (third axis) against non-mutated cells (Apc+/+ cells, top) to the cytotoxicity against cancer cells (CMT93 cells, bottom).

Principal Component Analysis led us to select [dark cooked meat, oxidized], [dark raw meat, anaerobic], [dark cooked meat with nitrite, oxidized] and [dark cooked meat with nitrite, anaerobic] processed meats, and CON-10 control diet, to be included into the 100 day carcinogenesis study.

The rationale was to choose contrasting groups according to their localization in the principal component analysis. The following groups were potential groups: CON-10 and [dark raw meat, anaerobic] (or [light raw meat with nitrite, oxidized]) were at both ends of axis 1, [dark raw meat, anaerobic] and [dark cooked meat, oxidized] (or [light cooked meat, oxidized]) at both ends of axis 2, and [dark cooked

meat with nitrite, oxidized] and [dark cooked meat with nitrite, anaerobic] on top of axis 3 (Fig. 1 ). In addition, fecal water TBARS and specific cytotoxicity on Apc+/+ cells have been independently linked to promotion in previous studies (16 –18 , 41 ). It supported the choice of [dark cooked meat, oxidized] (high TBARS, Table 2 ) and of [dark cooked meat with nitrite, anaerobic] (high cytotoxicity against Apc+/+, Table 2 ). Last, we decided to exclude light colored muscles, so that comparison could be made between groups differing by only one factor, and only dark groups were kept. In final, we decided to select: [dark raw meat, anaerobic], [dark cooked meat, oxidized], [dark cooked meat with nitrite, oxidized], [dark cooked meat with nitrite, anaerobic] and the control diet.

**2-One-hundred-day study**

***General observations***

The final body weight of rats was 212 ± 9 g, without significant difference between groups ($p>0.05$). As expected, diet intake was higher, and water intake lower, in processed meat-fed rats than in controls, a normal finding since meat-based diets were moist ($p<0.05$, data not shown).

***ACF***

All processed meat diets increased the number of ACF, and the number of aberrant crypts per colon, compared to control diet ($p = 0.002$, Table 4 ; and $p = 0.001$, data not shown). During scoring, some advanced ACF were noticed: in those ACF the number of aberrant crypts was difficult or impossible to count, and they were more elevated than usual above the mucosa. All these “advanced ACF” were found in oxidized diets [dark cooked meat with nitrite, oxidized] and [dark cooked meat, oxidized]-fed rats, but none in anaerobic diets [ dark cooked meat with nitrite, anaerobic] and [dark raw meat, anaerobic]-fed rats (Fisher exact test $p = 0.02$, data not shown).

***MDF***

Compared with control rats, [dark cooked meat with nitrite, oxidized]-fed rats had more Mucin Depleted Crypts per colon ($p = 0.046$) and more MDF per colon ($p = 0.02$, Table 4 ). No other group was different from control. [Dark cooked meat with nitrite, oxidized]-fed rats had significantly more Mucin Depleted Crypts per colon and more crypts per MDF than [dark cooked meat with nitrite, anaerobic]-fed rats ($p = 0.003$ and $p = 0.001$ respectively), which suggests that oxidized meat favors MDF growth. [Dark cooked meat with nitrite, oxidized]-fed rats had significantly more Mucin Depleted Crypts per colon than [dark cooked meat, oxidized]-fed rats ($p = 0.027$) which suggests that nitrite in meat favors MDF growth.

**Fecal and urinary biomarkers**

As expected, no heme was detected in fecal water from control rats (Table 4 ), in contrast to fecal waters from meat-fed rats. Surprisingly, heme in fecal water of [dark cooked meat with nitrite, oxidized]-fed rats was higher than in other meat-fed rats. Lipid oxidation markers, fecal TBARS and urinary DHN-MA, were correlated (Pearson $r = 0.5$, $p = 0.01$). Meat-based diets increased these oxidation markers, with a higher effect of [dark cooked meat, oxidized] than of [dark cooked meat with nitrite, oxidized] (Table 4 ). Fecal water from meat-fed rats was more toxic to CMT93 cells than fecal water from control rats, but the effect was significant only in [dark cooked meat, oxidized]- and [dark raw meat, anaerobic]-fed rats. Fecal level of ATNC was low in all groups of rats (e.g., control group, 47 ± 14 μM) except for [dark cooked meat with nitrite, oxidized]-fed rats (424 ± 92 μM, $p<0.001$, see Table 4 and Fig. 2 ).

To summarize, rats fed dark cooked meat with nitrite, oxidized, differed from the other meat-fed rats differed from the other meat-fed rats: they showed more heme and more ATNC in fecal water, but less lipid oxidation products and lower cytoxicity.

## Discussion

This study shows that a processed meat that contains heme and nitrite, and has been cooked at 70°C and exposed to air for five days at 4°C, can increase the number of preneoplastic lesions in rats, which suggests colon carcinogenesis promotion. This provides the first experimental evidence of promotion by cured meat, and it matches epidemiological results.

Promotion of carcinogenesis was evidenced on two putative precancerous endpoints: ACF and MDF (39 , 40 ). Results for ACF and MDF were partially discordant. Actually, all tested cured meat diets increased the number of ACF per colon compared to control diet with no meat, whereas only the [dark cooked meat with nitrite, oxidized] diet increased the number of MDF per colon (Table 4 ). Several cases of contradictory results between ACF and MDF results have already been published. Colonic MDF and tumors are suppressed by synbiotic, comprising the prebiotic Raftilose (a derivative of inulin) and two probiotic strains, a Lactobacillus and a Bifidobacterium , but ACF are not (40 ). Colonic MDF and tumors are promoted by cholic acid but ACF are not (42 ). MDF thus seem better predictors of colon carcinogenesis than ACF are. However, we decided to show both MDF and ACF data, since ACF have already been used in more than one thousand published studies (43 ). The MDF-promoting [dark cooked meat with nitrite, oxidized] meat was a cooked shoulder of pork supplemented with sodium nitrite, and kept unwrapped at 4°C for five days: it models a piece of cooked ham which has been kept in a refrigerator. Pork shoulder is used to make cooked hams and sausages, the most frequently consumed processed meats in the USA and in

Europe (44 , 45 ). No experimental study has been published yet on the effect of this kind of processed meat on colorectal carcinogenesis. Our team recently showed that freeze-dried cooked ham promotes colon carcinogenesis in carcinogen-injected rats (21 ), but the freeze-drying process dramatically enhances fat oxidation and 4-hydroxy-nonenal yield. The present study shows that fresh moist processed meat also can increase the number of preneoplastic lesions in rats, which suggests colon carcinogenesis promotion.

The oxidation of cured meat increased MDF promotion, compared with the same kind of cured meat directly packaged once it was processed: there were more MDF in rats given [dark cooked meat with nitrite, oxidized] than in rats given [dark cooked meat with nitrite, anaerobic] (Table 4 ). To make [dark cooked meat with nitrite, anaerobic], meat was directly packaged once processed, whereas to make [ dark cooked meat with nitrite, oxidized], meat was stored unwrapped for five days at 4°C in the dark. We speculated that the difference between [dark cooked meat with nitrite, anaerobic] and [dark cooked meat with nitrite, oxidized] effects on promotion of preneoplastic lesions could be due to oxygen radical species formed in [dark cooked meat with nitrite, oxidized] during the air exposure. Oxidation of polyunsaturated fat produces aldehydes such as malondialdehyde and 4-hydroxynonenal, which form mutagenic DNA-adducts and may explain the observed MDF promotion (46 , 47 ). Greater oxidation of fat occurs in unwrapped meat, which may lead to the formation of volatile compounds and to radical oxygen species such as peroxyl and alkoxy radicals. These are found when heme is added to oil rich in n-6 polyunsaturated fatty acids, the composition of which is close to pork fat (48 , 49 ). American country ham contains many volatile compounds due to fat oxidation such as hexanal (50 ). Animal-fat oxidation products such as oxysterols and aldehydes may act as a primary mechanism of cancer progression in the digestive tract through modulation TGF (transforming growth factor)-beta-1 signaling (51 ). However, in this study we did not detect any differences between [dark cooked meat with nitrite, anaerobic] and [dark cooked meat with nitrite, oxidized], regarding hexanal concentration and pro-oxidant activity of processed meat (Table 3 ) or TBARs and DHN-MA in rats feces and urine (Table 3 and 4 ). These results thus do not support the hypothesis that different peroxidation levels could explain the difference in MDF promotion in rats fed [dark cooked meat with nitrite, anaerobic] and rats fed [dark cooked meat with nitrite, oxidized].

There were more MDF in rats given [dark cooked meat with nitrite, oxidized] than in rats given the same nitrite-free meat (Table 4 ). Nitrites are nitrosating agents and can interact with secondary amino compounds to form N-nitroso-compounds. Parnaud et al. (11 , 52 ) showed that rats fed fried bacon excrete 10 to 20 times more ATNC in feces than controls, but these ATNC do not initiate ACF in rats, nor do they promote ACF in azoxymethane-initiated rats. Haorah et al. showed that hot-dogs contain 10 times more ATNC than fresh red meat (53 ). Mice given a 18% hot-dog diet had 5 times more ATNC in feces than no-meat fed controls (54 , 55 ), feeding heme increased ATNC levels in feces of mice also fed nitrite (56 ). Lewin et al. showed that in human volunteers fecal ATNC concentrations correlate with N-nitroso-specific adducts, $O^6$ -carboxymethylguanine (57 ). The [dark cooked meat with nitrite, oxidized] diet, which contained more nitrosyl heme than the other diets (Table 3 ), led to a nine-fold increase in fecal ATNC excretion (Table 4 , Figure 2 ). Fecal ATNC may not promote ACF formation (11 , 52 ), but may explain the MDF promotion observed here.

Red meat promotion is associated with lipid oxidation and cytotoxicity of fecal water (15 –18 , 58 ). We measured these biomarkers, assuming that red meat and cured meat would promote carcinogenesis by similar heme-induced mechanisms. Here, [dark cooked meat with nitrite, oxidized]-fed rats had much more heme in fecal water and more MDF per colon than the other groups, which supports the hypothesis that heme (or nitrosyl heme) is responsible for cured meat induced promotion. It also suggests that processing can change heme bioavailability in feces. Fecal water from [dark cooked meat with nitrite, oxidized]-fed rats also contained more lipoperoxides and cytotoxic activity than fecal water from control rats, but less than fecal water from other meat-fed rats. This means that these biomarkers do not predict promotion in cured meat-fed rats. Assuming the biomarkers would predict promotion, our choice of four processed meats for the 100-day study was based on ACP analysis of biomarker data from the 14-day study. Because these biomarkers did not correlate with MDF promotion it is likely that mechanism of cured-meat promotion differs from mechanism of red meat promotion. The hypothesis that N-nitroso compounds would be involved in the etiology of colorectal cancer has been first proposed by Kälble et al . and Rowland et al . (59 , 60 ). Bingham's and Mirvish's works supported this hypothesis and we agree that cured meat promotion can be in part due to heme-induced formation of N-nitroso compounds, measured as ATNC (10 , 61 , 62 ) Dietary heme enhances intestinal ATNC formation (9 , 10 , 56 ). According to Kuhnle et al (63 ) and Hogg (64 ), the major part of fecal ATNC following heme intake is nitrosyl-heme. However since nitrosyl-heme contains Fe-NO bound but not N-NO bound, it may not be part of ATNC (S. Mirvish, personal communication). Cured meat (like [dark cooked meat with nitrite, oxidized]) is a high nitrite/high heme food, and, as suggested by Hogg, "a high nitrite/high heme diet could be particularly problematic" (64 ). Lastly, ACF promotion by processed meat here seems similar to ACF promotion by red meat (16 , 17 ): all the tested processed meat diets ([dark cooked meat with nitrite, oxidized], [dark cooked meat with nitrite, anaerobic], [ dark cooked meat, oxidized] and [dark raw meat, anaerobic]) increased the number of ACF, fat peroxidation (fecal TBARs and urinary DHN-MA) and fecal water cytotoxicity (Table 4 ). In contrast, [dark cooked meat with nitrite, oxidized] was the only MDF-promoting diet, and the only fecal ATNC-enhancing diet: Although it was not our starting hypothesis, these data strongly support Bingham and Mirvish's hypothesis that the pro-cancer factor in processed meat belongs to N-nitroso-compounds (8 , 54 ). The experimental processed meats that have been given to rats have been made without ascorbate addition, but sodium ascorbate or erythorbate are currently added to most processed meat to reduce NOCs production in the meat (65 ). It would thus be interesting to test, in a future study, if ascorbate can prevent promoting effects of [dark cooked meat with nitrite, oxidized].

In conclusion, this study is the first to show that a moist model of cured meat diet can increase the number of preneoplastic lesions in carcinogen initiated rats, which suggests colon carcinogenesis promotion. This kind of oxidized cooked red meat with nitrite corresponds to badly packaged cooked ham. Packaging of processed meat seems to decrease, and addition of nitrite seems to increase, the promoting potency of cured meat. We are now searching processes and food additives which could suppress the promoting effect of cured meat on colorectal carcinogenesis.

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## Footnotes:

**Disclosure of Potential Conflicts of Interest** R.L. Santarelli and J.L. Vendeuvre are paid by the French Pig Institute. The other authors disclosed no potential conflicts of interest

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**Figure 1**

Principal Components score plot 1 vs 2 (A) and 2 vs 3 (B) related to data the matrix, and principal component loading plot 1 vs 2 (C) and 2 vs 3 (D). Groups are represented by the plot labels Abbreviations of biomarker names: TBARs = TBARs of fecal water; Cytotox. = Cytotoxicity of fecal water, Cytotox. Apc+/+ = Cytotoxicity of fecal water on Apc+/+ cells; Cytotox. Apc+/− = Cytotoxicity of fecal water on Apc+/− cells; Cytotox. CMT = Cytotoxicity of fecal water on CMT93 cells. Abbreviations of diet names: CON-10, control diet; All experimental diets contained 55% processed meat: LCNA, [light cooked meat with nitrite, anaerobic]; DCNA, [dark cooked meat with nitrite, anaerobic]; LRNA, [light raw meat with nitrite, anaerobic]; DRNA, [dark raw meat with nitrite, anaerobic]; LCZA, [light cooked meat, anaerobic]; DCZA, [dark cooked meat, anaerobic]; LRZA, [light raw meat, anaerobic]; DRZA, [dark raw meat, anaerobic]; LCNO, [light cooked meat with nitrite, oxidized]; DCNO, [dark cooked meat with nitrite, oxidized]; LRNO, [light raw meat with nitrite, oxidized]; DRNO, [dark raw meat with nitrite, oxidized]; LCZO, [light cooked meat, oxidized]; DCZO, [dark cooked meat, oxidized]; LRZO, [light raw meat, oxidized]; DRZO, [dark raw meat, oxidized].

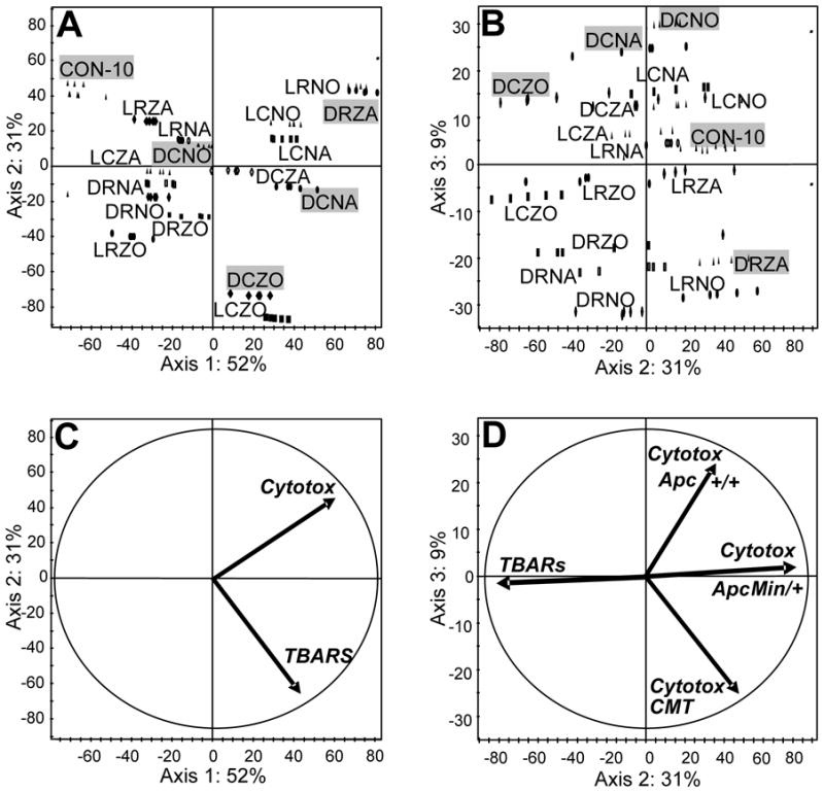


**Fig. 2**

Fecal N-nitroso-compounds (ATNC) in rats given experimental diets for 80d after a 1,2-dimethylhydrazine injection. Values are means ± SD, n = 5. DCNO significantly different from all other groups (P<0.001, n = 5) Diets contained 55% processed meat: DCNO, Dark-Cooked-With Nitrite and Oxidized; DCNA, Dark-Cooked-With Nitrite and kept Anaerobic; DCZA, Dark-Cooked-Without Nitrite Oxidized; DRZA, Dark-Raw-Without Nitrite and kept Anaerobic. Detailed compositions are given in the Material and Methods section. N-nitroso-compounds were assessed as apparent total N-nitroso compounds (ATNC) by Pollock (UK).

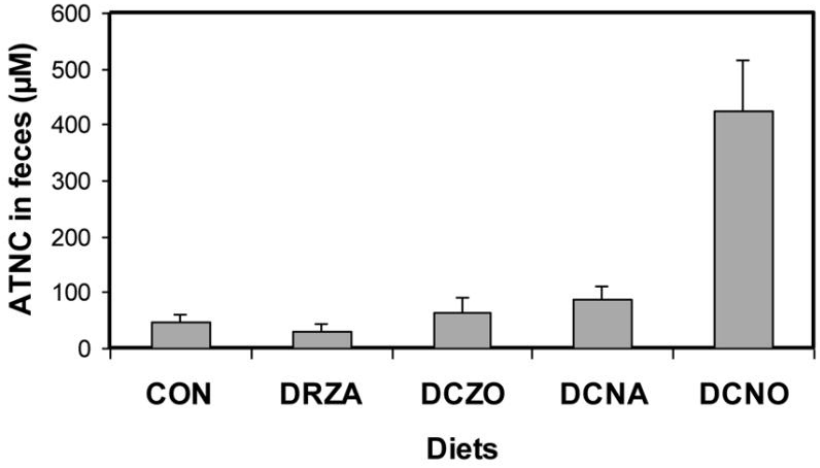

**Table 1**

Composition of the experimental diets (dry basis/100g of diet).

| | | Meat (g dry weight) | Modified AIN76 base (g) | Safflower oil (g) | Lard (g) | Casein (g) | Sucrose (g) | Ferric citrate (g) |
|---|---|---|---|---|---|---|---|---|
| 14-day study | Meat diets | 55[a] | 40[b] | 5 | 0 | 0 | 0 | 0.000 |
| | CON-10 | 0 | 50[c] | 5 | 5 | 40 | 0 | 0.012 |
| 100-day study | Meat diets | 47[d] | 43[e] | 5 | 0 | 5 | 0 | 0.000 |
| | CON-15 | 0 | 43[e] | 5 | 10 | 40 | 2 | 0.012 |

**a** 190 to 219 g of processed meat was added to each diet, depending on water content of the processed meat.

**b** Modified AIN76 base composition (g/100 g): sucrose, 59.5; corn starch, 15; cellulose, 12.5, AIN76 calcium free mineral mix, 8.7; AIN76 vitamin mix, 2.5; methionine, 0.75; calcium phosphate, 0.53; choline bitartrate, 0.5.

**c** Modified AIN76 base composition (%): sucrose, 67.5; corn starch, 12; cellulose, 10, AIN76 calcium free mineral mix, 7; AIN76 vitamin mix, 2; methionine, 0.6; calcium phosphate, 0.54; choline bitartrate, 0.4.

**d** 175 g of processed meat was added to each diet, depending on water content.

**e** Modified AIN76 base composition (%): sucrose, 64.6; corn starch, 11.6; cellulose, 11.6, AIN76 calcium free mineral mix, 8.1; AIN76 vitamin mix, 2.3; methionine, 0.70; calcium phosphate, 0.49; choline bitartrate, 0.47.

**Table 2**

Effect of the experimental diets on lipid oxidation products and cytotoxicity of fecal water, and urinary DHN-MA, in rats given one of 16 experimental diets for 14 days (values are means ± SD, n = 5 or 10 for CON-10).

| Diet | Number of rats | TBARS in fecal water (MDA eq., μM) | Cytotoxicity of fecal water on cells | | | Urinary DHN-MA (μg/24h) |
|---|---|---|---|---|---|---|
| | | | Apc+/+ (%) | Apc+/− (%) | CMT93 (%) | |
| CON-10 control | 10 | 34±19[*] | 0±8 | 1±5 | 0±9 | 0.2±0.05 |
| light cooked meat with nitrite, anaerobic | 5 | 84±13[a] | 68±8[a] | 44±4[a] | 32±6[a] | 6.5±1.2[a] |
| dark cooked meat with nitrite, anaerobic | 5 | 102±17[a] | 73±15[a] | 47±5[a] | 34±10[a] | 6.3±2.4[a] |
| light raw meat with nitrite, anaerobic | 5 | 72±7[a] | 30±10[a] | 22±3[a] | 22±9[a] | 3.7±0.9[a] |
| dark raw meat with nitrite, anaerobic | 5 | 85±23[a] | 0±18 | 16±4[a] | 26,±6[a] | 4.0±1.0[a] |
| light cooked meat, anaerobic | 5 | 78±10[a] | 25±6[a] | 8±4 | 14±6[a] | 3.4±0.6[a] |
| dark cooked meat, anaerobic | 5 | 88±25[a] | 49±14[a] | 33±7[a] | 26±3[a] | 3.5±0.8[a] |
| light raw meat, anaerobic | 5 | 58±16[a] | 12±19 | 22±6[a] | 15±12[a] | 2.3±0.6[a] |
| dark raw meat, anaerobic | 5 | 80±11[a] | 59±17[a] | 81±2[a] | 87±6[a] | 3.2±0.9[a] |
| light cooked meat with nitrite, oxidized | 5 | 81±21[a] | 68±12[a] | 47±10[a] | 48±9[a] | 4.0±0.7[a] |
| dark cooked meat with nitrite, oxidized | 5 | 71±12[a] | 45±10[a] | 40±6[a] | 3±12 | 4.0±3.0[a] |
| light raw meat with nitrite, oxidized | 5 | 81±17[a] | 53±15[a] | 85±2[a] | 85±3[a] | 2.7±1.4[a] |
| dark raw meat with nitrite, oxidized | 5 | 93,±19[a] | 0±17 | 0±5 | 5±14 | 3.4±1.0[a] |
| light cooked meat, oxidized | 5 | 156±25[a] | 36±7[a] | 41±3[a] | 31±4[a] | 6.3±1.7[a] |
| dark cooked meat, oxidized | 5 | 143±30[a] | 43±9[a] | 39±4[a] | 12±4[a] | 6.7±0.9[a] |
| light raw meat, oxidized | 5 | 100±24[a] | 3±11 | 6±5 | 0±6 | 5.6±1.6[a] |
| dark raw meat, oxidized | 5 | 124±17[a] | 13±8 | 10±2 | 33±4[a] | 3.7±0.6[a] |
| | | ANOVA p values | | | | |
| Factor | | | | | | |
| Color (light/dark) | | **p<0.01** | p>0.1 | p>0.5 | p>0.1 | p>0.05 |
| Cooking (cooked/raw) | | **p<0.01** | **p<0.01** | p>0.1 | **p<0.05** | **p<0.01** |
| Nitrite (with/without nitrite) | | **p<0.01** | **p<0.01** | p>0.1 | p>0.05 | p>0.2 |
| Oxidation (oxidized/anaerobic) | | **p<0.01** | p>0.1 | p>0.1 | p>0.05 | p>0.05 |

[*] Values are means ± SD. Processed meats name (e.g., [light cooked meat with nitrite, anaerobic]): with nitrite, sodium nitrite was added to meat; anaerobic, meat was anaeobically packaged; oxidized, meat pieces were kept at 4°C in the dark without any packaging for five days; see table 1 and Material and Methods section for more details.

**a** significantly different from control diet, CON-10 (p<0.05)

**Table 3**

pH, nitrosyl heme concentration, pro-oxidant activity, and hexanal concentration in processed meats (values are means ± SD, n=1 for pH and nitrosyl heme, n=2 for pro-oxidant activity and hexanal).

| Processed meat | pH | Nitrosyl heme (mg/kg) | Pro-oxidant activity (H to consume all oxygen) | Hexanal (μg/g) |
|---|---|---|---|---|
| light cooked meat with nitrite, anaerobic | 6.0 | 17 | 31 ± 3 | 15.0 ± 3.0 |
| dark cooked meat with nitrite, anaerobic | 6.3 | 40 | 24 ± 2 | 7.0 ± 2.0 |
| light raw meat with nitrite, anaerobic | 5.7 | 13 | 25 ± 1 | 11.0 ± 2.0 |
| dark raw meat with nitrite, anaerobic | 6.1 | 6 | 21 ± 2 | 14.0 ± 5.0 |
| light cooked meat, anaerobic | 5.9 | 2 | 92 ± 9 | 3.0 ± 1.0 |
| dark cooked meat, anaerobic | 6.2 | 3 | 47 ± 7 | 5.0 ± 2.0 |
| light raw meat, anaerobic | 5.7 | 3 | 54 ± 8 | 3.0 ± 2.0 |
| dark raw meat, anaerobic | 6.0 | 5 | 23 ± 2 | 2.0 ± 0.5 |
| light cooked meat with nitrite, oxidized | 5.9 | 21 | 35 ± 5 | 11.0 ± 4.0 |
| dark cooked meat with nitrite, oxidized | 6.4 | 51 | 22 ± 4 | 5.0 ± 2.0 |
| light raw meat with nitrite, oxidized | 5.7 | 4 | 39 ± 5 | 11.0 ± 5.0 |
| dark raw meat with nitrite, oxidized | 6.0 | 6 | 24 ± 1 | 18.0 ± 3.0 |
| light cooked meat, oxidized | 6.0 | 5 | 81 ± 5 | 11.0 ± 3.0 |
| dark cooked meat, oxidized | 6.3 | 4 | 41 ± 3 | 1.0 ± 0.4 |
| light raw meat, oxidized | 5.8 | 6 | 49 ± 9 | 6.0 ± 2.0 |
| dark raw meat, oxidized | 6.0 | 5 | 25 ± 4 | 8.0 ± 1.0 |
| | | ANOVA p values | | |
| Color (light/dark) | **p<0.01** | p>0.05 | **p<0.01** | p>0.05 |
| Cooking (cooked/raw) | **p<0.01** | p>0.05 | **p<0.01** | p>0.05 |
| Nitrite (with/without nitrite) | p>0.05 | **p<0.01** | **p<0.01** | **p<0.01** |
| Oxidation (oxidized/anaerobic) | p>0.05 | p>0.05 | p>0.05 | p>0.05 |

All experimental diets contained 55% processed meat. Detailed composition of diets is given in the Material and Methods section. P values in bold indicate that the process significantly influences processed meat characteristics.

**Table 4**

Effect of processed meat diets on ACF and MDF formation, in the colon of rats 106 d after the injection of 1,2-dimethylhydrazine, and fecal and urinary biomarkers after 80 d on experimental diets. (values are means ± SD, n=10 for each group)

| | ACF/colon | MDF/colon | Crypts/MDF | MD crypts/colon | Heme in fecal water (μM/24h) | TBARS in fecal water (μM MDA eq.) | DHN-MA in urine (μg/24h) | Cytotoxicity of fecal water on CMT93 (% dead cells) | ATNC Concentration (μM as NNO) |
|---|---|---|---|---|---|---|---|---|---|
| control diet | 81 ± 18 | 2.9 ± 1.9 | 3.9 ± 1.5 | 11 ± 8 | 0 ± 0 | 58 ± 9 | 0.5 ± 1.2 | 0 ± 30 | 47 ± 14 |
| dark cooked meat with nitrite, oxidized | 100 ± 16[a] | 4.1 ± 2.9[a] | 4.2 ± 1.2 | 18 ± 13[a] | 324 ± 112[a] | 88 ± 22[a] | 3.4 ± 3.9 | 11 ± 17 | 424 ± 92[a] |
| dark cooked meat with nitrite, anaerobic | 102 ± 25[a] | 2.1 ± 2.0 | 2.7 ± 1.7[a,b] | 8 ± 8[b] | 137 ± 50[a,b] | 102 ± 23[a] | 5.3 ± 4.2[a] | 25 ± 10 | 88 ± 25 |
| dark cooked meat, oxidized | 106 ± 21[a] | 2.8 ± 2.8 | 3.5 ± 1.2 | 10 ± 11[b] | 74 ± 43[a,b] | 130 ± 15[a,b] | 9.7 ± 4.4[a,b] | 35 ± 10[a] | 64 ± 25 |
| dark raw meat, anaerobic | 101 ± 17[a] | 3.4 ± 2.6 | 3.9 ± 1.9 | 14 ± 10 | 79 ± 92[b] | 104 ± 16[a] | 5.2 ± 3.7[a] | 53 ± 25[a,b] | 31 ± 14 |

All experimental diets contained 47% processed meat. Detailed composition of diets is given in the Material and Methods section.

[a] significantly different from control diet (p<0.05)

**b** significantly different from [dark cooked meat with nitrite, oxidized] ($p<0.05$)