O-6-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat

ARTICLE *in* MOLECULAR NUTRITION & FOOD RESEARCH · SEPTEMBER 2014

Impact Factor: 4.6 · DOI: 10.1002/mnfr.201400078 · Source: PubMed

9 AUTHORS, INCLUDING:

Stefaan De Smet
Ghent University
232 PUBLICATIONS **3,611** CITATIONS
SEE PROFILE

Lynn Vanhaecke
Ghent University
133 PUBLICATIONS **1,347** CITATIONS
SEE PROFILE

RESEARCH ARTICLE

O^6-carboxymethylguanine DNA adduct formation and lipid peroxidation upon in vitro gastrointestinal digestion of haem-rich meat

Julie Vanden Bussche[1], Lieselot Y. Hemeryck[1*], Thomas Van Hecke[2], Gunter G. C. Kuhnle[3,4], Frank Pasmans[5], Sharon A. Moore[6], Tom Van de Wiele[7], Stefaan De Smet[2] and Lynn Vanhaecke[1]*

[1] Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[2] Laboratory of Animal Nutrition and Animal Product Quality, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Melle, Belgium
[3] Department of Food and Nutritional Sciences, University of Reading, Reading, UK
[4] Department of Public Health and Primary Care, MRC Centre for Nutritional Epidemiology in Cancer Prevention & Survival, University of Cambridge, Cambridge, UK
[5] Faculty of Veterinary Medicine, Department of Pathology, Bacteriology and Poultry Diseases, Ghent University, Merelbeke, Belgium
[6] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK
[7] Laboratory of Microbial Ecology and Technology, Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Received: February 5, 2014
Revised: April 15, 2014
Accepted: May 14, 2014

Scope: Epidemiological and clinical studies have demonstrated that the consumption of red haem-rich meat may contribute to the risk of colorectal cancer. Two hypotheses have been put forward to explain this causal relationship, i.e. *N*-nitroso compound (NOC) formation and lipid peroxidation (LPO).

Methods and Results: In this study, the NOC-derived DNA adduct O^6-carboxymethylguanine (O^6-CMG) and the LPO product malondialdehyde (MDA) were measured in individual in vitro gastrointestinal digestions of meat types varying in haem content (beef, pork, chicken). While MDA formation peaked during the in vitro small intestinal digestion, alkylation and concomitant DNA adduct formation was observed in seven (out of 15) individual colonic digestions using separate faecal inocula. From those, two haem-rich meat digestions demonstrated a significantly higher O^6-CMG formation ($p < 0.05$). MDA concentrations proved to be positively correlated ($p < 0.0004$) with haem content of digested meat. The addition of myoglobin, a haem-containing protein, to the digestive simulation showed a dose–response association with O^6-CMG ($p = 0.004$) and MDA ($p = 0.008$) formation.

Conclusion: The results suggest the haem-iron involvement for both the LPO and NOC pathway during meat digestion. Moreover, results unambiguously demonstrate that DNA adduct formation is very prone to inter-individual variation, suggesting a person-dependent susceptibility to colorectal cancer development following haem-rich meat consumption.

Keywords:
In vitro gastrointestinal digestion / Malondialdehyde / Meat consumption / *N*-nitroso compounds / O^6-carboxymethylguanine

Correspondence: Dr. Julie Vanden Bussche, Laboratory of Chemical Analysis, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke 9820, Belgium
E-mail: Julie.VandenBussche@UGent.be
Fax: +32-9-2647492

Abbreviations: **ATNC**, apparent total *N*-nitroso compounds; **CRC**, colorectal cancer; **GIT**, gastrointestinal tract; **KDA**, potassium diazoacetate; **LPO**, lipid peroxidation; **MDA**, malondialdehyde; **MGMT**, O^6-methylguanine-DNA methyltransferase; **NOC**, *N*-nitroso compound; **O^6-CMdG**, O^6-carboxymethyl-2′-deoxyguanosine; **O^6-CMG**, O^6-carboxymethylguanine; **O^6-MeG**, O^6-methylguanine; **QqQ-MS**, triple quadrupole mass analyser; **SCFAs**, short-chain fatty acids; **TBARS**, thiobarbituric acid reactive substances

*These authors contributed equally to this work (shared first author).

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Epidemiological and clinical studies have demonstrated that the consumption of meat, and in particular red and processed meat is associated with certain health risks [1–3]. Regular or high consumption of red and processed meat has been linked to the risk of colorectal cancer (CRC) [1, 2], coronary heart disease and also type 2 diabetes [3–5]. The link between red meat consumption and CRC on the one hand and red meat and type 2 diabetes on the other might even be intertwined [6, 7]. As a plausible origin for the increased CRC risk, the formation of endogenous *N*-nitroso compounds (NOCs) has been hypothesised, since a dose–response relationship with the faecal excretion of NOCs for red and processed meat but not for white meat intake has been established [8–10]. The role of haem, abundantly present in red beef meat (~1.8 mg/100 g) [11], in the faecal excretion of NOCs has been confirmed [12]. Haem can become readily nitrosylated and act as a nitrosating agent and thus promote the formation of NOCs [13]. A high-haem diet has also been suggested as a mechanistic basis in the observed correlation between lipid peroxidation (LPO) and increased CRC risk [14].

NOCs, such as nitrosamines, nitrosamides or nitrosoguanidines, are known to be potent carcinogens. The alkylation of DNA is likely to be a major effect of metabolised *N*-alkyl-NOCs, which are able to interact with the nucleophilic centres of DNA bases [15–17]. In addition, several nitrosated glycine derivatives have been shown to react with DNA in vitro to give NOC-derived, i.e. carboxymethyl (e.g. O^6-carboxymethyl-2'-deoxyguanosine, O^6-CMdG) and to a lesser extent methyl adducts (e.g. O^6-methyl-2'-deoxyguanosine) [16, 18, 19]. Since glycine is one of the most common dietary amino acids, it would appear likely that nitrosation products of glycine would constitute a major source of alkylating agents in the human gastrointestinal tract (GIT) [20, 21]. This was reinforced by the detection of O^6-CMdG and O^6-methyl-2′-deoxyguanosine in colonic biopsies and human blood DNA [19, 22, 23]. Additionally, O^6-CMdG is not repaired by either bacterial or mammalian O^6-methylguanine-DNA methyltransferase (MGMT) under in vitro conditions suggesting that this adduct is likely to accumulate in cellular DNA of GIT tissue and possibly represents a promutagenic lesion [16, 23]. The identification and quantification of very low NOC-derived DNA adduct levels, in vivo or in vitro requires ultrasensitive methodologies. This is in particular true for the analysis of human samples or in vitro applications on cell lines, where only small amounts of sample, and therefore DNA, are available. The different methods currently used for DNA adduct analysis include, i.e. immunoassays [24, 25], ^{32}P-postlabelling [26], GC-ECD [27] and HPLC with fluorescence detection [18, 28]. For several years now, mass spectrometric detection has been playing an increasingly important role in the field of DNA adduct detection [29–32], since it achieves a perfect balance between a high specificity and sensitivity. Because of this, we recently developed an UHPLC-MS/MS method for the simultaneous detection of O^6-methyl and O^6-carboxymethylguanine (O^6-CMG) adducts, with LODs of 30 and 50 fmol/mg DNA, respectively [33].

Lipids are biological targets of various reactive oxygen and nitrogen species. This oxidative stress leads to free radical chain reactions and subsequent formation of a vast array of by-products including aldehydes [34, 35]. The main aldehyde formed from the peroxidation of polyunsaturated fatty acids is malondialdehyde (MDA), a DNA-reactive product capable of forming exocyclic DNA adducts, of which most of these are anticipated to be highly mutagenic [36]. The mutagenicity and carcinogenicity of MDA has been confirmed in mammalian cells as well as in animals [36], and has been put forward as a plausible hypothesis for the link between high red meat consumption and CRC [14]. A variety of methods described in literature allow the detection and quantification of MDA in biological matrices. Nowadays, the most commonly employed method utilises the reaction of MDA with thiobarbituric acid, resulting in the well-known thiobarbituric acid reactive substances (TBARS) assay [37]. Its product can be detected by colorimetry (532–535 nm) or fluorimetry (excitation at 532 nm and emission at 553 nm) [38]. It is an easy and inexpensive method, however MDA may be bound to matrix molecules and therefore undetectable without an adequate step to liberate it, such as alkaline hydrolysis [37].

Mechanistic studies investigating the correlation between red meat consumption and LPO as well as NOC-derived DNA adduct formation are scarce. Therefore, during this study, in vitro gastrointestinal digestive simulations of different meat types were undertaken to determine which specific digestive processes are involved in the formation of the harmful NOC-derived DNA adducts O^6-methylguanine (O^6-MeG) and O^6-CMG and the LPO product MDA. DNA adduct formation was assessed by quantitatively measuring O^6-MeG and O^6-CMG using LC-QqQ-MS/MS. The LPO product MDA was directly measured by means of a TBARS assay.

2 Materials and methods

2.1 Reagents and chemicals

(Caution: Potassium diazoacetate (KDA) is carcinogenic. It should be handled in a well-ventilated fume hood with extreme care and personal protective equipment.)

The chemical standard O^6-MeG was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the internal

standard O^6-methyl-d3-guanine was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). O^6-CMG standard was derived via acidic hydrolysis with 0.1 M formic acid at 70°C for 1 h, of O^6-carboxymethyl-2′-deoxyguanosine (O^6-CMdG, purity >96%) [24]. The stock solutions of the chemical standards O^6-MeG and O^6-CMG were prepared in ethanol at a concentration of 6.06 and 4.8 μmol/mL, respectively, and diluted with deionised water for obtaining working solutions of 818/81.8/8.18 fmol/μL and 646/64.6/6.46 fmol/μL, respectively. A working solution of O^6-methyl-d3-guanine (118.9 fmol/μL) in deionised water was prepared, as internal standard (IS). All solutions were stored in dark glass bottles at −20°C.

KDA, a known nitrosated glycine derivative [16, 19], was synthesised via alkaline hydrolysis of ethyl diazoacetate (Sigma-Aldrich) [18]. The stock solution of 800 mM KDA was made up of 1.14 g of ethyl diazoacetate and 11.4 mL of 1.8 M KOH, and mixed for 4 h at room temperature in the dark. Working solutions were obtained through dilution with PBS buffer [18].

Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification, and of Optima® MS grade for MS application (Fisher Scientific UK, Loughborough, UK), respectively.

2.2 Incubation conditions

2.2.1 Collection and preparation of human faecal samples

Faecal samples were obtained from 15 healthy volunteers (ten males and five females) between the ages of 20 and 60. Donors were on a Western-type diet and none had a history of digestive pathology nor had received antibiotics during 6 months prior to the sampling.

Faecal slurries of 20% w/v fresh inocula were prepared by homogenising (stomacher 400 Classic Laboratory Blender, Seward, West Sussex, UK) the faeces with PBS (0.1 M, pH 7), containing 1 g/L sodium thioglycolate as reducing agent. The particulate matter and biomass were removed by centrifugation for 2 min at 500 × *g*. For storage purposes at −80°C, 20% (v:v) of glycerol (99.5%; Analar Normapur, Fontenay-sous-Bois, France) was added to the supernatant as a cryoprotectant.

2.2.2 Simulated gastrointestinal digestion

Static in vitro incubation experiments of meat varying in haem content (chicken, beef and to a small extent pork) were performed in autoclaved penicillin flasks. The meat was therefore subjected to a sequential simulation of stomach, small and large intestinal digestion according to Van de Wiele et al. [39]. The different meat samples were obtained at a local butcher shop and their haem content was measured according to Hornsey [40]. A typical in vitro stomach digestion consisted of an incubation of 4 g of prepared meat (cooked for 10 min at 80°C to mimic a Western meal preparation) with pepsin (10 mg/L) for 2 h at pH 1.5 at a 1:10 ratio (v:v). Next, bile salts (6.0 g/L) and pancreatic enzymes supplemented as pancreatic powder of bovine origin (0.9 g/L) were added to the stomach suspension at a 1:2 ratio (v:v) to simulate small intestinal conditions. Small intestinal digestive simulation consisted of an incubation for 4 h at pH 7. Finally, for the colonic digestion, faecal microbiota (1:3 ratio (v:v)) and Simulator of the Human Intestinal Microbial Ecosystem (SHIME) broth (1:4 ratio (v:v)) were added to the small intestinal incubation mixture and incubation was continued for another 72 h after 1 h of N_2 flushing. The colon suspension contained in vitro cultured microbiota that was isolated from human faeces and is considered representative of the in vivo colon microbial ecology after a growth stabilisation period in Brain Heart Infusion (BHI) broth at a 1:9 ratio (v:v) in N_2 atmosphere. As a control, meat samples were incubated with inactive faecal microbiota. To this extent, faecal microbiota was autoclaved for 30 min (121°C, 1 bar overpressure). For investigating the haem-iron hypothesis, an additional in vitro digestion was performed with the faecal inoculum of an O^6-CMG-producing volunteer. Besides the beef, different amounts of myoglobin were supplemented at the start of the digestion, i.e. 0, 2.8, 14.1 and 28.3 nmol of myoglobin per millilitre of digestive fluid, respectively.

To avoid photocatalytic effects, all digestions were performed in amber flasks. Each batch culture was sealed with butylrubber stops and anaerobiosis was obtained by flushing the flasks with N_2 alternating every 2 min with vacuum suction during 1 h. Resazurin (2 mg/L) was added as redox indicator to the faecal slurry and to the colonic stage of the digestion. A pink colour indicated a redox potential higher than −80 mV, a colourless solution showed a redox potential below this limit, i.e. anaerobic. The redox potential in the large intestine typically ranges between −150 and −280 mV [41]. During the in vitro digestion, sampling of stomach, small intestine and large intestinal digestion (at 0, 24, 48 and 72 h of the colonic incubation) was done using syringes, causing as little disturbance as possible to the bacterial environment. The samples were then stored at −20°C before analysis. All experiments were performed in triplicate. A schematic overview of the in vitro gastrointestinal digestion of meat and consecutive analyses are presented in Fig. 1.

2.3 Cell culture

The human colon carcinoma cell line Caco-2 (American Type Culture Collection, Manassas, VA, USA) was cultured as a monolayer in DMEM (Gibco Invitrogen cooperation) containing 10% foetal calf serum, 1% non-essential amino acids and penicillin (100 U/mL) and streptomycin (0.1 mg/mL; all from Sigma-Aldrich), at 37°C in a humidified 5% CO_2 atmosphere. Cells were passaged weekly, using 0.25% trypsin-EDTA. For the experiments, Caco-2 cells were harvested at 80% confluency.

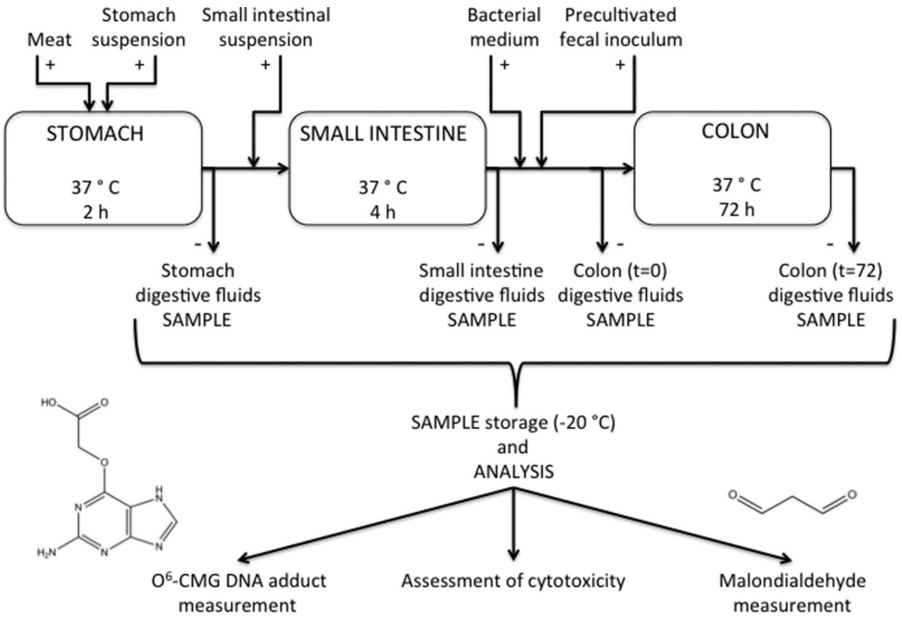

Figure 1. Schematic overview of the experimental setup of the in vitro gastrointestinal digestions of meat and consecutive analyses.

2.4 DNA extraction

Caco-2 cells, grown in a monolayer culture (80% confluency), were collected after a 5 min incubation at 37°C with 0.25% trypsin-EDTA. Prior to cell lysis, a cell viability rate of 99% was confirmed by trypan blue exclusion. For DNA extraction, cells were centrifuged for 5 min at 10 000 × *g* at 4°C to obtain a pellet. Subsequently, 1 mL of DNAzol® reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) was added per 1×10^7 of cells. Lysis of the cells was obtained by gently pipetting the mixture or inverting the assay tube. DNA was precipitated from the lysate by adding 0.5 mL of 100% ethanol per millilitre of DNAzol® reagent used for the isolation. Next, the sample was mixed by inversion and stored at room temperature for 1–3 min. DNA, visible as a cloudy precipitate, was collected by spooling with a pipette tip, while carefully decanting the supernatant. In case the resulting sheared DNA would not spool, 5 min centrifugation at 5000 × *g* was applied to obtain a DNA pellet. Finally, the pellet was resuspended in 250–500 μL of TE (Tris-EDTA) buffer. The concentration of the collected DNA was determined by a Nanodrop ND-1000 Spectrophotometer (Isogen Lifescience, IJsselstein, The Netherlands).

2.5 Assessment of cell injury

A colorimetric cell proliferation assay, WST-1 (Roche Diagnostics, Switzerland) was used to assess the cell proliferation and viability of the Caco-2 cells. The tetrazolium salt WST-1 is a ready-to-use substrate that is cleaved by mitochondrial dehydrogenase, which is present in viable cells [42], to formazan dye [43]. The total activity of this mitochondrial dehydrogenase correlates with the number of viable cells, which is in turn quantified by absorbance at 450 nm with a scanning multi-well spectrophotometer. This is a quick and easy manner to investigate the cytotoxic effect of digestion samples [44].

Caco-2 cells (200 μL; 1.25 e^5 cells/mL) were seeded on flat-bottom 96-well plates and incubated at 37°C, 5% CO_2 for 24 h. The next day, the DMEM medium was removed and replaced by 100 μL of the digest filtrates diluted in DMEM at 1:10, 1:50 and 1:100. The adhered cells were incubated for 24 h with the digestive fluid. Afterwards, 10 μL of the ready-to-use WST-1 reagent was added to each well and incubated for 2 h at 37°C, UV absorption was measured at 450 nm. Cell cytotoxicity was determined in quadruplicate and calculated accordingly:$[((A_{\text{Negative control}} - A_{\text{Blank control}}) - (A_{\text{Experimental value}} - A_{\text{Blank control}}))/(A_{\text{Negative control}} - A_{\text{Blank control}})] \times 100$; where $A_{\text{Experimental value}}$ is the absorbance of the sample, $A_{\text{Blank control}}$ is the absorbance of DMEM medium and $A_{\text{Negative control}}$ is the absorbance of cells incubated with DMEM medium. Additionally, some wells were treated with 10% TritonX-100 solution as a positive control (no cell viability).

For the WST-1 test, a sample selection was made based on the results of the chemical analysis of the NOC-derived DNA adducts. To this end, the cytotoxicity of the colonic digestive samples was investigated. In total, six faecal inocula were selected, of which three that displayed DNA adduct formation during colonic digestion and three with no to low DNA adduct concentrations upon colonic digestion.

2.6 Chemical analysis

2.6.1 Extraction protocol for DNA adducts

As a preparative step, all digestive samples were filtersterilised (0.22 μm) to ensure the absence of bacterial cells. Afterwards,

182 μL of the filtersterilised sample was supplemented with 100 μg Caco-2 DNA and 5.95 pmol of O^6-Me-d3-G (internal standard at 118.9 fmol/μL). Prior to the incubation step (18 h at 37°C, stirred at a constant speed of 150 RPM), the volume of the samples was standardised to 500 μL by means of TE buffer. The next day an acidic hydrolysis (2 mL of 0.1 M formic acid for 30 min) at 80°C was performed on all samples to release DNA bases, allowing detection of the individual targeted DNA adducts. Before applying the hydrolysate to a SPE cartridge which was conditioned with 2 mL of 100% MeOH and equilibrated with 2 mL deionised water, it was cooled down in crushed ice. The Oasis HLB SPE (30 mg, 1 mL) was, after loading the hydrolysate, eluted with 2 mL of 100% MeOH. The collected fraction was then evaporated to dryness (90 min, 20°C) using a SpeedVac® Plus (Savant, Holbrook, NY, USA). Finally, the dried residue was redissolved in a total volume of 100 μL of mobile phase consisting of 90/10 0.05% acetic acid in deionised water/MeOH [33]. As positive and negative control, 100 μg Caco-2 DNA in TE buffer was dissolved and incubated with and without the addition of 2 mM KDA, respectively.

2.6.2 HPLC-MS/MS measurement of DNA adducts

An extensive validation of the LC-MS/MS analysis method for the detection of the NOC-derived DNA adducts O^6-MeG and O^6-CMG, characterised with an LOD of 30 and 50 fmol/mg DNA, respectively, as well as the selection of a proper internal standard was previously described by Vanden Bussche et al. [33].

Before every analysis, injection of a standard mixture of the target compounds checked the operational conditions of the chromatographic device. Concentration of the analytes was calculated by fitting area ratios in a 12-point calibration curve, established in Caco-2 DNA fortified with O^6-MeG and O^6-CMG in the range of 0–817 and 0–645 pmol/mg DNA, respectively, with the IS at 118.9 fmol/μL. Instrument control and data processing were carried out with Xcalibur Software (Thermo Electron, San José, USA).

2.6.3 Apparent total *N*-nitroso compound analysis

The digestive samples were analysed by thermal energy analysis for the determination of the apparent total *N*-nitroso compounds (ATNCs). A selective chemical de-nitrosation with iodine/iodide reagent was performed to detect the ATNCs according to Kuhnle et al. [45]. A digestive homogenate of 100 μL was taken and incubated with 500 μL of aqueous sulphanilamide (50 mg/mL in 1 M HCl) for 5 min to remove unbound nitrite. Afterwards, the sample was injected into a custom-made purge-vessel containing the iodine/iodide reagent kept at 60°C. The released NO was transferred by nitrogen to the NOA chemiluminescence analyser (Model 88 et, Eco Physics, Duernten, Switzerland), via a condenser consisting of a NaOH (1 mol/L) trap. For differentiation between the *N*-nitroso, nitrosothiol and nitrosohaem compounds, mercury (II) stable (nitrosothiols) and potassium ferricyanide stable (nitrosyl iron) compounds were determined under exactly the same way as described above after an additional incubation with $HgCl_2$ (53 mmol/L) and $K_3Fe(CN)_6$ (4 mM), respectively. Results were expressed as millimoles of NO released per litre of digestive sample.

2.6.4 TBARS assay

The MDA concentration in the digestive samples was measured by a modified method in accordance with Grotto et al. [37]. TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. The absorbance of the coloured complex was measured spectrophotometrically (Genesis 8 UV-VIS Spectrometer, Spectronic-instruments, UK) at 532 nm with an LOD of 0.05 nmol/mL of digestive sample. The TBARS concentration was expressed in nanomole MDA per millilitre of digestive fluid and determined by means of a 7-point calibration curve with 1,1,3,3-tetramethoxypropane (0–50 nmol/mL).

2.6.5 Metabolic activity

Liquid digestion samples (2 mL) were collected and frozen at −20°C to inhibit further bacterial activity. For analysing short-chain fatty acids (SCFA), the samples were extracted with diethyl ether prior to the analysis on a Di200 gas chromatograph (Shimadzu, Hertogenbosch, The Netherlands). The GC was equipped with a capillary free fatty acid packed column (EC-1000 Econo-Cap column, Alltech, Laarne, Belgium; 25 m × 0.53 mm, film thickness 1.2 μm), a flame ionisation detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL/min. The column temperature and the temperature of the injector and detector were set at 130 and 195°C, respectively.

For the analysis of phenolic compounds (indol, phenol and *p*-cresol), a liquid/liquid extraction with n-hexane was performed prior to the GC-MS detection (PolarisQ, Thermo Fischer Scientific, San Jose, CA, USA). The Trace GC 2000 Gas Chromatograph was fitted with a PolarisQ quadrupole ion trap mass spectrometer. Helium (99.99% purity, Air Liquide, France) was used as carrier gas at a flow rate of 3 mL/min. A volume of 1 μL was injected (split flow 10 mL/min, splitless time 1 min). The GC was equipped with a Bpx-5 column (SGE, Victoria, Australia; 25 m × 0.22 mm, film thickness 0.25 μm), injector, ion source and transfer line temperature were, respectively, 290, 260 and 250°C. The column temperature consisted of a three-step temperature gradient starting at 40°C and went up to 350°C.

Ammonia analysis was performed on a 1026 Kjeltec Auto Distillation apparatus (FOSS Benelux, Amersfoort, The

Netherlands). Ammonium in the sample was liberated as ammonia by the addition of MgO. The released ammonia was distilled from the sample into a boric acid solution, which was subsequently back-titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm).

2.7 Statistics

Linear mixed model analysis (TIBCO Spotfire S+® 8.2, Göteborg, Sweden) provided a powerful and flexible approach to handle the correlated data (i.e. repeated measurements on each faecal inoculum over time, etc.). In this model, both fixed as well as random effects were incorporated, respectively, the meat type and the variability between the different volunteers. Mixed models can also be extended to non-normal outcomes. Additionally, a paired *t*-test and an ANOVA fixed effect model (TIBCO Spotfire S+® 8.2) were applied to investigate the individual response variable per volunteer.

3 Results

To investigate the influence of different meat types on specific metabolic and biological end points relevant to the gastrointestinal health status, in vitro digestive simulations were performed. Three different meat types (chicken, pork and beef) varying in haem content were selected, with the main focus on beef and chicken. To obtain representative microbial communities during the colonic in vitro digestion, 15 faecal inocula, obtained from healthy volunteers, were used in separate fermentation experiments.

3.1 Haem content of meat

The haem contents of the different meat types were determined at 2.57 ± 0.08, 4.97 ± 0.11 and 34.92 ± 0.35 nmol/g for chicken, pork and beef, respectively (Hornsey) [40]. Based on the haem analysis of the different meat types, the following haem concentrations were initially present in the incubation flasks: 1.40, 0.20, 0.10 nmol/mL for beef, pork and chicken, respectively.

3.2 NOC derived DNA adducts

The digestive fluids obtained during the different stages of the in vitro digestion were incubated with the extracted DNA of the colonic epithelial Caco-2 cell line to investigate the potential formation of O^6-CMG and O^6-MeG. After incubation and extraction, the samples were quantitatively analysed for the presence of NOC-derived DNA adducts by LC-MS/MS. As a positive control, Caco-2 DNA was incubated with 2 mM KDA, a reactive intermediate derived from nitrosated glycine, capable of inducing the NOC-derived DNA adducts [16,18,19]. This resulted in the detection of O^6-CMG at 10.4 ± 1.1 pmol/mL and of O^6-MeG at 1.4 ± 0.3 pmol/mL. Omitting KDA resulted in the absence of both DNA adducts.

Table 1. Concentrations of O^6-carboxymethylguanine (pmol/mL) formed during in vitro colonic digestive simulation of chicken and beef with faecal inocula from 15 different individuals

ID	Sampling time (h)	O^6-CMG (pmol/mL digestive fluid)	
		Poultry	Beef
1	0	33.7 ± 1.2	41.1 ± 4.5
	72	226.4 ± 6.5	539.3 ± 77.5*
2	0	ND	ND
	72	548.0 ± 33.2	1594.8 ± 119.2**
3	0	9.7 ± 0.5	14.0 ± 0.3**
	72	174.0 ± 10.9	231 ± 14.1**
4–6	0	ND	ND
	72	ND	ND
7	0	29.9 ± 1.2	32.3 ± 2.1
	72	619.8 ± 21.7*	492.3 ± 25.6
8	0	50.6 ± 2.0	54.4 ± 4.9
	72	1324.4 ± 103.4	1254.8 ± 75.1
9	0	ND	ND
	72	37.7 ± 0.8	39.8 ± 1.7
10	0	ND	ND
	72	46.7 ± 1.3	39.6 ± 3.9
11–15	0	ND	ND
	72	ND	ND

ND, not detected; significantly higher with a *p-value < 0.10 or with a **p-value < 0.05; mean ± SE, performed in triplicate.

Both DNA adducts could not be detected in the simulated digestive fluids of the stomach and small intestine after overnight incubation with Caco-2 DNA. In samples taken at the end of the colonic digestion (after 72 h of incubation), seven out of 15 colonic digests contained the NOC-derived DNA adduct O^6-CMG. The two positive female inocula and five positive male inocula all belonged to different age categories. At the start of the colonic digestion (0 h), five out of seven digests that were positive at 72 h, already contained low amounts (<55 pmol/mL) of O^6-CMG (Table 1). The O^6-CMG concentration in the positive digestive fluid samples increased significantly ($p < 0.05$) from 0 to 72 h for six faecal inocula, whereas one inoculum showed a borderline significant decrease ($p = 0.094$).

Upon linear mixed effect modelling of means, a borderline significant difference ($p = 0.055$) was observed in O^6-CMG DNA adduct formation between the different digested meats at the end of the colonic digestion. When looking at each of the seven O^6-CMG positive faecal inocula individually (by means of paired *t*-test), the beef indicated a significantly higher genotoxic effect compared to the digested chicken ($p < 0.05$) for two out of seven inocula. The inoculum of one volunteer displayed a borderline significantly higher genotoxic effect for beef ($p < 0.1$; Table 1). For one other inoculum, chicken proved to generate more DNA adducts

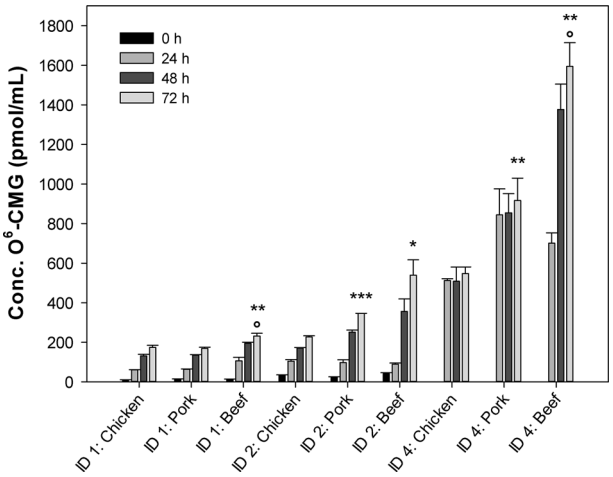

Figure 2. LC-MS/MS data of O^6-carboxymethylguanine (pmol/mL ± SE) in the different colonic digestive samples (0, 24, 48, 72 h) for three different faecal inocula, performed in triplicate with three different meat types. (Significantly higher than chicken with **p*-value < 0.1 or ***p*-value < 0.05 or ****p*-value < 0.01; significantly higher than pork with °*p*-value < 0.1.)

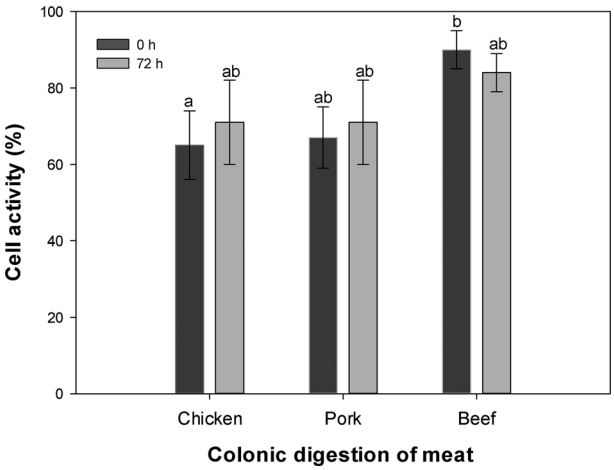

Figure 3. Results of the WST-1 cell proliferation assay of Caco-2 cells incubated (24 h) with the colonic digestive fluids of different meat types (dilution 1:10; 0 and 72 h), calculated as the average of six faecal inocula incubations, each performed in triplicate (a, b: a bar with a different letter differs with a 90% confidence interval).

compared to beef ($p < 0.1$; Table 1). Noteworthy was that O^6-MeG was never detected in any of the digestive fluids of the 15 volunteers.

During a second experiment, additional digestions of pork (cfr *cytotoxicity of meat*) with six different faecal inocula were performed. To this purpose, three O^6-CMG-producing and three non-O^6-CMG-producing faecal inocula were randomly selected. In Fig.2, the O^6-CMG concentrations of the three (out of six selected) faecal inocula, which produced the NOC-derived DNA adduct upon colonic digestion, are displayed. Two of these showed a significant difference ($p < 0.05$) between the generated O^6-CMG adducts derived from the digested pork and chicken, whereas the third volunteer only displayed a borderline significant difference ($p < 0.1$). The differences between the digested pork and beef were less pronounced ($p < 0.1$, only for two out of three inocula; Fig.2).

Additionally, an investigation into the involvement of the colonic microbiota in the DNA adduct formation process was envisaged. To this extent, the six selected faecal inocula underwent autoclavation (121°C, 1 atm, 15 min) and in vitro digestive simulation of the different meat types. During this experimental set-up, it was not possible to detect O^6-CMG or O^6-MeG in any of the different digestive samples (data not shown).

The addition of a faecal inoculum to the colonic stage of the in vitro digestion results in the presence of a large quantity of bacterial DNA, 7–8 log 10 colony forming units/mL digestive fluids. This bacterial DNA might undergo alkylation due to ongoing *N*-nitrosation taking place during the digestive process. In order to investigate the contribution of bacterial DNA to the formation of the O^6-CMG DNA adduct, the addition of Caco-2 DNA to the incubation step of the digestive samples upon LC-MS/MS analysis was omitted. No significant differences ($p > 0.05$) in DNA adduct levels were observed in the presence or absence of Caco-2 DNA.

3.3 Cytotoxicity of digested meat

The WST-1 proliferation assay was used to investigate the cytotoxic effect of the different meat types during in vitro colonic digestion. The digested meat samples were diluted 1:10, 1:50 and 1:100. The latter two displayed only a small decrease in total cell activity (14.1 ± 0.6%) compared to the control. The 1:10 dilution affected the enzymatic cell activity the most (25.3 ± 1.4%). At the beginning of the colonic digestion (0 h), a limited but significantly higher cytotoxic effect could be observed for the digested chicken as compared to the beef ($p = 0.053$). For the digested beef samples, a non-significant increase in cytotoxicity ($p > 0.1$) was observed after colonic digestion (0 versus 72 h), in all six of the selected faecal inocula (Fig. 3).

3.4 Total apparent *N*-nitroso compound analysis

The main focus of the ATNC analysis was to single out the NOC fraction from the other NO-contributing compounds, e.g. nitrosothiol, nitrosyl iron, by means of selective chemical denitrosation and chemiluminescence detection [45]. The presence of the NOC-derived DNA adduct O^6-CMG was detected during the colonic digestion and peaked towards the end, however no NOCs were found during this stage of the colonic in vitro digestion simulation. The only digestive fluids positive for NOCs were the stomach samples, this however at very low concentrations (<35 nmol of NO derived from NOCs detected per litre of digestive sample), and with the highest

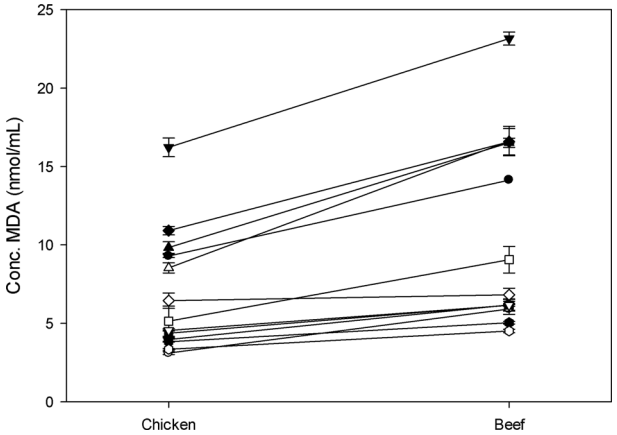

Figure 4. MDA concentrations (mean ± SE, performed in triplicate) for all 15 volunteers measured at the beginning of the in vitro colonic digestive simulation (0 h) for a low and high haem-containing meat, chicken and beef, respectively.

concentration found for the digested beef samples (data not shown).

3.5 TBARS assay

To determine the levels of the main LPO end product MDA in the digestive samples, a TBARS analysis was performed on all digestive fluids. The highest MDA concentrations (15 faecal inocula performed in triplicate, mean ± SE) were observed at the beginning of the colonic simulation (0 h: 22.3 ± 4.9 nmol/mL and 17.5 ± 3.3 nmol MDA/mL in digestive fluids of beef and chicken, respectively) and decreased towards the end (72 h: 10.5 ± 6.1 nmol/mL and 6.9 ± 3.9 nmol MDA/mL for digested beef and chicken, respectively).

By mixed linear effect modelling of the means, a significant difference was observed between the MDA results ($p < 0.0001$) between 0 and 72 h of colonic digestion, but also between the different meat types ($p < 0.0004$). As displayed in Fig. 4, the direction of response for MDA formation appeared to be consistent towards the high-haem meat.

3.6 Haem involvement

It is speculated by Corpet [14] that haem iron could play a major role in cancer promotion [46]. This hypothesis is supported by a meta-analysis of epidemiological studies that shows a suggestive association between dietary haem and risk of colon cancer [47]. For this reason, an additional in vitro gastrointestinal digestive simulation comprising a sequential stomach, small intestinal and large intestinal digestion was performed. For the large intestinal digestion, a faecal inoculum of an O^6-CMG- and high-MDA-producing volunteer was selected and added. Different amounts of myoglobin, the major haem-containing protein in mammalian muscle tissue, were added at the start (i.e. stomach) of the in vitro gastrointestinal beef simulation, 0, 2.8, 14.1 and 28.3 nmol of myoglobin per millilitre of digestive fluid were supplemented, respectively. As depicted in Table 2, a clear positive dose response could be noticed for the O^6-CMG DNA adduct formation when haem-containing myoglobin was added to the digestion. An increase in the myoglobin dose (28.3 nmol/mL) resulted in significant higher concentration levels of O^6-CMG ($p = 0.004$, by ANOVA).

All of the digestive samples of this experiment were also analysed for their MDA levels, which peaked at 0 h of the colonic digestion. A positive dose response of MDA towards higher myoglobin concentration levels was also observed, with the most pronounced effect at the beginning (0 h) of the colonic digestion ($p = 0.008$) compared to the end (72 h) ($p = 0.015$).

3.7 Metabolic activity

To evaluate the microbial fermentation during the three different meat incubations (bovine, porcine and poultry), SCFAs, indol, *p*-cresol, phenol and ammonia were monitored during the in vitro colonic digestive simulation (Supporting Information Table 1). An increasing amount of SCFAs was produced during the colonic digestion approximating the acetic acid:propionic acid:butyric acid molar ratio of 60:20:20 as put forward by Cummings [48]. The digestion of the different meat types did not influence the SCFA profile significantly, the faecal inocula of the different volunteers however did as shown by the beef data (Supporting Information Table 1). Similar results were obtained for the other meat types. Noteworthy is that elevated butyric acid profiles were observed for three out of six faecal inocula (four to six) at the end of the colonic digestion. The measured concentrations of indol, phenol, *p*-cresol and ammonia did not display significant differences between the different meat types and the different faecal inocula (data not shown).

4 Discussion

It has been suggested that haem iron may play a major role in CRC promotion. Two independent pathways have been hypothesised in explaining the link between haem and CRC promotion. Fat peroxidation, on the one hand, resulting in the formation of potent aldehydes (i.e. MDA), capable of producing mutagenic DNA adducts, and the *N*-nitroso pathway, on the other hand, leading to DNA alkylation and DNA adduct formation [14]. The aim of this study was to explore both pathways in order to assess the potential genotoxicity and molecular mechanisms involved in different meat types varying in haem content (beef, chicken and pork) under simulated GIT conditions.

To investigate the genotoxicity of the digested meat types, in terms of NOC-derived DNA adduct formation, two adducts

Table 2. Obtained concentrations of O^6-CMG and MDA per millilitre of digestive fluid after the addition of different amounts of myoglobin to in vitro digestive simulations of beef

Time of sampling	Amount of myoglobin added (nmol/mL digestive fluids)			
	0.0	2.8	14.1	28.3
O^6-CMG (pmol/mL digestive fluids)				
Colonic digestion: 0 h	ND	ND	ND	ND
Colonic digestion: 72 h	2.3 ± 0.7[a)]	7.8 ± 1.2[a)]	12.4 ± 0.6[a)]	36.4 ± 7.5[b)]
MDA (nmol/mL digestive fluids)				
Colonic digestion: 0 h	15.8 ± 0.6[a)]	17.4 ± 0.9[a)]	19.7 ± 1.0[a)b)]	19.8 ± 0.5[b)]
Colonic digestion: 72 h	6.0 ± 0.1[a)]	7.6 ± 0.7[a)b)]	7.8 ± 0.3[a)b)]	8.4 ± 0.2[b)]

a), b) means within a row with a different superscript differs significantly (p-value < 0.05); ND, not detected; mean ± SE, performed in triplicate.

were selected, namely O^6-CMG and O^6-MeG. Previous research has demonstrated their presence in colonic cell tissue, indicating their relevance in CRC promotion [22, 23]. To investigate the NOC-derived DNA adduct formation potential of the different digestive fluids, the latter were incubated with extracted DNA of the human colorectal adenocarcinoma cell line Caco-2 prior to LC-MS/MS analysis. This approach was preferred over the incubation with whole cells, which displayed unacceptable inter-batch variation between the different passages of the cell cultures and a low sample throughput [33]. Furthermore, in previous research, we were able to demonstrate that DNA adduct concentrations did not significantly differ if a known NOC (KDA) was added to Caco-2 cells or to pre-extracted Caco-2 DNA [33].

The incubation results demonstrated that the O^6-CMG DNA adduct was formed for seven out of 15 faecal inocula during the large intestinal (colonic) simulation and significantly increased towards the end of the colonic digestion ($p < 0.05$).

When assessing the role of the different GIT compartments in the DNA alkylation pathway, the data of the ATNC measurements with nitrosyl iron as mean contributor and O^6-CMG, both peaking at the end of the colonic digestion, reflect the significance of the large intestine. The key factor in the endogenous alkylation appears to be the presence of an active microbial community, which was acknowledged by the absence of O^6-CMG when autoclaved faecal inocula were added to the batch cultures. Massay et al. [49] reported the same observation in germ-free rats, where *N*-nitrosation did not occur due to the absence of a normal microbial population. Moreover, the essential role of the individual microbiota is reflected by the observed inter-individual differences [50] in the formation of the O^6-CMG DNA adduct (Table 1). It is clear from literature that the composition and metabolic activity of the colonic microbiota can differ considerably between and even in individuals during the course of their lifetime [50]. With respect to NOC formation, the *N*-nitrosating properties of certain bacteria colonising the individual human gut (and a possible connection to carcinogenesis) have been demonstrated before [51–53]. Furthermore, the possible role (of the exact composition) of the individual gut microbiota in health and disease was already pointed out decades ago [54], and has been brought to the surface even more in recent years [55, 56]. Other examples of significant inter-individual differences in the transformation of dietary compounds or contaminants such as, phytoestrogens [57], heterocyclic aromatic amines [58], polycyclic aromatic hydrocarbons [39], etc. have been reported.

For five out of seven O^6-CMG-generating faecal inocula, the O^6-CMG DNA adduct could already be detected at the mere beginning of the colonic digestion. The only difference between the small intestinal fluids, where O^6-CMG was never detected, and the initial colonic digestive fluids (before 72 h of incubation), is the addition of the individual faecal inoculum. We concluded that for those five volunteers, O^6-CMG was already present in the faecal inoculum, most probably due to in vivo formation of this DNA adduct prior to sampling. O^6-CMG quantities in the faecal inocula of the two other volunteers may have been absent or below the detection limit of the utilised LC-MS/MS method. Indeed, the presence of O^6-CMG in certain human faecal samples further demonstrates the in vivo relevance of O^6-CMG since O^6-CMG has previously been detected in various biological samples (i.e. blood, colonic biopsies and exfoliated colonocytes) [19, 22, 23, 33].

To investigate the genotoxicity of the different meat types (i.e. chicken and beef) with respect to the NOC pathway, a mixed model analysis of the entire dataset was performed. This statistical analysis demonstrated a borderline significantly higher ($p = 0.055$) O^6-CMG concentration for beef versus chicken at the end of the colonic digestion (72 h).

The suggested higher genotoxicity of red meat is in line with previous in vivo studies, indicating that a higher *N*-nitrosation occurs when haem-rich meat has been digested [8, 9, 23, 45]. Most of these studies used the faecal ATNC concentration to compare the dose response of haem-rich versus low-haem meat. Accordingly, ATNC analyses were performed on the digestive samples obtained from our in vitro stomach, small and large intestinal digestions to correlate the *N*-nitrosation process during the incubations with the obtained O^6-CMG values. No NOCs were detected in the digestive fluids of the small and large intestinal digestion. This

finding might also suggest as reported by Mirvish [20] that besides NOCs, other nitrite-derived alkylating agents may be responsible for the formation of O^6-CMG. Moreover, these agents might be unable to form O^6-MeG, providing a potential additional explanation for the absence of O^6-MeG. The largest fraction of the ATNC measured (data not shown) however consisted of nitrosyl iron, reinforcing the involvement of haem in the link between a high red meat diet and CRC [12, 14, 23, 45].

To mimic the human in vivo gastrointestinal digestion of meat, this study relied on sequential in vitro incubations. These in vitro models take into account the human physiology by simulating the transit through the human digestive tract, which can be executed either by using separate GI compartments or by sequential exposure of the food source to simulated mouth, gastric, small and large intestinal conditions [59–61]. Though the use of these types of batch cultures has its limitations, i.e. absence of gastrointestinal absorption, lack of interaction with the host colonic mucosa, etc., in vivo studies were not considered here due to lack of versatility in terms of mechanistic explorative work as well as time consuming and costly nature [62]. Furthermore, whenever possible, in vivo studies should be avoided due to ethical considerations. For this study, the advantage of sequential batch cultures lies with the fact that they allow to investigate which element or mechanism within the gastrointestinal digestion is involved in the studied process. To enable the use of different faecal inocula in a shorter time frame in combination with a consecutive multi-phase simulation of the GIT, static over dynamic batch cultures were preferred [59].

The addition of pre-cultured microbiota (faecal inoculum) to the batch cultures resulted in the presence of a large quantity of bacterial DNA (350 pmol DNA/mL digestive fluids for 7–8 log10 colony forming units/mL). Analysing the colonic digestive samples for O^6-CMG without the incubation of Caco-2 DNA did not result in a significant difference in O^6-CMG levels. This finding suggests that alkylation of the DNA occurred during the in vitro digestion, indicating the highly reactive nature of either NOCs, possibly explaining the absence of NOCs in the ATNC measurements of the colonic digestive fluids [63] or of other nitrite-derived alkylating agents. Under in vivo circumstances, alkylating agents that are produced in the colon will most likely not only bind to bacterial DNA, but through the intimate contact that exists between the microbiota and the colonic epithelium [64], also to human DNA, and result in the formation of DNA adducts. As a result, the measurement of the O^6-CMG DNA adducts, although originating from bacterial or faecal exfoliate DNA in this study, is considered as a biomarker for potential DNA adduct formation in vivo.

Previously reported observations [16, 18] indicate that carboxymethylation at the O^6 atom of 2'-deoxyguanosine appeared to be a common feature when nitrosated glycine derivatives are formed. The reported concomitant methylation however appeared absent in our digestive simulations, and no O^6-MeG adducts could be detected in the digestive fluids. O^6-MeG can be repaired under in vitro circumstances by MGMT [16, 65], a repair mechanism expressed by the Caco-2 cell line [66]. Nevertheless, in our experiments, pre-extracted Caco-2 DNA was used, which is most likely depleted of any MGMT molecules as a result of the DNA extraction procedure [33]. Therefore, it is believed that MGMT could not have been responsible for the absence of O^6-MeG. Indeed, in literature it has been consistently reported that the formation rate of O^6-MeG compared to O^6-CMG is significantly lower when incubating DNA with KDA [16, 18, 33], which could explain its absence. As for O^6-CMG, only very recently a report was published of its ability to act as a MGMT substrate in synthetic oligodeoxyribonucleotides [67]. This finding could possibly undermine O^6-CMG biomarker capacities for CRC. However, in a previously reported study by Vanden Bussche et al. [33], Caco-2 cells as well as the pre-extracted DNA thereof were incubated with KDA and similar levels of O^6-CMG DNA adducts were formed, which might suggest that the MGMT repair system is not potent enough to repair a considerable amount of O^6-CMG adducts. Based on this finding and the presence of O^6-CMG in biological samples [22, 23, 33], it would still seem acceptable to consider O^6-CMG as an adequate biomarker and potential causative agent for CRC.

Next to the alkylation pathway, the peroxidation of polyunsaturated fatty acids with MDA as predominant product has been suggested by Corpet [14], as an additional mechanistic hypothesis for the existing link between dietary haem intake and CRC risk. The MDA measurements demonstrated significant differences ($p = 0.0004$) between the digestion of beef and chicken, suggesting the higher genotoxic potential of red meat consumption, with the highest levels generated before colonic digestion (Table 2). These MDA levels may be correlated to the data of the WST-1 cell proliferation assay where only at the beginning of the colonic digestion a borderline significant difference was observed between the digested beef and chicken ($p = 0.053$). The latter effect disappeared towards the end of the colonic digestion, which could be linked to the decline in MDA level. However, it needs to be stated that the results of the cytotoxicity test proved inconclusive and tests based on different mechanisms, e.g. detection of caspase positive cells (marker for apoptosis) [68], checking membrane permeability by propidium iodides staining [69], might be advisable for future experiments. As for the significant MDA decrease during colonic digestion, this could be attributed to different factors: i.e. degradation into volatile compounds, formation of Schiff bases through reaction with protein chains, reaction with bacterial DNA resulting in several DNA adducts (e.g. 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2-α]purin-10(3H)-one (M_1dG) [25]), oxidation by bacterial aldehyde dehydrogenase activity [70] and presence of dietary fibre in the faecal inocula, which are known to bind MDA [71].

An additional incubation experiment was performed to substantiate the haem iron hypothesis as potential causative CRC agent [14] by adding myoglobin to the in vitro digestion. Both O^6-CMG and MDA increased significantly upon addition of 28.3 nmol myoglobin per millilitre of digestive

fluid. These results indicate the involvement of the myoglobin protein and haem molecule, which are more present in red than white meat, in the active formation of O^6-CMG and MDA. Next to the involvement of haem in the gastrointestinal formation of cyto- and genotoxic compounds upon red meat digestion, it is very likely that other factors may play a role in the formation pathway(s) of both MDA and the O^6-CMG DNA adduct. As a first factor, the colonic microbiota consist of a complex mixture with several attributes that differ inter- and intra-individually and even by anatomical site along the colon and their location within the lumen [50,56]. The colonic bacteria are constantly influenced by their surroundings; haem-Fe can, e.g. increase the prevalence of *Enterobacteria* and *Bacteroidetes* spp. and decrease *Lactobacilli* and *Firmicutes* spp. [72]. Additionally, it has been reported that certain *Lactobaccilli* and *Bifidobacteria* spp. are capable of producing nitric oxide (• NO) from nitrite [73]. Depending on the •NO to reactive oxygen species ratio, •NO can enhance or inhibit oxidation processes [74] and thus MDA formation. Of course, nitrite and •NO may play their part in the nitrosation pathway as well. Furthermore, high-fat diets give rise to a decrease in *Bifidobacteria*. *Bifidobacteria* are involved in the production of SCFAs such as butyrate, which is known for its beneficial anticarcinogenic attributes. Another important and possibly interfering factor that is linked to diet is the remaining amount of polyunsaturated fatty acids (PUFAs) in faeces [75] and thus also in the digestive flasks. Since PUFAs initiate the LPO process, variation in PUFA content (both in faeces and meat) may reflect directly upon MDA formation. These and other modulating factors in both the O^6-CMG and MDA formation pathway will influence the haem-Fe dose response.

To evaluate whether proper microbial fermentation occurred during the in vitro digestion simulation, SCFA levels were monitored, since these are the main end products resulting from colonic bacterial fermentation of dietary carbohydrates. The most important SCFAs are acetic acid, propionic acid and butyric acid, which occur in molar ratios of about 60:20:20 in the colon [48]. The SCFA profiles of the conducted in vitro digestions (Supporting Information Table 1) indicated proper microbial fermentation, approximating the in vivo molar ratios. The SCFA profiles obtained from three out of six selected faecal inocula showed a significantly higher production of butyric acid. It is not uncommon that butyric acid increases or even exceeds the levels of propionic acid as a result of peptide supplementation [76], which may be considered as a probable cause since meat is an important protein source. Additionally, some human faecal bacteria are known as net consumers of acetic acid, resulting in the production of butyric acid [77], which could have been the case in our experimental set-up due to the extensive batch culture duration of 72 h. Butyric acid is known to exert important effects on cell differentiation and gut health [78] and is thought to play a protective role against CRC [79]. Indeed, the batch cultures with high butyric acid profiles were those with almost no O^6-CMG formation (<15 pmol/mL). In this case, one could propose a potential butyrogenic effect, although further research would be required to confirm this. The other digestive products, i.e. phenol, indo, *p*-cresol and ammonia did not significantly differ between the different meat types or the faecal inocula.

In this study, two independent pathways have been set forward to mechanistically explain the reported correlation between red meat consumption and CRC, namely the alkylation and LPO pathway. The in vitro experiments clearly demonstrated that both pathways depend on different mechanisms, but may both require the presence of myoglobin (representing the meat-specific haem iron fraction) as a catalytic agent. While endogenous alkylation was observed during colonic fermentation, MDA formation peaked during the small intestinal digestion. Moreover, the formation of the alkylated DNA adduct O^6-CMG appeared to depend on the microbial composition, since the inter-individual variability of the faecal inocula influenced the DNA adduct formation considerably and autoclavation completely inhibited the process. A contributing factor in the MDA production was most likely the inherent fat content of the meat [9], since MDA is the main by-product of the peroxidation of polyunsaturated fatty acids, which is known to be prone to iron-mediation [80]. Both factors play a role in the MDA formation, which significantly differed between the digested beef and chicken ($p < 0.0004$). To conclude, our in vitro digestions confirmed that the consumption of high-haem meat (beef) may exert genotoxic effects in the GIT. As for the role of the gastrointestinal digestion, it was demonstrated that the duodenum displayed the highest LPO rate with subsequent MDA formation, while for the alkylated DNA adduct O^6-CMG, the colon and its innate microbial biota were proven to be vital and the basis for the significant observed variability between the individual faecal inocula.

L. Vanhaecke is a postdoctoral fellow from the Research Foundation Flanders (Fonds voor Wetenschappelijk Onderzoek (FWO-Vlaanderen). Part of this study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (RT 11/6250 MEATNOX). The authors would like to thank M. Naessens, L. Dossche, J. Goedgebeur, I. Opsteyn and D. Stockx for their practical assistance in the laboratory and K. Goethals for the support of the statistical matters.

The authors have declared no conflict of interest.

5 References

[1] Norat, T., Bingham, S., Riboli, E., Re: meat, fish, and colorectal cancer risk: the European prospective investigation into cancer and nutrition – Reply. *J. Natl. Cancer Inst.* 2005, *97*, 1787–1788.

[2] Norat, T., Chan, D., Lau, R., Aune, D., Vieira, R., The associations between food, nutrition and physical activity and the risk of colorectal cancer. *WCRF/AICR Systematic Literature Review Continuous Update Project Report*, Imperial College London, London, UK 2010.

[3] Micha, R., Wallace, S. K., Mozaffarian, D., Red and processes meat consumption and risk of incident coronary heart disease, stroke, and diabetes mellitus: a systematic review and meta-analysis. *Circulation* 2010, *121*, 2271–2283.

[4] Pan, A., Sun, Q., Bernstein, A. M., Manson, J. E. et al., Changes in red meat consumption and subsequent risk of type 2 diabetes mellitus. *JAMA Intern. Med.* 2013, *173*, 1328–1335.

[5] Männistö, S., Kontto, J., Kataja-Toumola, M., Albanes, D. et al., High processed meat consumption is a risk factor of type 2 diabetes in the ATBC study. *Br. J. Nutr.* 2010, *103*, 1817–1822.

[6] Larsson, S. C., Orsini, N., Wolk, A., Diabetes mellitus and risk of colorectal cancer: a meta-analysis. *J. Natl. Cancer Inst.* 2005, *97*, 1679–1687.

[7] Giovanucci, E., Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. *Am. J. Clin. Nutr.* 2007, *86*, 836S–842S.

[8] Bingham, S. A., Pignatelli, B., Pollock, J. R. A., Ellul, A. et al., Does increased endogenous formation of *N*-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis* 1996, *17*, 515–523.

[9] Hughes, R., Cross, A. J., Pollock, J. R. A., Bingham, S., Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. *Carcinogenesis* 2001, *22*, 199–202.

[10] Bingham, S. A., Hughes, R., Cross, A. J., Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. *J. Nutr.* 2002, *132*, 3522s–3525s.

[11] Lombardi-Boccia, G., Martinez-Dominguez, B., Aguzzi, A., Total heme and non-heme iron in raw and cooked meats. *J. Food Sci.: Food Chem. Toxicol.* 2002, *67*, 1738–1741.

[12] Cross, A. J., Pollock, J. R. A., Bingham, S. A., Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer Res.* 2003, *63*, 2358–2360.

[13] Bonnett, R., Charalambides, A. A., Martin, R. A., Sales, K. D. et al., Reactions of nitrous acid and nitric oxide with porphyrins and haems. Nitrosylhaems as nitrosating agents. *J. Chem. Soc., Chem. Commun.* 1975, *21*, *884–885*.

[14] Corpet, D. E., Red meat and colon cancer: should we become vegetarians, or can we make meat safer? *Meat Sci.* 2011, *89*, 310–316.

[15] Mirvish, S. S., Formation of *N*-nitroso compounds: chemistry, kinetics, and in vivo occurrence. *Toxicol. Appl. Pharmacol.* 1975, *31*, 325–351.

[16] Shuker, D. E. G., Margison, G. P., Nitrosated glycine derivatives as a potential source of O^6-methylguanine in DNA. *Cancer Res.* 1997, *57*, 366–369.

[17] Wang, J., Wang Y., Chemical synthesis of oligodeoxyribonucleotides containing N_3- and O^4-carboxymethylthymidine and their formation in DNA. *Nucleic Acids Res.* 2009, *37*, 336–345.

[18] Harrison, K. L., Jukes, R., Cooper, D. P., Shuker, D. E., Detection of concomitant formation of O^6-carboxymethyl- and O^6-methyl-2′-deoxyguanosine in DNA exposed to nitrosated glycine derivatives using a combined immunoaffinity/HPLC method. *Chem. Res. Toxicol.* 1999, *12*, 106–111.

[19] Cupid, B. C., Zeng, Z. T., Singh, R., Shuker, D. E. G., Detection of O^6-carboxymethyl-2′-deoxyguanosine in DNA following reaction of nitric oxide with glycine and in human blood DNA using a quantitative immunoslot blot assay. *Chem. Res. Toxicol.* 2004, *17*, 294–300.

[20] Mirvish, S. S., Role of *N*-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett.* 1995, *93*, 17–48.

[21] Challis, B. C., Chemistry and biology of nitrosated peptides. *Cancer Surv.* 1989, *8*, 363–384.

[22] Hall, C. N., Badawi, A. F., O'Connor, P. J., Saffhill, R., The detection of alkylation damage in the DNA of human gastrointestinal tissues. *Br. J. Cancer* 1991, *64*, 59–63.

[23] Lewin, M. H., Bailey, N., Bandaletova, T., Bowman, R. et al., Red meat enhances the colonic formation of the DNA adduct O-6-carboxymethyl guanine: implications for colorectal cancer risk. *Cancer Res.* 2006, *66*, 1859–1865.

[24] Moore, S. A., Xeniou, O., Zeng, Z. T. T., Humphreys, E. et al., Optimizing immunoslot blot assays and application to low DNA adduct levels using an amplification approach. *Anal. Biochem.* 2010, *403*, 67–73.

[25] Nair, U., Bartsch, H., Nair, J., Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: a review of published adduct types and levels in humans. *Free Radic. Biol. Med.* 2007, *43*, 1109–1120.

[26] Phillips, D. H., Arlt, V. M., The P-32-postlabeling assay for DNA adducts. *Nat. Protoc.* 2007, *2*, 2772–2281.

[27] van Sittert, N. J., Boogaard, P. J., Natarajan, A. T., Tates, A. D. et al., Formation of DNA adducts and induction of mutagenic effects in rats following 4 weeks inhalation exposure to ethylene oxide as a basis for cancer risk assessment. *Mutat. Res.* 2000, *447*, 27–48.

[28] Gottschalg, E., Scott, G. B., Burns, P. A., Shuker, D. E. G., Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O^6-carboxymethyl- and O^6-methyl-2′-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer. *Carcinogenesis* 2007, *28*, 356–362.

[29] Farmer, P. B., Brown, K., Tompkins, E., Emms, V. L. et al., DNA adducts: mass spectrometry methods and future prospects. *Toxicol. Appl. Pharmacol.* 2005, *207*, 293–301.

[30] Singh, R., Farmer, P. B., Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection. *Carcinogenesis* 2006, *27*, 178–196.

[31] Himmelstein, M. W., Boogaard, P. J., Cadet, J., Farmer, P. B. et al., Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage. *Crit. Rev. Toxicol.* 2009, *39*, 679–694.

[32] Zhang, F., Bartels, M. J., Pottenger, L. H., Bhaskar Golapudi, B. et al., Simultaneous quantitation of 7-methyl- and O^6-methylguanine adducts in DNA by liquid chromatography-positive electrospray tandem mass spectrometry. *J. Chrom. B* 2006, *833*, 141–148.

[33] Vanden Bussche, J., Moore, S. A., Pasmans, F., Kuhnle, G. G. et al., An approach based on ultra-high pressure liquid chromatography-tandem mass spectrometry to quantify O^6-methyl and O^6-carboxymethylguanine DNA adducts in intestinal cell lines. *J. Chromatogr. A* 2012, *1257*, 25–33.

[34] Niki, E., Lipid peroxidation: physiological levels and dual biological effects. *Free Radic. Biol. Med.* 2009, *47*, 469–484.

[35] Del Rio, D., Stewart, A. J., Pellegrini, N., A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* 2005, *15*, 316–328.

[36] Marnett, L. J., Oxy radicals, lipid peroxidation and DNA damage. *Toxicology* 2002, 181–182, 219–222.

[37] Grotto, D., Santa Maria, L. D., Boeira, S., Valentini, J. et al., Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. *J. Pharm. Biomed. Anal.* 2007, *43*, 619–624.

[38] Knight, J. A., Pieper, R. K., McClellan, L., Specificity of the thiobarbituric acid reaction: its use in studies of lipid peroxidation. *Clin. Chem.* 1988, *34*, 2433–2438.

[39] Van de Wiele, T., Vanhaecke, L., Boeckaert, C., Peru, K. et al., Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites. *Environ. Health Perspect.* 2005, *113*, 6–10.

[40] Hornsey, H. C., The color of cooked cured pork. I. Estimation of the nitric oxide-haem pigments. *J. Sci. Food Agr.* 1956, *7*, 534–540.

[41] Jonas, C. R., Estivariz, C. F., Jones, D. P., Gu, L. H. et al., Keratinocyte growth factor enhances glutathione redox state in rat intestinal mucosa during nutritional repletion. *J. Nutr.* 1999, *129*, 1278–1284.

[42] Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K. et al., A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharm. Bull.* 1996, *19*, 1518–1520.

[43] Slater, T. F., Sawyer, B., Sträuli, U., Studies on succinate-tetrazolium reductase systems. III. Points of coupling of four different tetrazolium salts. *Biochim. Biophys. Acta* 1963, *77*, 383–393.

[44] Carlson, M. A., Technical note: assay of cell quantity in the fibroblast-populated collagen matrix with a tetrazolium reagent. *Eur. Cells Mater.* 2006, *12*, 44–48.

[45] Kuhnle, G. G., Story, G. W., Reda, T., Mani, A. R. et al., Diet-induced endogenous formation of nitroso compounds in the GI tract. *Free Radic. Biol. Med.* 2007, *43*, 1040–1047.

[46] Sawa, T., Akaike, T., Kida, K., Fukushima, Y. et al., Lipid peroxyl radicals from oxidized oils and heme-iron: implication of a high-fat diet in colon carcinogenesis. *Cancer Epidemiol. Biomarkers Prev.* 1998, *7*, 1007–1012.

[47] Bastide, N. M., Pierre, F. H., Corpet, D. E., Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. *Cancer Prev. Res.* 2011, *4*, 177–184.

[48] Cummings, J. H., Short chain fatty acids in the human colon. *Gut* 1981, *22*, 763–779.

[49] Massay, R. C., Key, P. E., Mallett, A. K., Rowland, I. R., An investigation of the endogenous formation of apparent total N-nitroso compounds in conventional microflora and germ-free rats. *Food Chem. Toxicol.* 1988, *26*, 595–600.

[50] Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M. et al., The human microbiome project. *Nature* 2007, *449*, 804–810.

[51] Leach, S. A., Thompson, M., Hill, M., Bacterially catalysed N-nitrosation reactions and their relative importance in the human stomach. *Carcinogenesis* 1987, *8*, 1907–1912.

[52] Engemann, A., Focke, C., Humpf, H.-U., Intestinal formation of N-nitroso compounds in the pig cecum model. *J. Agric. Food Chem.* 2013, *61*, 998–1005.

[53] Suzuki, K., Mitsuoka, T., N-nitrosamine formation by intestinal bacteria. *IARC Sci. Publ.* 1984, *57*, 275–281.

[54] Hill, M. J. (Ed.), *The Role of Gut Bacteria in Human Toxicology and Pharmacology*, Taylor & Francis, London 1995.

[55] Sekirov, I., Russell, S. L., Antunes, I. C., Finlay, B., Gut microbiota in health and disease. *Physiol. Rev.* 2010, *90*, 859–904.

[56] Culpepper, T. B. S., Volker, M., Evidence for contributions of gut microbiota to colorectal carcinogenesis. *Curr. Nutr. Rep.* 2013, *2*, 10–18.

[57] Decroos, K., Cattoir, S., Boon, N., Verstraete, W., In vitro production of Equol by human intestinal microbiota. *J. Nutr.* 2004, *134*, 1283S–1284S.

[58] Kassie, F., Rabot, S., Kundi, M., Chabikovsky, M. et al., Intestinal microflora plays a crucial role in the genotoxicity of the cooked food mutagen 2-amino-3-methylimidazo[4,5-f]quinolone (IQ). *Carcinogenesis* 2001, *22*, 1721–1725.

[59] Wickham, M., Faulks, R., Mills, C., In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Mol. Nutr. Food Res.* 2009, *53*, 952–958.

[60] Hur, S. J., Lim, B. O., Decker, E. A., McClements, D. J., *In vitro* human digestion models for food applications. *Food Chem.* 2011, *125*, 1–12.

[61] Van de Wiele, T., Gallawa, C. M., Kubachka, K. M., Creed, J. T. et al., Arsenic metabolism by human gut microbiota upon in vitro digestion of contaminated soils. *Environ. Health Perspect.* 2010, *118*, 1004–1009.

[62] Oomen, A. G., Hack, A., Minekus, M., Zeijdner, E. et al., Comparison of five *in vitro* digestion models to study the bioaccessibility of soil contaminants. *Environ. Sci. Technol.* 2002, *36*, 3326–3334.

[63] Stuff, J. E., Goh, E. T., Barrera, S. L., Bondy, M. L. et al., Construction of an N-nitroso database for assessing dietary intake. *J. Food Compost. Anal.* 2009, *22*, S42–S47.

[64] Manco, M., Putignani, L., Bottazzo, G. F., Gut microbiota, lipopolysaccharides, and innate immunity in the pathogenesis of obesity and cardiovascular risk. *Endocr. Rev.* 2010, *31*, 817–844.

[65] O'Driscoll, M., Macpherson, P., Xu, Y. Z., Karran, P., The cytotoxicity of DNA carboxymethylation and methylation by the model carboxymethylating agent azaserine in human cells. *Carcinogenesis* 1999, *20*, 1855–1862.

[66] Ramos, A. A., Pedro, D. F. N., Lima, C. F., Collins, A. R. et al., Development of a new application of the comet assay to

assess levels of O^6-methylguanine in genomic DNA. *Free Radical Biol. Med.* 2013, *60*, 41–48.

[67] Senthong, P., Millington, C. L., Wilkinson, O. J., Marriott, A. S. et al., The nitrosated bile acid DNA lesion O^6-carboxymethylguanine is a substrate for the human DNA repair protein O^6-methylguanine-DNA methyltransferase. *Nucleic Acids Res.* 2013, *41*, 3047–3055.

[68] Jerome, K. R., Sloan, D. D., Aubert, M., Measurement of CTL-induced cytotoxicity: the caspase 3 assay. *Apoptosis* 2003, *8*, 563–571.

[69] Liao, T. T., Jia, R. W., Shi, Y. L., Jia, J. W. et al., Propidium iodide staining method for testing the cytotoxicity of 2,4,6-trichlorophenol and perfluorooctane sulfonate at low concentrations with Vero cells. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng.* 2011, *46*, 1769–1775.

[70] Nosova, T., Jokelainen, K., Kaihovaara, P., Jousimies-Somer, H. et al., Aldehyde dehydrogenase activity and acetate production by aerobic bacteria representing the normal flora of human large intestine. *Alcohol Alcohol.* 1996, *31*, 555–564.

[71] Hur, S. J., Lim, B. O., Park, G. B., Joo, S. T., Effects of various fibre additions on lipid digestion during in vitro digestion of beef patties. *J. Food Sci.* 2009, *74*, C653–C657.

[72] Ijssennagger, N., Derrien, M., van Doorn, G. M., Rijnierse, A. et al., Dietary heme alters microbiota of mouse colon without changes in host-microbe cross-talk. *PLoS One* 2012, *7*, e49868.

[73] Sobko, T., Reinders, C. I., Jansson, E. A., Norin, E.et al., Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. *Nitric Oxide* 2005, *13*, 272–278.

[74] Darley-Usmar, V., Wiseman, H., Halliwell, B., Nitric oxide and oxygen radicals: a question of balance. *FEBS Lett.* 1995, *2–3*, 131–135.

[75] Amate, L., Gil, A., Ramirez, M., Dietary long-chain polyunsaturated fatty acids from different sources affect fat and fatty acid excretions in rats. *Am. J. Nutr.* 2001, *131*, 3216–3221.

[76] Walker, A. W., Duncan, S. H., Leitch, E. C. M., Child, M. W. et al., pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. *Appl. Environ. Microbiol.* 2005, *71*, 3692–3700.

[77] Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H. et al., Phylogenetic relationships of dominant butyrate-producing bacteria from the human gut. *Appl. Environ. Microbiol.* 2000, *66*, 1654–1661.

[78] Csordas, A., Butyrate, aspirin and colorectal cancer. *Eur. J. Cancer Prev.* 1996, *5*, 221–231.

[79] McIntyre, A., Gibson, P. R., Young, G. P., Butyrate production from dietary fiber and protection against large bowel cancer in a rat model. *Gut* 1993, *34*, 386–391.

[80] Janero, D. R., Burghardt, B., Thiobarbituric acid-reactive malondialdehyde formation during superoxide-dependent, iron-catalyzed lipid peroxidation: influence of peroxidation conditions. *Lipids* 1989, *24*, 125–131.

