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Cyto- and genotoxic effects of meat in vitro digestion products on HT29 colon cancer cell lines

Tazkiyah Izzati

Promoters:
Prof. Dr. Stefaan De Smet
Dr. Winnok H. De Vos

Supervisor: Thomas Van Hecke

Master’s dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science in Nutrition and Rural Development, main subject: Human Nutrition
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Gent, June 2013

The promoters

1. Prof. Dr. Stefaan De Smet
   Signature:

2. Dr. Winnok H. De Vos
   Signature:

The author

Name: Tazkiyah Izzati
Signature:
Dedication

To my beloved parents, Akhmad Setiobudi and Dewi Widya, who are always there to pray and support me from the very beginning

To all my friends who always believe in me, even when I don’t

“In the name of Allah, the Most Beneficent, the Most Merciful”

“So, high (above all) is Allah, the Sovereign, the Truth. And O’ Muhammad, do not hasten with recitation of the Qur’an before its revelation is completed to you, and say, “My Lord, increase me in knowledge”. (Q.S Taha (20) : 114)
Acknowledgements

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<th>Definition</th>
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<tbody>
<tr>
<td>5-YSR</td>
<td>5-year survival rate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangectasia mutated</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>DSBs</td>
<td>Double Strand Breaks</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>LAOOH</td>
<td>Linoeic acid hydroperoxide</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>NOCs</td>
<td>N-nitroso compounds</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SHIME</td>
<td>Simulator of Human Intestinal Microbial Ecosystem</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SSBs</td>
<td>Single Strand Breaks</td>
</tr>
<tr>
<td>TNM</td>
<td>primary Tumour, regional lymph Nodes and distant Metastasis</td>
</tr>
<tr>
<td>UDS</td>
<td>Unscheduled DNA Synthesis</td>
</tr>
<tr>
<td>•OH</td>
<td>Hydroxyl radical</td>
</tr>
</tbody>
</table>
Abstract

An increasing body of evidence suggests that there is a correlation between red meat consumption and colon cancer. However, the exact mechanisms are not yet fully understood. The aim of this study was to assess the adverse effects of in vitro digestion products of various types of meat on HT29 colon cancer cell lines. Various end-points, including cell viability, cell and nuclear morphology, lipid peroxidation and DNA damage were measured in cell lines incubated with in vitro digestion products derived from duodenal and colon digestion of different meat types, i.e. beef, pork (with and without nitrite) and chicken.

After optimization of a dedicated fluorimetric assay, we found a clear and significant effect on cell viability in cells treated with 10% meat digests. This effect was confirmed by transmission microscopy, which revealed a reduced cell density (disaggregation). More detailed inspection revealed additional morphological changes including darkening of cells at the periphery and alteration of the nuclear shape. Additional assays for gauging lipid peroxidation and DNA damage as yet yielded inconsistent results and require further optimization.

However, based on the current data we concluded that meat digests may contain a specific cytotoxic effect, higher in red meat (pork and beef), compared to white meat (chicken), which may be attributed to the presence of heme.
1. LITERATURE REVIEW

1.1 Introduction

Cancer is a multifactorial disease that is associated with alterations in gene expression patterns resulting from a complex interplay between genetic and environmental factors. The major hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, including angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2000, 2011). According to statistical data from the American Cancer Society in the year 2008 (Karp, 2005), cancer is the second most deadly disease in the world after cardiovascular disease. The most lethal cancer type is lung cancer (31% for men and 26% for women), followed by breast cancer for women (15%) and prostate cancer for men (13%). The third most lethal cancer type is colorectal cancer (8% for men and 9% for women). When placed under scrutiny, it appears that only 10% of the colorectal cancer has a hereditary cause, meaning that the other 90% is caused by exogenous factors such as diet (Forman, 1992). This also implies that a change of diet could dramatically affect colon cancer incidence.

Colorectal cancer (CRC) develops in the colon or rectum and originates from epithelial cells (colonocytes), which delineate the bowel and which are the first cells to interact with exogenous compounds from the diet and byproducts of digestion. Since colon and rectum are mostly composed of glandular tissue, 96% of CRC are adenocarcinomas (American Cancer Society 2011). Development of CRC usually starts with the formation of noncancerous polyps called adenomatous polyps or adenomas, that become increasingly malignant and during metastasis can spread to lymph nodes through lymph vessels and to the liver, lungs or ovary through blood vessels (American Cancer Society 2011). Colorectal cancer stages are described by the extent of cancer spreading. The most common staging systems used in clinical settings is the TNM classification system (primary Tumor, regional lymph Nodes and distant Metastasis) which is mostly based on the depth of invasion, lymph node status and degree of distant metastasis, factors that are essential for prognosis and determining the choice of treatment (Sobin et al., 2009; Weitz et al., 2005).
According to the TNM system, the following stages can be discriminated (Sobin et al., 2009; Weitz et al., 2005):

- **Stage 1**: the tumor invades the submucosa and/or muscularis propria, without metastasis in both regional lymph nodes nor distant metastasis. 80-95% 5-year-survival rate (5-YSR) after treatment.

- **Stage 2**: the tumor invades through the muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissue (stage 2A) or directly invades other organs or structures and/or perforates visceral peritoneum (stage 2B), without metastasis in both regional lymph nodes nor distant metastasis. 72-75% 5-YSR for stage 2A and 65-66% 5-YSR for stage 2B.

- **Stage 3**: the tumor invades submucosa and/or muscularis propria (stage 3A) or invades through the muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissue and directly invades other organs or structures and/or perforates visceral peritoneum (stage 3B), with metastasis in 1 to 3 regional lymph nodes, or any tumor invasion with metastasis in 4 or more regional lymph nodes (stage 3C). 55-60%, 35-42%, and 25-27% 5-YSR for stage 3 A, B and C respectively.

- **Stage 4**: tumor invasion and metastasis to lymph nodes, with distant metastasis. Only 0-7% 5-YSR.

Given the rapid decrease in 5-YSR with cancer stage, early detection and treatment is essential in reducing CRC-related mortality (Weitz et al., 2005). Early detection followed by treatment with combination of surgical resection and chemotherapy will show better prognosis with higher survival rate. Next to traditional surgery, the minimally invasive surgical technique termed laparoscopy is also recommended for its advantages of less intraoperative blood loss, decreased post-operative pain and shorter length of hospitalization. For patients with later stage CRC (with distant metastasis), liver resection (the most common metastasized tissue in CRC) combined with chemotherapy allows to achieve 58% 5-YSR. However, by the time the patients reach this CRC stage (with distant metastasis to liver), only 15-20% of them are candidates for surgical resection at the time of diagnosis and the other 75-80% patients will still have 5-YSR of 0-7% (Hayat, 2009). From this evidence we can conclude that treatment at early stage of CRC will increase survival rate of patients and therefore early
diagnosis with better, higher sensitivity and specificity, as well as less invasive screening and detection of CRC is really important.

Several screening methods are available such as fecal-occult blood testing (FOBT), flexible sigmoidoscopy and colonoscopy. These screening methods have major drawbacks such as dietary and drug restrictions, demand for multiple samples and bowel preparation. In choosing screening methods, sensitivity exceeds specificity for detecting the highest proportion of true positive individuals with CRC and to date, colonoscopy (with sensitivity reported between 55% to 92%) is still considered as the gold standard for the detection of colorectal neoplasia (Hayat, 2005; American Cancer Society, 2011). However, some studies show failure in the detection of neoplastic lesions in colonoscopy (van Rijn et al., 2006).

Molecular screening methods are rising to overcome the limitations (e.g. patient discomfort) of the aforementioned methods, DNA-based stool testing for multiple DNA markers (K-ras, p53, APC and Bat26) or methylation-specific PCR testing of p16 detection in patients’ serum increase sensitivity for adenomas and early carcinomas (Ahlquist and Shuber, 2002; Zou et al., 2002). Despite the increased sensitivity, the high cost for DNA-based stool testing and low specificity for p16 testing are still major limitations for these approaches. Hence, to improve diagnosis, there is need for novel specific and sensitive biomarkers.

1.2 Colon cancer and the potential role of red meat

CRC is considered as an environmental disease (Haggar and Boushey, 2009), with a broad range of environmental causal factors including socio-cultural background and lifestyle. Geographically, CRC incidence is higher in the United States of America, Australia, and Europe, whereas it is very low in Japan and Africa (Haggar and Boushey, 2009; Norat et al., 2005). Some studies show increased risk among migrants from countries with low incidence of CRC to a country with high CRC incidence. This has been linked to high consumption of red meat and processed meat, refined carbohydrates, and food with high level of fats, known as “western diet”, because 90% of CRC is caused by dietary factors (Giovannucci, 2002). Several population studies connect red and processed meat with colorectal cancer development.

In a large epidemiological study on a cohort of 478040 men and women in 10 European countries, Norat and colleagues (2005) built a linear regression model based
on gender and lifestyle (diet, physical activity and smoking behaviour), that showed a
significant correlation between red meat and processed meat intake with colorectal
cancer incidence (Norat et al., 2005). Moreover, more recent studies give evidence for a
direct correlation between red meat consumption with colon cancer, but not with rectal
cancer (Chan et al., 2011; Haggar and Boushey, 2009; Magalhaes et al., 2012;
Zandonai et al., 2012). In the meta-analysis study of 8 cohort and 8 case-control
studies, Magalhaes et al. (2012) showed an increased risk of colon cancer (not rectal
cancer) with high intake of red and processed meat. In line with this study, another
meta-analysis study by Zandonai et al. (2012) showed that increased consumption of
red meat from 100 grams to 120 grams per day increased risk of CRC from 28% to
35%, while increased processed meat consumption from 25-30 grams/day increased
CRC risk from 20% to 49%.

1.3 Digestion and in vitro digestion of meat

The most faithful way to study digestion is to use animal models. However, in vivo
models have the disadvantage that they are complex, elaborate and difficult to
standardize. In addition there are ethical considerations associated with their use. To
circumvent those issues, in vitro digestion models have been conceived. Obviously, an
in vitro model represents a simplified situation and offers a means to perform digestion
in a systematic and reproducible manner (Oomen et al., 2003; Versantvoort et al.,
2005). In vitro models should approximate human physiological conditions as close as
possible. In the context of human digestion this means that various factors need to be
taken into account, including: compartments, temperature, concentration and
composition of digestive juices, pH and transit time. Table 1 summarizes some of the
most important conditions of the in vivo human digestion system that need to be
simulated in an in vitro digestion model.

Miller et al. (1981) first introduced the in vitro digestion as a method to measure
bioavailability of iron and other trace elements. Later, in vitro digestion models were
developed to estimate bio-availability of active compounds, such as protein, starch,
minerals (calcium and selenium) or carotenoid (Hedren et al., 2002) from vegetable or
to gauge toxic contaminants (such as lead) in food (Oomen et al., 2003). Other
examples of bio-accessibility studies include measurements of aflatoxins in peanut
samples or ochratoxin in buckwheat or heterocyclic amines from cooked meat (Kulp et
al., 2003; Versantvoort et al., 2005).
Krul et al. (2000) developed an integrated *in vitro* model for meat digestion to measure the mutagenic activity of fried chicken breast or fried beef. The model mimics enzymatic digestion in the stomach and small intestine as well as the mechanic digestion through peristaltic movements with physiological conditions similar to those in the human body (pH, temperature, digestive enzymes, pancreatic movements, bile and pancreatic juices, and absorption of digested products by removal via dialysis). Kulp et al. (2003) developed a model to determine the bio-accessibility of Heterocyclic Aromatic Amines in cooked chicken, by simulating the digestion in the mouth (using amylase and CaCl$_2$), in the stomach (using HCl, NaCl and pepsin) and in the small intestine (using NaHCO$_3$ and pancreatin). The *in vitro* digestion process was performed in resealable plastic bags. Peristaltic movement, transit along GI layer and absorption were not taken into account.

Table 1. Digestion process *in vivo* as mentioned by Oomen et al. (2003), Guven et al. (1996), Dabrowska-Ufniarz et al. (2002) based on human physiology (Guyton and Hall, 2006) and Rotard model (Rotard et al. 1995) in (Oomen et al., 2003)

<table>
<thead>
<tr>
<th>Compartments</th>
<th>Temperature</th>
<th>pH</th>
<th>Transit time</th>
<th>Constituents and components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouth</strong></td>
<td></td>
<td>6.2 – 7.4</td>
<td>5 minutes</td>
<td>Saliva:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Inorganic solution (KCl, KSCN, NaH$_2$PO$_4$, NaCl, NaOH)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Organic solution (Urea)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• a-amylase</td>
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<td>• mucin</td>
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<td></td>
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<td></td>
<td>• Peroxidase</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td>37°C</td>
<td>1.5 – 2.0</td>
<td>2 hours</td>
<td>Gastric juice:</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Inorganic solution (NaCl, NaH$_2$PO$_4$, KCl, CaCl$_2$, H$_2$O, NH$_4$Cl, HCl)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Organic solution (Glucose, Glucoronic acid, Urea)</td>
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<td></td>
<td>• Pepsin, mucin</td>
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<td>• Ascorbic acid</td>
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<td></td>
<td>• H$_2$O2</td>
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<td></td>
<td></td>
<td>• Iron</td>
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<tr>
<td><strong>Small Intestine</strong></td>
<td></td>
<td>4.5 – 7.5</td>
<td>2 hours</td>
<td>Duodenal Juice:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Inorganic solution (NaCl, NaHCO$_3$, KH$_2$PO$_4$, KCl, MgCl$_2$, HCl)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Organic solution (Urea)</td>
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<td></td>
<td></td>
<td>• Pancreatin</td>
</tr>
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<td></td>
<td>• Lipase</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>• Bile salts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Inorganic solution (NaCl, NaHCO$_3$, KCl, HCl)</td>
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<td></td>
<td>• Organic solution (Urea)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Bile</td>
</tr>
<tr>
<td><strong>Large Intestine</strong></td>
<td></td>
<td>8.0</td>
<td>72 hours</td>
<td>Human bacterial inoculum and SHIME medium</td>
</tr>
</tbody>
</table>
A more generic and complete model was established by Versantvoort et al. (2005). He built on the model proposed by Oomen et al. (2003) to examine the bio-accessibility of toxic compounds present in the food matrix. This static method takes into account the retention time in the most dominant gastrointestinal compartments: mouth, stomach and small intestine, and simulates physiological conditions such as the composition of digestive fluid and pH. However, like many of the aforementioned in vitro models, simulations of digestion in the large intestine are limited. Especially with an eye on understanding CRC development, it is important to also take into account protein fermentation processes by intestinal microflora in the colon, since they play a major role in the formation of potentially hazardous metabolites such as ammonia and nitrosamines that could interact with colonocytes and thus trigger carcinogenesis (Cross and Sinha, 2004; Cummings and MacFarlane, 1997a). For this purpose, it is important to include microflora fermentation in the in vitro models, for instance by using a SHIME installation (simulator of human intestinal microbial ecosystem) (Molly et al., 1994). This in vitro model is able to maintain the diverse microbial communities of the intestinal tract.

1.4 Cell-based assays in carcinogenicity/mutagenicity

Epidemiological findings about the correlation between certain carcinogenic/mutagenic compounds and the emergence of cancer demand fundamental research to shed a light on the actual working mechanisms. To do so, several bioassays have been established, ranging from long-term animal bioassays to molecular/cell-based snapshot assays (Bajpayee et al., 2005). In the first type of assays the development of neoplastic lesions is observed in test animals during a major portion of their lifespan after exposure of various doses of test substances. Such an experiment can take up to years, is labor-intensive and therefore expensive.

Around 1969, a group of researchers tested the effects of a classic carcinogenic compound, dimethyl nitrosamine, on a yeast, Neurospora crassa and discovered DNA mutation, which later become a tool to study mutational events caused by carcinogenic compounds. This study opened possibilities for studying carcinogens in vitro as a rapid and efficient alternative to the laborious and expensive long-term animal bioassays (Weisburger, 1999). Once it was clear that DNA and the genetic apparatus might be a key target of potentially hazardous compounds, the term of genotoxicology arised, which refers to the study of various chemical and physical agents that can react with
hereditary material in living organisms, thus having the potential of being mutagenic, cell transforming and carcinogenic (Li and Heflich, 1991; Weisburger, 1999).

Currently, there are direct and indirect in vitro and in vivo genotoxicity assays designed to detect agents that can induce genetic alterations with various modes of action. Typically DNA damage is identified in the form of point mutations, single strand or double strand breaks (SSBs/DSBs), chromosome rearrangements or, more recently also epigenetic alterations (Baylin and Herman, 2000; Cox et al., 1973; Tice et al., 2000). Usually a combination of 3 or 4 tests are required for carcinogen/mutagen classification (Bajpayee et al., 2005).

One of the first genotoxicity assays that is still often used, is the Ames test. The principle of this test is based on the usage of a strain of Salmonella typhimurium that carries mutations in a gene involved in histidine synthesis, rendering it only viable in histidine-supplemented medium. Mutagenic compounds, however can induce point mutations in the histidine operon, and thereby create bacteria that can grow in histidine-free medium. Comparison of growth in both media offers simple and efficient tool for screening potential mutagenic compounds (Phillips and Venitt, 1995).

Other methods that are often used to detect DNA damage are the chromosome aberration assay, micronucleus and sister chromatid exchange in proliferated cell population; detection of DNA repair synthesis or unscheduled DNA synthesis (UDS) in individual cells; and detection of DNA breaks (single strand breaks or SSBs and double strand breaks or DSBs) and alkali labile sites with COMET assay. The principle of some of these assays is described in table 2.

In UDS detection, damaged cells have to be incubated with radioactive nucleotides and sensitivity is limited because not all DNA damage can be repaired equally. Alkaline elution or alkaline gel electrophoresis evaluate DNA damage on the cell population level and need a large amount of cells (Phillips and Venitt, 1995). Scoring chromosome aberrations, micronuclei or sister chromatid exchanges has many disadvantages, such as the fact that it can only be used with proliferating cells and DNA damage has to be rendered microscopically visible. COMET assay is a widely used technique based on gel-electrophoresis to detect DNA strand breaks (SSBs, DSBs) and alkali-labile lesions in individual cells. While considered to be sensitive, rapid and inexpensive (Phillips and Venitt, 1995), the recently developed immunofluorescence
assay for detecting double-strand breaks (DSBs) by specific labeling of the phosphorylated histone protein H2AX (\(\gamma\)-H2AX) surpasses the COMET assay in sensitivity and specificity and allows for localizing the damage \textit{in situ} (Kuo and Yang, 2008; Redon et al., 2011).

**Table 2.** Principle of numerous cell-based genotoxicity assay: UDS, chromosome aberration assay, micronucleus, COMET assay and \(\gamma\)-H2AX (Kelly and Latimer, 2005; Kohn and Grimek-Ewig, 1973; Phillips and Venitt, 1995; Tucker and Preston, 1996)

<table>
<thead>
<tr>
<th>Genotoxicity assay</th>
<th>Principle</th>
<th>Endpoint measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unscheduled DNA synthesis (UDS)</td>
<td>Measuring cell’s ability to perform global genomic nucleotide excision repair (NER) in living cells by creating 6-4 photoproducts and pyrimidine dimers using UVC irradiation and then allowing for their repair.</td>
<td>Amount of radioactive thymidine incorporated after UVC radiation and length of time allowed for the incorporation.</td>
</tr>
<tr>
<td>Chromosome aberration assay</td>
<td>Scoring cells with fragmented chromosomes in mitotic cells during anaphase or telophase.</td>
<td>Chromosome fragments, anaphase bridges, aneuploidy, telomere fusions, telomere loss, polycentrometric chromosomes.</td>
</tr>
<tr>
<td>Micronucleus</td>
<td>Scoring micronuclei formed by the loss of whole chromosome or portions of chromosomes from daughter nuclei at mitosis and exist separately from the main nucleus of a cell.</td>
<td>Number of cells with micronuclei.</td>
</tr>
<tr>
<td>COMET assay</td>
<td>Intact cells are fluorescently labeled for DNA and put under an electrical field. The principle of gel electrophoresis is used to determine the amount of DNA fragments. The rationale is that DNA fragments will move out of the nucleus, generating a tail or comet-like occurrence.</td>
<td>Comet tail and size.</td>
</tr>
<tr>
<td>Alkaline elution</td>
<td>Selective elution of single strands DNA from filters.</td>
<td>Single strand breaks.</td>
</tr>
<tr>
<td>(\gamma)-H2AX immunofluorescence staining</td>
<td>Immunostaining for phosphorylated H2AX ((\gamma)-H2AX) and subsequent detection by fluorescent microscopy.</td>
<td>(\gamma)-H2AX spot number and intensity.</td>
</tr>
</tbody>
</table>

Even though cell-based genotoxicity assays only provide a snapshot of the current state of a biological system, they can be used at different stages of carcinogenesis and allow for detecting early events in the carcinogenesis process (the initiation phase).

### 1.5 Cellular stress response and cellular damage

Cells are threatened continuously with various compounds either from the environment (exogenous) or from metabolic processes that occur within the cells (endogenous). These compounds might induce cellular damage, directly or indirectly, for instance by causing oxidative stress (Fulda et al., 2010). Depending on the level and
mode of stress, cellular responses towards these potential hazards are highly variable, ranging from activation of survival pathways to initiation of cell death (Fulda et al., 2010; Martindale and Holbrook, 2002). In the following paragraphs we will discuss two major types of cellular damage, oxidative stress and DNA damage, and focus on the potential cellular responses.

a. Oxidative stress and stress-activated pathways

Organisms living in an aerobic environment are constantly and inevitably exposed to reactive oxygen species (ROS) that comprise various partially reduced oxygen metabolites with higher reactivity than molecular oxygen (e.g., superoxide anions, hydrogen peroxide, and hydroxyl radicals). ROS can be generated endogenously by normal cell function, such as normal aerobic metabolism predominantly within the electron transport chain of the mitochondria or NADPH oxidases (Liu et al., 2002) or exogenously (Martindale and Holbrook, 2002). In low amounts, ROS have been attributed important signaling functions (e.g. ROS production by NADPH oxidase, activated by Vascular Endothelial Growth Factor (VEGF) that stimulating angiogenesis) but in higher amounts, ROS become a constant threat for cell viability (Finkel, 2003; Ushio-Fukai, 2006). To counteract the damage from ROS, cells possess numerous antioxidants, such as non-enzymatic molecules (e.g. glutathione and vitamin A) or enzymatic scavengers (e.g., superoxide dismutase (SOD), catalase and glutathion peroxide). When there is an imbalance in favor of ROS production, the cell experiences a condition called oxidative stress (Fulda et al., 2010). Depending on the degree of oxidative stress the cell continues proliferation, it enters temporary or permanent growth arrest (senescence) or initiates cell death. Martindale and Holbrook (2002) explains several pathways activated by oxidative stress that determine cell’s fate. ROS can modulate the functions of several enzymes (e.g. ataxia-telangectasia mutated (ATM) kinase, ERK, JNK and p38) and transcription factors (e.g. p53 and NFκB), resulting in enhanced survival or cell death.

Upon increase of cellular ROS, ERK and SAPK (Stress Activated Protein Kinase: JNK and p38) become activated by a phosphorylation cascade involving MAPK, in which its activation contributes to either cell-cycle arrest or apoptosis depending on intensity of the insult. However, the predominant outcome of ERK pathway activation is cell survival, while JNK pathway activation predominantly
leads to cell death (Finkel and Holbrook, 2000; Martindale and Holbrook, 2002). The outcome of the p38 kinase pathway is more variable, with mitotic arrest being induced in low oxidative stress conditions, and agent-specific induction of apoptosis (e.g. by singlet oxygen, but not by hydrogen peroxide) (Kurata, 2000).

Tumor suppressor protein p53 is a universal sensor for genotoxic stress and transcription factor which regulates gene expression involved in mediating hydrogen peroxide-induced growth arrest, replicative senescence (p21) and/or apoptosis (Bax, Noxa, p53 AIPI, PUMA, Fas). The commitment of a cell to undergo apoptosis is a result of achieving a critical threshold of ROS, and a positive feedback loop in which p53 activation by ROS leads to more generation of ROS, and so on (Martindale and Holbrook, 2002; Waldman et al., 1995).

ROS also activates NFκB transcription factor which has both apoptotic and anti-apoptotic effects. Several NFκB-dependent genes that have anti-apoptotic functions are TNF receptor-associated factor 1 (TRAF1), TRAF2, cellular inhibitors of apoptosis protein (CIAPs), MnSOD and A20 zinc finger protein. Activity of Fas ligand and p53 is also correlated with NFκB (Martindale and Holbrook, 2002).

Ataxia Telangiectasia Mutated (ATM) kinase is required for optimal induction and activation of p53 protein in the presence of oxidative stress. ATM acts as a sensor of ROS and/or oxidative damage in human cells and has protective roles by triggering cell-cycle arrest to allow repair (Guo et al., 2010).

In general, there is an intricate interplay of different pathways in response to various oxidative insults, whereby one pathway modulates activities of the other pathway and vice versa. The combined effects of individual pathways can be either synergetic or antagonistic (Martindale and Holbrook, 2002).

b. DNA damage and DNA damage repair

Upon infliction of DNA damage, the cell initiates a multifaceted response of gene induction and protein mobilization that leads to cell cycle arrest and DNA repair. A typical hallmark of DNA repair is the rapid accumulation of repair proteins at damage sites (also referred to as damage foci). The underlying signaling cascades consist of sequential and reciprocal activation of DNA damage sensors, transducers, mediators and effectors.
Two major causes of DNA damage are ROS and environmental conditions. They differ in the damage they cause and repair mechanisms they elicit. ROS compounds such as superoxide anions, hydroxyl radicals and hydrogen peroxide derived from oxidative respiration and products of lipid peroxidation may trigger subtle changes in DNA structure called oxidative modifications in DNA. Abnormal environmental conditions such as absence of specific nutrient or growth factors may induce ROS formation in innoxious amount, in which the cell is still able to repair such base modifications (e.g. abasic sites) (Hoeijmakers, 2001). However, when there are excessive ROS or environmental agents such as ultraviolet, ionizing radiation and genotoxic chemicals more severe damage can occur, like DSBs, which are more difficult to repair (Jackson, 2002).

The consequences of DNA damage include cell-cycle arrest at specific checkpoints in G1, S, G2 and M to allow DNA repair (Bunz et al., 1998; Hoeijmakers, 2001; Waldman et al., 1995). p53 is responsible for regulating cell-cycle arrest at G1. Subsequently, histone protein H2AX becomes phosphorylated (by ATM kinase) to provide a local chromatin state that favors complex repair reactions and/or for recruiting repair proteins (Hoeijmakers, 2001). Successful repair will allow the cell to resume its normal metabolism and continue with cell cycle progression, while misrepair may induce mutations that can lead to tumorigenesis and irreparable damage may induce cell death.

c. Cell states

In general, when confronted with damage, a cell will arrest its cycling behavior and try to repair the damage, but if the damage is too severe and cannot be repaired, the cells will enter senescence or induce apoptosis (Takekawa et al., 2011). The following paragraphs will discuss the signaling pathways leading to different cell states as the results of cellular damage. The effects of disturbance on those pathways will also be mentioned.

Cell cycle arrest

According to Pietenpol and Stewart (2002), 2 checkpoints (G1/S and G2 checkpoints) maintain the fidelity of DNA replication, repair and division. Upon infliction of DNA damage, p53 will be activated to induce synthesis of the cell cycle inhibitor p21 which inhibits production of cyclin-Cdk complexes, which are
required for the transition from G1 to S phase. The p21 protein is also synthesized during G2; therefore, p53 and p21 are important to sustain G2-M cell cycle arrest after DNA damage (Bunz et al., 1998; Waldman et al., 1995). When there is a mutation in the p53 gene, p21 protein will not be synthesized and the cell cycle will continue without repairing DNA damage. In this scenario, the cell will accumulate and propagate mutations, chromosome damage and aneuploidy; in brief such mutations herald genomic instability.

**Cellular Senescence**

Triggered by events such as telomere uncapping, DNA damage, oxidative stress and oncogene activation, cells can also enter a permanent form of cell cycle arrest termed senescence. In this state, cells will show a completely different, reduced metabolic activity and no longer divide. Once cells enter senescence, some morphological and nuclear changes will take place, such as enlarging and flattening of the cells, changes in chromatin structure and gene expression (Ben-Porath and Weinberg, 2005; Collado et al., 2007).

Entry of senescence mainly depends on p53 and Rb protein activation. Ben-Porath and Weinberg (2005) proposed two senescence pathways:

1) linear senescence pathway, in which p53 is activated, leading to downstream activation of Rb by p21 that inhibits CycE/Cdk2

2) parallel pathway involving p19ARF and p16INK4a, which are both inhibitors of CycD/Cdk4,6. This inhibition activates Rb.

Senescence blocks cells in a state of irreversible growth arrest, which is why it is also known as cellular aging. Inability of cells to enter senescence pathway may cause excessive or aberrant cellular proliferation and development of cancer (Ben-Porath and Weinberg, 2005; Collado et al., 2007).

**Apoptosis**

When all repair mechanisms fail or damage is too severe, a cell will initiate a suicidal program termed apoptosis. Apoptotic cells show distinct morphological alterations such as cellular rounding and shrinkage, autophagy and blebbing, chromatin condensation and nuclear fragmentation. Apoptosis is a form of controlled self-destruction, in which a set of cutting enzymes, also known as caspases are sequentially activated.
There are 3 main routes for caspase activation (Taylor et al., 2008). The extrinsic pathway is initiated by FasL/TNFα (Tumor Necrosis Factor α) binding to death receptors at the cell membrane. The binding of death receptor and its ligand provokes the recruitment of adaptor proteins, such as Fas-associated death domain protein (FADD) which then leads to caspase-8 recruitment and aggregation, promoting its activation. Activated caspase-8 will activate downstream executioner caspases, leading to induction of substrate proteolysis and finally cell death.

The intrinsic pathway is triggered by various stimuli inducing cell stress or DNA damage that activates BH-3 only proteins. BH-3 only proteins have a tendency to cluster but this is inhibited by BCL-2 family proteins. However, upon external stress, this inhibitory effect will be gone leading to BH-3 aggregation and subsequent pore formation in the mitochondrial membrane. As a consequence cytochrome c leaks to cytoplasm where it serves as seed for the apoptosome, a complex of pro-caspase 9 and APAF1. The activated apoptosome initiates the caspase activation cascade.

The third route or Granzyme B pathway is based on the activity of granzyme B and perforin, an enzyme injected by cytotoxic T lymphocytes and natural killer cells (NKC), with similar activity as caspases.

Cell death is an intrinsic protective mechanism, as it allows a biological system to remove cells with severe unrepaired damage (Takekawa et al., 2011; Taylor et al., 2008). Therefore, abnormalities in the apoptosis signaling pathway, such as mutation of genes that play role in apoptosis or disruption in the cascade, can contribute to a variety of diseases, including cancer, autoimmunity and degenerative disorders (Strasser et al., 2000).

1.6 Cellular effects of meat components

The exact working mechanism of red meat-induced carcinogenesis has not yet been fully resolved, but several components of red and processed meat have been shown to induce oxidative or DNA damage in colonocytes, such as fat, heme iron, hemoglobin, N-nitroso compounds and protein fermentation products such as ammonia, phenol and p-cresol (Angeli et al., 2011; Cross and Sinha, 2004; Howe et al., 1997; Visek, 1978; Wynder et al., 1969). Those components and metabolites are considered to inflict damage to colonocytes and may enhance carcinogenesis. Even though the exact carcinogenic mechanisms are not yet known, some plausible hypotheses have been
conjectured to explain the association of red meat and colon cancer. This is discussed in the following paragraphs and a scheme on the hypothetical mechanism of red meat causing DNA damage is shown in figure 1.

The association of fat and colon cancer incidence is considered to arise from the formation of lipid peroxide and aldehyde products (e.g. malondialdehyde (MDA) and 4-hydroxynonenal (HNE)) during lipid oxidation of polyunsaturated fatty acids (PUFAs) in meat. MDA is formed by oxidation of PUFAs with 2 or more double bonds and these compounds are mutagenic with ability to form DNA adducts with deoxyguanosine, deoxyadenosine and deoxycytidine (Bastide et al., 2011).

![Scheme on the hypothetical mechanism of red meat causing cytotoxic and genotoxic effect.](image)

**Figure 1.** Scheme on the hypothetical mechanism of red meat causing cytotoxic and genotoxic effect. (1) Ishikawa et al. (2010); (2) Glei et al. (2006); (3) Cross and Sinha (2004).

NOCs: N-Nitroso Compounds  
MDA: Malondialdehyde  
SSBs/DSBs: Single Strand Breaks or Double Strand Breaks DNA damage

Myoglobin, a component in red meat that gives rise its red color, contains a prosthetic group called heme (Bastide et al., 2011), which could be an important
carcinogenic factor because its absorption in the small intestine is low. Most ingested heme is therefore delivered to the colon and rectum. Enzymatic degradation of heme by heme oxygenase (HO) and microsomal cytochrome P450 reductase in colon and rectum could increase the production of reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$) as an unwanted byproduct (Ishikawa et al., 2010). Heme iron can further cause cellular toxicity and promutagenic lesions (Ishikawa et al., 2010) by a combination of ferrous iron (Fe$^{2+}$) with H$_2$O$_2$ as result of enzymatic degradation of heme iron that will produce hydroxyl radical (•OH) under Haber-Weiss Fenton-type (Equations (1), (2) and (3)) reaction as follow (Kehrer, 2000):

$$\text{Fe}^{3+} + \cdot \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2$$  
(1)

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$$
(Fenton reaction)  
(2)

$$\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \cdot\text{OH} + \text{OH}^-$$  
(3)

Hydroxyl radical (•OH) is a very reactive radical that can attack and damage various biological components in cells such as DNA, protein and lipid. Moreover, heme ingested with fat from meat or other food substances can generate lipid peroxyl radicals, which in turn induce DSBs or SSBs by cleaving DNA or modifying DNA bases, which could increase carcinogenesis (Ishikawa et al., 2010).

Protein in meat is not always fully digested and absorbed when it enters the large intestine. Unabsorbed protein is then fermented by microbes in descending colon, resulting in various metabolites such as Short Chain Fatty Acids (SCFA: butyrate, not as much as carbohydrate fermentation), phenols, gasses (H$_2$, CO$_2$ and methane) and ammonia (Cummings and Macfarlane, 1997b). Visek (1978) proved that a low concentration of ammonia (5-10 mM) can alter morphology, DNA synthesis, intermediary metabolism of intestinal cells and reduce life span, which may make ammonia an important compound in meat-induced carcinogenesis. However, Corpet et al. (1995) reported that protein fermentation products such as ammonia, phenols and p-cresol do not play an important role in colon carcinogenesis for its minor promotion of Aberrant Crypt Foci (ACF) in rats.

Nitrite is often used as an anti-bacterial agent against Clostridium botulinum or to maintain the reddish color of meat. But several studies reported that dietary nitrites are considered as human carcinogens, because they can be converted exogenously with
acidic pH during meat storage or endogenously to N-nitroso compounds (NOCs), alkylating agents that can alter DNA bases by inducing transitions of Guanine to Adenine (Cross and Sinha, 2004). NOCs can be produced during acidic digestion in the stomach or fermentation by bacteria in the large bowel. The probability of NOC formation is affected by the pH in the digestion system, microflora and other dietary components such as heme. Several studies confirmed that endogenous N-nitrosation, which results in the production of NOCs in the large bowel rises from heme iron ingestion (Cross et al., 2003; Kuhnle and Bingham, 2007; Lunn et al., 2006). These studies support that NOC formation is not merely due to nitrite addition to meat, but also due to the combination with heme, supporting epidemiological studies about the higher association of red meat with CRC than white meat (chicken).

Several toxicity studies of meat-related compounds such as heme iron, lipid hydroperoxyde have been conducted on human cell lines. An experimental study by Glei et al. (2006) showed that hemoglobin (Hb) and hemin can induce DNA damage, as measured by COMET assay, in both primary cell lines and human colon tumor cell lines. Another study by Ishikawa et al. (2010), demonstrated that heme has genotoxic and hyperproliferative effects on Caco-2 cells. Angeli et al. (2011) in turn showed that the preincubation of SW480 colon cancer cell lines with Hb and linoeic acid hydroperoxide (LAOOH) increased cell death, oxidation, MDA formation and DNA fragmentation. This study also reported that >100 μM of Hb + LAOOH exerted toxic effects on cells, lipid peroxidation and DNA or/and act as enhancer of NOC-induced genotoxicity.

In parallel, an in vivo study demonstrated significantly higher colonic DNA damage in rats fed with dietary red meat than when fed with white meat (Toden et al., 2007). These data further corroborate the hypothesis of heme as enhancer of fatty acid hydroperoxide-induced genotoxicity.

1.7 Scope

An increasing body of evidence supports a correlation between red meat consumption and colon cancer. While epidemiological studies show a strong, significant relation, they fail to provide the exact working mechanism. Toxicity studies of individual components on biological models may provide better insight, but represent an unnatural situation as there may be antagonistic or synergistic effects
between food components and the digestion process may have an additional modulatory role. While *in vivo* studies represent the most faithful situation, they often suffer from poor statistics and also are associated with ethical considerations. Therefore, we aim to assess the adverse effects of *in vitro* meat digestion products by integrating the *in vitro* digestion process of meat with a multiparametric cyto- and genotoxicity analyses in HT29 colon cancer cell lines. Furthermore, we investigated the effects of *in vitro* digestion products from different types of meat differing in the content of heme (beef, pork and chicken) and whether or not cured with nitrite (pork), at cellular level (cell morphology and viability) to determine cytotoxicity, nuclear level (nuclear shape and chromatin condensation), lipid peroxidation, as well as DNA damage by measuring double strand breaks (DSBs) in cell lines.
2. MATERIALS AND METHODS

2.1 In vitro digestion and fermentation of meat

In the in vitro digestion model, physiological conditions of human digestion were simulated in a simplified manner such as chemical composition of digestive fluids, pH and residence time. The model expands on the method developed by Oomen et al. (2003).

Two types of experiments were carried out: the first one simulated in vitro digestion up to the duodenum stage, and the second included the fermentation process with fecal microbial samples from a human volunteer to fully simulate the digestion process up to the colon stage. Before starting the in vitro digestion process, preparation of meat samples and human bacterial inoculum were carried out as described below.

2.1.1 Preparation of meat samples

Fresh, lean meat samples were collected from the m. Pectoralis profundus from chicken, m. Longissimus dorsi from pig and m. Biceps femoris from beef. Subcutaneous pork fat from one batch was added to the sampled meat to obtain a total fat content of 5% in order to exclude a possible confounding effect arising from different fat profiles. Nitrite-curing was obtained by adding 20 g 0.6% nitrite salt/kg meat, corresponding to an added concentration of 120 mg nitrite/kg meat. All meat samples were heated in a warm water bath for 15 minutes after the core temperature had reached 65°C. After manufacturing, all meat samples were minced, vacuum packed and stored at -20°C until the start of the incubation.

2.1.2 Preparation of human bacterial inoculum

Fresh fecal material was collected from a volunteer without known gastrointestinal diseases and without intake of antibiotics for at least 3 months. The volunteer was male, meat-eater on a “western diet”, and aged 49 years. Fresh fecal material was diluted in pre-heated PBS solution (1/4; w/v), to which sodium thioglycolate (1g/l) was added as a reducing agent. The fecal slurry was filtered by a 1 mm metal sieve to remove the particulate matter. Afterwards, the microbiota were stored at -80°C as glycerol stock (20%) in different aliquots. Before use in the colonic fermentation phase, bacterial inoculum was cultured during 24 hours at 37°C to obtain the necessary
macrobiotic culture. For this purpose, fecal inoculum was diluted with BHI broth (37 g/l Brain Heart Infusion and 0.5 g/l L-cysteine-HCl) at 1/9 ratio. Consequently, anaerobic conditions in the flask were reached by flushing the head space with N₂ during 1 hour.

2.1.3 The in vitro digestion of meat

For the in vitro digestion, the protocol described by Versantvoort et al. (2005) was adapted by adding oxidants and antioxidants that are normally present in digestive juices (Table 1). Hence, saliva also contained peroxidase Guven et al. (1996) and NaNO₂ Takahama et al. (2003) while ascorbic acid (Dabrowska-Ufniarz et al., 2002), H₂O₂ and FeSO₄ (Nalini et al., 1992) were added to the gastric juice. During each incubation, 4 replicates of each meat sample (4.5g) were incubated 5 minutes with 6 ml saliva, 2 hours with 12 ml gastric juice, 2 hours with 2 ml bicarbonate buffer (1M), 12 ml duodenal juice and 6 ml bile. After duodenum digestion, 2 replicates of each meat sample were diluted with 44 ml H₂O to obtain the same solid/liquid ratio as in colon (see further in table 3). Duodenum samples were stored at -20°C in aliquots after homogenizing with an ultraturrax. The 2 remaining replicates of each meat sample entered the colon digestion. SHIME medium (22 ml) (Molly et al., 1994) and human fecal microbiota (22 ml) were added to the digests. Closed vessels were flushed with N₂ for 30 minutes to obtain an anaerobic environment. Subsequently, vessels were incubated for 72 hours while stirring at 37°C. All digestive mixtures were then homogenized with ultraturrax, and while stirring, 1.3 mL aliquots of the samples were taken in 1.5 mL tube and stored in -20°C.
### Table 3. Composition of digestive juices used for in vitro incubation of meat samples

<table>
<thead>
<tr>
<th></th>
<th>Mouth</th>
<th>Stomach</th>
<th>Duodenum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva (1 L)</strong></td>
<td>Product</td>
<td>Product</td>
<td>Product</td>
<td>Product</td>
</tr>
<tr>
<td>KCl</td>
<td>0,90 g</td>
<td>2,75 g NaCl</td>
<td>7,01 g NaCl</td>
<td>5,26 g NaCl</td>
</tr>
<tr>
<td>KSCN</td>
<td>0,20 g</td>
<td>0,27 g NaH₂PO₄</td>
<td>3,39 g NaHCO₃</td>
<td>5,79 g NaHCO₃</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0,90 g</td>
<td>0,82 g KCl</td>
<td>0,08 g KH₂PO₄</td>
<td>0,38 g KCl</td>
</tr>
<tr>
<td>NaSO₄</td>
<td>0,57 g</td>
<td>0,40 g CaCl₂·2H₂O</td>
<td>0,56 g KCl</td>
<td>0,15 ml HCl 37%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0,30 g</td>
<td>0,31 g NH₄Cl</td>
<td>0,05 g MgCl₂</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1,69 g</td>
<td>5,60 ml HCl 37%</td>
<td>0,51 g KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>SHIME medium² (1 L)</td>
<td>Product</td>
<td>Product</td>
<td>Product</td>
<td>Product</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>6,8 g</td>
<td>2,75 g NaCl</td>
<td>3,39 g NaHCO₃</td>
<td>5,79 g NaHCO₃</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>8,8 g</td>
<td>0,27 g NaH₂PO₄</td>
<td>0,08 g KH₂PO₄</td>
<td>0,38 g KCl</td>
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<tr>
<td>NaHPO₄·12H₂O</td>
<td>0,23 g</td>
<td>0,31 g NH₄Cl</td>
<td>0,05 g MgCl₂</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>0,20 g</td>
<td>0,31 g NH₄Cl</td>
<td>0,05 g MgCl₂</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1,69 g</td>
<td>5,60 ml HCl 37%</td>
<td>0,51 g KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>Bacterial inoculum* (1 L)</td>
<td>Product</td>
<td>Product</td>
<td>Product</td>
<td>Product</td>
</tr>
<tr>
<td>NaCl</td>
<td>0,51 g</td>
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<tr>
<td>KH₂PO₄</td>
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<td>0,31 g NH₄Cl</td>
<td>0,05 g MgCl₂</td>
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</table>

**DIGESTION¹**: based on Versantvoort et al. (2005) unless otherwise indicated; ¹Molly et al. (1994); Peroxidase²: Guven et al. (1996); NaNO₂⁴: Takahama et al. (2003); Ascorbic acid⁵: Dabrowska-Ufnarz et al., 2002); H₂O₂⁶ (Nalini et al., 1992); FeSO₄⁷ (Nalini et al., 1992); * Bacterial inoculum was cultured in anaerobic conditions for 24 hours at 38°C and used immediately in fermentation procedure.

---

**Add**

<table>
<thead>
<tr>
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<th>Duodenum</th>
<th>Colon</th>
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<td>0,200 g CaCl₂·2H₂O</td>
<td>0,222 g CaCl₂·2H₂O</td>
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<tr>
<td>FeSO₄·7H₂O⁷</td>
<td>11,2 mg</td>
<td>11,2 mg FeSO₄·7H₂O⁷</td>
<td></td>
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</tbody>
</table>

**DIGESTION¹**: based on Versantvoort et al. (2005) unless otherwise indicated; ¹Molly et al. (1994); Peroxidase²: Guven et al. (1996); NaNO₂⁴: Takahama et al. (2003); Ascorbic acid⁵: Dabrowska-Ufnarz et al., 2002); H₂O₂⁶ (Nalini et al., 1992); FeSO₄⁷ (Nalini et al., 1992); * Bacterial inoculum was cultured in anaerobic conditions for 24 hours at 38°C and used immediately in fermentation procedure.
2.2 Digests preparation

For cell treatment, meat and blanco digests were prepared according to the method proposed by Lux et al. (2012). Beef, chicken and pork digests with 5% fat, 0 ppm nitrite and 120 ppm nitrite-cured pork digests were used in this experiment. The last suspension from in vitro digestion was centrifuged for 30 minutes at 4200 xg, followed by centrifugation of the supernatant fraction for 15 minutes at 4200 xg. The supernatant fraction was then centrifuged for 15 minutes at 16000 xg. All centrifugation steps were performed at 4°C. Subsequently, the digests were sterilised by filtration (pore size 0.22 mm) to obtain the final digests to be used in cell-based assays.

2.3 Cell culture

HT29 colon cancer cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Life Technologies, Merelbeke, Belgium) supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The cells were cultured in 25 cm² culture flasks. For experimental purposes, cells were cultured in 12-well plates for genotoxicity assay, 96-well plates (Greiner Bio One, Wemmel, Belgium) for cytotoxicity assays or glass-bottom dishes (MatTek, Massachusetts, USA) for lipid peroxidation assay at density of 50,000 cells/well, 5000 cells/well or 50,000 cells/well respectively. Cells were allowed to attach for 24 h before treatment and then treated with 2.5%, 5% or 10% meat digests (pork, beef or chicken), blanco digests (digestive juices without meat), or medium only as negative control.

2.4 Morphological screening: transmission microscopy

HT29 cell lines were seeded in 12-well plates at a density of 50,000 cells/well, and grown for 48 hours. Subsequently, cells were incubated for 24 hours with 2.5%, 5% or 10% of the meat digests, with 3 biological replications per experiment, using digests (without meat) as negative control and non-treated samples (only growth medium) as reference. After 24 h incubation, cell morphology was observed using a widefield microscope type Nikon-TI (Nikon Instruments, Paris, France) using a 10x (NA=0.5) objective. Per condition, a minimum of 15 images (5 images per well) were recorded.
2.5 Cytotoxicity assay

For measuring cell viability, we made use of PrestoBlue® reagent (Life Technologies, Merelbeke, Belgium). The PrestoBlue® assay is based on the reduction of resazurin by living cells. The original compound is blue and nonfluorescent, but the reducing environment of living cells converts the dye into a red and highly fluorescent form which can be detected for instance using fluorescence with 560 nm excitation and 600 nm emission.

In brief, the assay was performed as follows: HT29 were seeded in 96-well plates and allowed to grow (with or without meat digests) for 24 hours. Hereafter, 11 μl of PrestoBlue® was added to each well and then incubated for 25 minutes at 37°C. Subsequently, fluorescence intensity was measured using a plate reader (TECAN, Mechelen, Belgium) and software i-control, with 560 nm excitation and 600 nm emission wavelength. An optimization experiment was performed to know the optimal cell seeding density (cfr. Results section). For the actual viability experiments, cells were incubated for 24 hours with 2.5%, 5% or 10% of the meat digests with 4 biological replications per experiment, and three technical replications using blanco digests (without meat) as control and non-treated samples (only growth medium) as negative control. Because there is a possibility that the meat digests contain autofluorescent or highly absorbing components, a number of internal controls were built in to the assay (cfr. Results section). The experiments were set up as shown in figure 2: four replicates of cells incubated with medium only, blanco digests or meat digests for 24 h or 0 h (= the treatment group), 2 replicates of wells without cells incubated with medium only, blanco digests or meat digests for 24 h or 0 h (= control). Subsequently, the concentration of live cells were analysed using Presto Blue Assay, by adding 11 μl PrestoBlue® Cell Viability reagent to each well and the fluorescence intensity were measured using TECAN and software i-control, with 560 nm and 600 nm as excitation and emission wavelength respectively. Fluorescence intensity data, then normalized with equation:

\[ I_{\text{norm}} = \frac{I_{24\text{h}}^{\text{treat}} - I_{10\text{h}}^{\text{contr}}} {I_{0\text{h}}^{\text{treat}} - I_{10\text{h}}^{\text{contr}}} \]

With \( I_{\text{norm}} \), the normalized intensity and \( I \) the intensity measured in wells either with (treat) or without (control) cells after 0h or 24h of incubation.
Figure 2. Setup of cell viability assay in 96-well plate

Each plate can hold up to 2 samples either meat digests or control digests with various concentration (2.5%, 5% and 10%). Control 0 representing untreated sample with growth medium only added in wells.

A: cells incubated with medium only, blanco digests or meat digests for 24 h. Intensity measured = $I_{24h}^{treat}$

B: cells incubated with medium only, blanco digests or meat digests for 0h. Intensity measured = $I_{0h}^{treat}$

C: wells without cells incubated with digests for 24 h. Intensity measured = $I_{24h}^{contr}$

D: wells without cells incubated with digests for 0h. Intensity measured = $I_{0h}^{contr}$

2.6 Lipid peroxidation assay

In the experiment of lipid peroxidation, Image-iT® lipid peroxidation kit from Life technologies (Merelbeke, Belgium) was used. Basically, the kit is composed of BODIPY 581/591 C11 reagent which is a sensitive fluorescent reporter for lipid peroxidation. Upon oxidation in live cells, the fluorescence of the phenylbutadiene segment of the fluorophore shifts from red (590 nm) to green (510 nm). For the lipid peroxidation assay, cells were seeded in glass-bottom dishes at density of 50,000 cells/well and incubated for 48 hours before treatment. Besides treatment with meat digests, 100 μM cumene hydroperoxide was also used as a positive control. Cumene was added to the cells and incubated for 2 hours at 37°C. Subsequently, Image-iT® lipid peroxidation reagent was added to the cells at a concentration of 10 μM and incubated for 30 minutes at 37°C, followed by 2 washes with imaging buffer (1x HT Buffer; NaCl, HEPES, KCl, MgCl$_2$, H$_2$O, CaCl$_2$; pH 7.4). Cell imaging was done with HT Buffer (1x
HT Buffer; NaCl, HEPES, KCl, MgCl$_2$, H$_2$O, CaCl$_2$; pH 7.4) using a widefield microscope type Nikon-TI (Nikon Instruments, Paris, France) using a 40x (NA=1.3) plan apo oil objective. Per condition, a minimum of 30 images was recorded, capturing two channels per field, one with a TRITC filter (540/25 nm excitation, 565 nm dicroic, 605/55 nm emission), and the other with a GFP-BP filter cube (472/30 nm excitation, 495 nm dicroic, 520/35 nm emission). A higher ratio of green/red signal intensity indicates higher lipid oxidation in cells.

2.7 Genotoxicity assay: Nuclear morphology and γ-H2AX immunofluorescence

HT29 cell lines were seeded in two 12-well plates at a density of 50,000 cells/well, incubated for 72 hours to let them grow. Subsequently, cells were incubated with 2.5%, 5% or 10% of the meat digests (beef, chicken or pork, from colon or duodenum in vitro digestion) or control digests (digestive juice without meat) or control (medium only), in triplicate. At 24 hour time point, cells were fixed with 2% paraformaldehyde for 15 minutes and washed 3 times for 5 minutes with PBS to remove residues of paraformaldehyde. Then cells were permeabilized for 5 minutes with 0.05% Triton-X, followed by another washing step with PBS. Next, cells were blocked 20 minutes with 50% FCS and incubated with 60 μl of primary antibody (Rabbit pAb for γ-H2AX, phospho S139, ab 2893-50, abcam, Cambridge, UK, 1:1500) at room temperature for 1 hour. After 3 PBS washes, cells were blocked again and incubated with secondary antibody (Goat anti-Rabbit Alexa-555, Life Technologies, Merelbeke, Belgium; 1:600) for 30 minutes at room temperature. After another 3 washing steps, coverslips were dehydrated in an ethanol series (70%, 90% and 100%, 3 minutes each) and finally mounted onto glass slides with 8 μL of Vectashield and DAPI (1 mg/mL) and sealed with nailpolish. Images were acquired using a Nikon-TE 2000 microscope equipped with a 40x (NA=1.3) plan apo oil objective and a -80°C cooled EM-CCD camera. Per slide 3 mosaic acquisitions of 5x5 fields were acquired at 7 different axial positions, separated by 1 μm, capturing two channels per region: one with a DAPI filter (377/50 nm excitation, 409 nm dichroic, 447/61 nm emission), and the other with a TRITC filter cube (472/30 nm excitation, 409 nm dichroic, 520/35 nm emission). Mosaic acquisitions were automatically analyzed for various metrics such as γ-H2AX spot number and γ-H2AX intensity per nucleus, using INSCYDE software (De Vos et al., 2010) for ImageJ freeware.
Nuclear morphology was analyzed using similar mosaic acquisitions for $\gamma$-H2AX with different metrics such as nuclear DAPI intensity, nuclear area and nuclear circularity. From the image acquisition results (3 mosaic acquisitions from each sample), the amount of mitotic and aberrant nuclei (nuclei that have high DAPI intensity, are small in size, and irregular in shape) were also manually counted.

The average data for $\gamma$-H2AX spot number, $\gamma$-H2AX intensity, DAPI intensity, nuclear area and nuclear circularity in cells treated with meat digests from 3 mosaic acquisitions were all normalized to respective concentration of meat digests.

2.8 Statistical analysis

All data were normally distributed, and assumption on variance equality was fulfilled according do Kolmogorov-Smirnov test and Modified Levene Test, respectively. A statistical comparison between meat digests and blanco digests for cell viability, lipid peroxidation (ratio green/red), and other parameter for nuclear morphology and DNA damage (nuclear area, circularity, $\gamma$-H2AX spot number and DAPI, $\gamma$-H2AX intensity) with respective concentration (2.5%, 5% and 10%) were done with one-way ANOVA, with Tukey post-hoc tests at 5% level of significance. For comparison of mitotic index and aberrant nuclei, a two-sample T-test was conducted, comparing blanco digests and meat digests with respective concentration. All the statistical analysis were carried out by S+ 8.2 (TIBCO Software Inc., USA).
3. RESULTS

To assess cytotoxicity and genotoxicity of meat digests on HT29 colon cancer cell lines, a top-down approach was used in which we first focused on the coarse effects such as impact on cell viability and cell morphology and subsequently investigated more subtle effects on nuclear morphology, ROS levels, lipid peroxidation, and finally DNA damage (double strand breaks). Besides comparing effects of different meat digests, pork, beef and chicken, we also compared meat digests from different digestive compartments (duodenum and colon).

3.1 Cell Morphology

Morphologically, normal subconfluent HT29 cultures appear under the light microscope as patches of 10-100 cells with smooth transitions between cell boundaries and little to no individual cells. Initially we used PBS as a negative control. In PBS-treated wells, no change in cell morphology was observed (data not shown). However, PBS is not a proper negative control, since it does not reflect the composition of the actual digests. Therefore, it was replaced by a more appropriate control, namely a solution that has the composition of the final physiological destination (duodenum or colon), but without addition of meat. We refer to this as a blanco digest. Using this control, we performed an experiment with different concentrations (2.5%, 5% and 10%) of meat digests (pork, beef and chicken). Cells incubated with the blanco digests showed modest morphological changes, compared to non-treated cells, i.e. cells that were kept in growth medium throughout the experiment. These morphological changes (indicated by orange arrows in figure 3), included a darkening of cells at the edges of the patches. This darkening became more pronounced in cells treated with meat digests in a concentration-dependent manner, and was most dominant in cell cultures treated with pork digests. A closer look revealed that the darkening occurred in a cytoplasmic region that surrounds the nucleus, possibly the endoplasmic reticulum (ER). The exact cause of this darkening and its location however, remain to be determined.

At the highest concentration (10%), all meat digests also induced a reduction in cell density and disaggregation of the cell patches, yielding more rounded, individualized cells (figure 3).
Similar morphological changes were also observed in cells treated with control and meat digests from duodenum samples (figure 4). Here, changes were more dramatic with the typical disaggregation and darkening already being overt at concentrations of 5%.

**Figure 3.** Representative images of HT29 cells after 24 hour incubation with various concentrations (2.5%, 5% or 10%) of control digests or meat digests (beef, chicken or pork) from colon samples. Control 0 means no digesta added (medium only). Orange arrow shows darkening at perinuclear region of cells.

**Figure 4.** Representative images of HT29 cells after 24 hour incubation with 5% and 10% concentration of control digests or meat digests (beef, chicken or pork) from duodenum samples. Pork (120 ppm nitrate) is 120 ppm nitrite-cured pork digests. Orange arrow shows darkening at perinuclear region of cells.

Note: During the optimization process, we wondered whether it was necessary to include all centrifugation steps, considering potential loss of specific substances (such as lipid peroxides, NOCs, heme iron) during centrifugation. Therefore, a comparison was made between centrifuged and non-centrifuged digests-treated cells.
Figure 5. Representative images of HT29 cells after 24 hour incubation with various concentrations (2.5%, 5% and 10%) of control and meat digests from colon samples with different digests preparation.

Control digests mean digestive juices without meat (blanco digests), Control 0 means no digesta added (medium only).

(S) : cells treated with sterilized only digests
(S+C) : cells treated with centrifuged and sterilized digests.
Orange arrow shows darkening at perinuclear region of cells.

As demonstrated on figure 5 and 6, digests without centrifugation elicit more severe morphological abnormalities than the centrifuged ones, indicating that there must be substances that aggravate the effects. However, since the differences also occur in blanco digests, this points to an aspecific effect, plausibly due to the presence of debris and bacterial residues in the crude digests. For this reason, it was decided to continue with the centrifuged digests.
Control digests mean digestive juices without meat, control 0 means no digesta added (medium only).
(S) : cells treated with sterilized only digests
(S+C) : cells treated with centrifuged and sterilized digests.

3.2 Cell Viability

To measure the impact on cell viability, we made use of the PrestoBlue® reagent, according to the protocol described in the Materials and Methods section. Before running the actual experiment, the assay was optimized by tuning the cell density and by measuring the potential interference of digests with specific PrestoBlue® fluorescence.

For gauging the optimal density, cells were seeded at 6 different densities (2500, 5000, 7500, 10000, 15000, 20000 cells/well) and incubated for 24 hours before measuring viability by means of the PrestoBlue® assay. As depicted in figure 7, a linear increase in fluorescence signal was observed ($R^2=0.975$) for the first 4 concentrations, but there is a deviation from linearity at the cell densities beyond, suggestive of a saturation effect. Based on this result, an intermediate concentration of 5000 cells/well was selected for the actual viability/ cytotoxicity experiments.
Figure 7. Graph of fluorescence intensity at various cell density: 2500, 5000, 7500, 10000, 15000, 20000 cells/wells in 96-well plate measured by PrestoBlue®.

The “Black line” shows a linear fit for the first 4 data points across 4 dots (2500 to 10000). The “Red line” represents a polynomial fit for all data points.

Since the digests contain biological components, it could very well be that the treatment interferes with PrestoBlue® fluorescence readings due to absorption, scattering or autofluorescence effects. Therefore, we performed an experiment in which we measured signals from different controls, including empty wells (without cells) but with digests as well as wells with cells in which digests were added right before the actual measurements. We found increased fluorescence readings in wells with cells treated with pork digests in concentration-dependent manner. Autofluorescence effects were also detected in wells without cells treated with blanco and meat digests (Figure 8). Therefore, we decided it was necessary to take into account all these effects, by including all the aforementioned controls in the actual experiment. This means that the readout from the PrestoBlue® experiments was expressed as follows:

$$I_{norm} = \frac{I_{treat}^{24h} - I_{contr}^{24h}}{I_{treat}^{0h} - I_{contr}^{0h}}$$

With $I_{norm}$ the normalized intensity and I the intensity measured in wells either with (treat) or without (control) cells after 0h or 24h of incubation. With this optimized procedure, all meat digests from colon or duodenum were screened at different concentrations for their cytotoxic effect (Figure 9, 10).
Allover we noticed a large variability between replicates, which may be due to variations in cell seeding density (cell counting was done manually so gives a rough estimation). As demonstrated in figure 9, cell growth seems to be promoted in cell cultures treated with blanco in a concentration-dependent manner, possibly due to the presence of growth factors in the raw digests (e.g. glucose, glucosamine, BSA and potato starch... cfr. Table 3). Cells treated with meat digests, experienced the opposite effect, i.e. higher concentrations impeded cell growth. The effect was statistically significant for concentrations of 10% of chicken and beef digests, while for pork digests the difference was already significant from 5% on. There was no significant difference between pork without (0 ppm) or with (120 ppm) nitrite. A similar trend was observed for cells treated with duodenum samples, as shown in figure 10, but the significance was only observed in 10% concentration of all meat digests.
Figure 9. Cell viability of colon blanco digests (digests without meat), or meat digests treated-cells, including (pork, pork with 120 ppm nitrite, chicken and beef), presented as relative change of fluorescence intensity to negative control (C(-)).

C(-) means negative control with growth medium only. Pork (120 ppm nitrate) is 120 ppm nitrite-cured pork digests.
* indicates statistically difference (p<0.05) from the corresponding concentration of control digests.

Figure 10. Cell viability of duodenum blanco digests (digests without meat), or meat digests treated-cells, including (pork, pork with 120 ppm nitrite, chicken and beef), presented as relative change of fluorescence intensity to negative control (C(-)).

C(-) means negative control with medium only. Pork (120 ppm nitrate) is 120 ppm nitrite-cured pork digests.
* indicates statistically difference (p<0.05) from the corresponding concentration of control digests.
3.3 Nuclear Morphology

Next, we zoomed in on the cell nucleus, focusing on nuclear shape and chromatin condensation as proxies for cellular health status. These features can be revealed by means of a DAPI staining and automatic image analysis (see Materials and Methods section), allowing discrimination between healthy cells in interphase from mitotic and (pre-) apoptotic cells. First we performed an experiment with all colon samples, after which we repeated the experiment for the highest concentrations (10%), but including duodenum samples and 120 ppm nitrite-cured pork meat.

![Figure 11. Representative images of HT29 nuclear stained with DAPI (1 µg/mL) after 24 hour incubation with various concentration of different digests (blanco digests, beef, chicken and pork) from colon samples. Control 0 means no digests added (medium only, for negative control). Green arrow indicates mitotic cells while orange arrow indicates aberrant cells.](image)

Figure 11 shows representative images of HT29 cell nuclei after treatment with different samples. Clearly, the number of aberrant nuclei, i.e. nuclei with very high intensity, was higher in cell cultures treated with >5% meat digests than respective blanco digests, but not statistically significant, due to high variation of the data (high standard deviation). At lower concentrations, mitotic cells were observed (green arrows) at all different stages, from prophase, metaphase, anaphase to cytokinesis. While metaphase and prophase stages were still found in cells treated with 5% beef and pork digests, in 10% meat digests, mitotic cells could no longer be detected (table 4).
Table 4. Results for mitotic index and percentage of aberrant cells

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<th>Sample</th>
<th>Mitotic Index</th>
<th>Percentage of Aberrant cells</th>
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<tr>
<td>Control 0%</td>
<td>0.009 ± 0.003</td>
<td>4.01 ± 3.99</td>
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<tr>
<td>Blanco 2.5%</td>
<td>0.01 ± 0.1</td>
<td>1.22 ± 0.37</td>
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<tr>
<td>Blanco 5%</td>
<td>0.006 ± 0.004</td>
<td>1.38 ± 1.88</td>
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<tr>
<td>Blanco 10%</td>
<td>0.005 ± 0.001</td>
<td>3.98 ± 1.67</td>
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<tr>
<td>Chicken 2.5%</td>
<td>0.008 ± 0.006</td>
<td>0.71 ± 0.48</td>
</tr>
<tr>
<td>Chicken 5%</td>
<td>0.005 ± 0.005</td>
<td>2.92 ± 2.12</td>
</tr>
<tr>
<td>Chicken 10%</td>
<td>0.005 ± 0.006</td>
<td>11.51 ± 9.03</td>
</tr>
<tr>
<td>Beef 2.5%</td>
<td>0.007 ± 0.002</td>
<td>2.40 ± 1.98</td>
</tr>
<tr>
<td>Beef 5%</td>
<td>0.009 ± 0.006</td>
<td>2.40 ± 2.54</td>
</tr>
<tr>
<td>Beef 10%</td>
<td>0.007 ± 0.002</td>
<td>16.02 ± 9.27</td>
</tr>
<tr>
<td>Pork 2.5%</td>
<td>0.01 ± 0.003</td>
<td>1.03 ± 1.09</td>
</tr>
<tr>
<td>Pork 5%</td>
<td>0.009 ± 0.006</td>
<td>0.53 ± 0.29</td>
</tr>
<tr>
<td>Pork 10%</td>
<td>0.009 ± 0.006</td>
<td>9.19 ± 2.67</td>
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</table>

Quantification of projected nuclear area, nuclear circularity and nuclear DAPI intensity is shown in figure 12 (A and B), and 13 respectively. There is a statistically significant decrease in nuclear circularity of cells treated with 10% beef and chicken digests, suggesting that those concentrations induce an increase in nuclear irregularity. For nuclear area, a slight non-significant decrease was observed in cells treated with 10% meat digests, suggesting a modest decrease in nuclear volume (i.e. nuclei become smaller).

![Figure 12](image_url)

**Figure 12.** Relative change (RC) of the average values of nuclear area (A) and circularity (B) to control digests 2.5%, 5% and 10%, with meat digests (pork, beef and chicken) from colon sample in the same concentration with control digests. Significant differences (calculated by one-way ANOVA with Tukey post-hoc test) are highlighted by a *.

Quantification of DAPI signal intensity revealed that there was a gradual intensity increase in cells treated with increasing concentrations of chicken digests.
Except for the 10% chicken digest, intensity changes for other digests were modest and in fact lower than those of blanco digests (relative change <1).

**Figure 13.** Relative change of the average values of nuclear intensity (mean DAPI intensity) to corresponding concentration of control digests (2.5%, 5% and 10%), with meat digests (pork, beef and chicken) from colon samples in the same concentration with control digests.

In the second experiment we included duodenum samples as well as a sample from nitrite-cured pork. Figure 14 represents results for duodenum samples, while figure 15 demonstrates results for colon samples and shows both mitotic and aberrant nuclei in cells treated with 10% blanco and meat digests. Quantitative analysis of aberrant nuclei (figure 16), shows a significantly higher amount of aberrant nuclei in cell cultures treated with 10% pork digests, both without (0 ppm) or with (120 ppm) nitrite, chicken digests from colon samples, and 10% beef digests from duodenum samples. As shown in figure 16 A, B and C, aberrant nuclei, indicated with orange arrows, are nuclei with irregular shape and chromatin condensation.

In line with our earlier observations, all cell cultures treated with 10% of all meat digests (pork, nitrite-cured pork, beef and chicken) showed a similar amount of aberrant cells (figure 13, 14, 15). However, we could not show a significant difference in nuclear area and we could only show that 10% pork and chicken colon digests alter the nuclear shape (circularity) significantly (figure 18). Despite a strong impact on the average circularity from other digests, including 120 ppm nitrite-cured pork duodenum samples, we could not show a significant effect (due to the high variations).
Figure 14. Representative images of HT29 nuclear stained with DAPI (1 μg/mL) after 24 hour incubation with 10% concentration of different digests (control digests, beef, chicken and pork) from duodenum samples. Control 0 means no digesta added (medium only, for negative control). Green arrows indicate mitotic cells while orange arrows indicate aberrant cells.

Figure 15. Representative images of HT29 nuclear stained with DAPI (1 μg/mL) after 24 hour incubation with 10% concentration of different digests (control digests, beef, chicken and pork) from colon samples. Control 0 means no digesta added (medium only, for negative control). Pork 120 Nitrate is Nitrate-cured pork digests with 120 ppm of nitrate. Green arrows indicate mitotic cells while orange arrows indicate aberrant cells.
Figure 16. Percentage of aberrant nuclei in cells treated with 10% blanco digests and meat digests from colon (Col) and duodenum (Duo), with pork digests without (0 ppm) or with (120 ppm) nitrite. Figures at the right side shows aberrant nuclei indicated with red arrows (strong intensity) and yellow arrows (aberrant circularity) in cells treated with pork (120 ppm nitrite) digests (A), pork (0 ppm nitrite) digests (B) and chicken digests (D).

* indicates statistically significant difference (p<0.05) compared to 10% blanco digests according to one-way ANOVA with Tukey post-hoc test.

|                | Control | Blanco | Pork   | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beef | Col 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| Beef | Col | Beef | Col | Beef | Col | Beef | Col | Beach digests and pork digests without (0 ppm) or with (120 ppm) nitrite. Figures at the right side shows aberrant nuclei indicated with red arrows (strong intensity) and yellow arrows (aberrant circularity) in cells treated with pork (120 ppm nitrite) digests (A), pork (0 ppm nitrite) digests (B) and chicken digests (D).

* indicates statistically significant difference (p<0.05) compared to 10% blanco digests according to one-way ANOVA with Tukey post-hoc test.

Figure 17. Mitotic index in cells treated with 10% blanco digests and meat digests from colon (Col) and duodenum (Duo), with pork digests without (0 ppm) or with (120 ppm) nitrite. Figures at the right side shows different stages of mitosis (Prophase, Metaphase and Anaphase) in cells without treatment (growth medium only).

The mitotic index was calculated as the ratio between mitotic cells and the total number of cells in the acquired images. Mitotic cells at different mitotic stages were observed in almost all samples, but especially in cells without treatment (figure 17). Cells treated with 10% chicken colon samples showed little proliferating activity with
mitotic index of 0.004, whereas cells treated with 10% beef and pork colon samples showed absolutely no proliferating activity (figure 17). For cells treated with digests from duodenum samples, a higher mitotic index was observed compared to colon samples, even though it was not statistically significant due to high standard deviation for duodenum samples.

**Figure 18.** Relative change of the average values of nuclear area (A), nuclear circularity (B) and nuclear DAPI intensity (C) in cells treated with 10% meat digests compared to 10% blanco digests, from different samples, colon (C) and duodenum (D) with 120 ppm nitrate-cured pork digests. * indicates statistically significant compare to control 10% (p<0.05) according to ANOVA with Tukey post-hoc test.
3.4 Lipid Peroxidation

Presence of oxidation and lipid peroxidation products in meat digests might explain the reduction in cell viability, and changes in cell and nuclear morphology. We tested CM-DCFDA, a probe that measures general ROS levels, but unfortunately, this probe was not internalized properly (accumulated in vesicles) impeding proper quantitative analysis. We also measured lipid peroxidation using image-iT® reagent, a ratiometric probe that shifts its fluorescence emission from red (590 nm, reduced) to green (510 nm, oxidized). Two approaches were tried for this assay: one setup using 96-well plates and another with glass-bottom dishes. Whilst the first option would allow opportunities for upscaling, the other was expected to be more sensitive given its better compatibility with high numerical aperture (NA) lenses (glass vs. Plastic). Typical examples of the color-coded ratio images are given in figure 19.

We first performed the assay in a 96-well plate format, using a 40x (NA=1.3) plan apo oil objective. Quantification of the green-to-red signal ratio did not show any significant difference for any of the samples, except for cumene, the positive control which caused a shift in ratio from 1 to 1.37. There are limitations to working with 96-well plates for live-cell imaging because all conditions are on the same plate and cannot be measured simultaneously, they will have different incubation times with consequent different physiological conditions (lower temperature and low CO₂ environment). Therefore, to increase reproducibility of this assay we repeated the experiment with glass-bottom dishes; performing measurements one condition at a time, so as to have exactly the same incubation time for all samples. The result presented in figure 20 shows almost similar results as the experiment in 96-well plate (figure 19), with only cells treated with cumene showing a significant increase in green/red ratio, almost doubled from 0.25 (negative control) to 0.4 (cumene). This significant increase of lipid peroxidation in cumene is more pronounced compared to previous experiment with 96-well plate, even though it is still not sensitive enough for analysing lipid peroxidation in digests-treated cells.
Figure 19. Ratio graphs between green signal (590 nm) and red signal (510 nm) of cells treated by meat digests (chicken, beef and pork) from colon samples with various concentration (2.5%, 5% or 10%) in 96-well plates. Blanco digests used as internal control, while control 0% (cont) means negative control with medium only, and positive control with cumene hydroperoxide (cum). Figures at right side show results for image analysis in cells treated with cumene, with highly oxidized (High Ox=1.78) cells (indicated by red signal), and negative control with no oxidation (Low Ox=0.8) observed (purple to blue signal).

Figure 20. Ratio graphs between green signal (590 nm) and red signal (510 nm) of cells treated by beef digests from colon samples with various concentration (2.5%, 5% or 10%) in glass-bottom dishes. Control 0% means negative control with medium only, and positive control with cumene hydroperoxide.

3.5 DNA Damage

DNA damage might occur as a result of exposure to exogenous or endogenous compounds, in this case the digestion products of meat. Here, we focused on the most detrimental form of DNA damage, i.e. double strand breaks, measuring the amount of γ-
H2AX, a histone protein that becomes phosphorylated upon infliction of DNA double strand breaks. Specifically, we measured \( \gamma \)-H2AX intensity as well as the \( \gamma \)-H2AX spot number, a reflection of the number of double strand breaks in one cells. Figure 20 shows representative composite images obtained after immunostaining of \( \gamma \)-H2AX (green) and nuclear counterstaining with DAPI (red). The highest intensity of \( \gamma \)-H2AX signal can be seen at 5% concentration of beef and chicken digests. In line with this result, quantitative image analysis confirmed that cells treated with 5% beef and chicken digests gave the strongest \( \gamma \)-H2AX intensity and \( \gamma \)-H2AX spot number (figure 21). As seen in figure 23, cells treated with pork digests barely have spots, especially in 10% pork digests, and figure also shows the lowest \( \gamma \)-H2AX spot number in pork digests. This result is not consistent with previous results on cell viability and cell and nuclear morphology.

As we originally expected the 10% concentration to give the most pronounces effects (supported by viability and morphology results), we performed a pilot experiment with 10% duodenum samples and 10% colon samples.

![Figure 21](image)

**Figure 21.** Representative images of HT29 colon cancer cell lines exposed to control and meat digests (beef, chicken and pork) from colon samples at various concentration. Control 0 indicates negative control, with medium only added. Red color shows nucleus stained by DAPI, while green dots represent \( \gamma \)-H2AX formed in cells.
Figure 22. Relative change of the average value of $\gamma$-H2AX intensity in cells treated with meat digests (pork, beef and chicken) from colon samples in various concentration (2.5%, 5% and 10%), relative to corresponding concentration of control digests.

Figure 23. Relative change of the average value of $\gamma$-H2AX spot number in cells treated with meat digests (pork, beef and chicken) with various concentration (2.5%, 5% and 10%), relative to corresponding concentration of control digests.
Here, we found highly saturated images for γ-H2AX signal in cells treated with colon samples, but not with duodenum samples (figure 25 and 24, respectively). Due to the saturation problem, quantification of γ-H2AX signal including γ-H2AX intensity and spot number could not be conducted for colon samples, and we can only proceed with image acquisition results from duodenum sample.

As demonstrated in figure 26, none of the meat digests from duodenum samples seems to induce additional DNA damage, as lower level of both γ-H2AX intensity (Figure 26A) and spot number (Figure 26B) were measured, compared to 10% blanco digests (relative change < 1). However, it should be noted that the amount of DNA damage in control samples was unexpectedly high, especially compared to the previous experiment. Furthermore, the presence of saturation in part of the data set and the potential presence of staining differences makes the results not completely reliable. Further optimization of γ-H2AX immunofluorescence assay for detecting DNA damage in cells treated with meat digests is therefore advised.
Figure 25. Representative images of HT29 colon cancer cell lines exposed to 10% control and meat digests (beef, chicken and pork) from colon samples. Includes 120 ppm nitrite-cured pork. Control 0 indicates negative control, with medium only added. Red color shows nucleus stained by DAPI, while green dots represent γ-H2AX formed in cells.

Figure 26. Relative change of the average value of γ-H2AX intensity (A) and spot number (B) in cells treated with 10% meat digests (pork, beef and chicken) from duodenum samples, relative to 10% blanco digests.
4. DISCUSSION

The presented work fits in a study, MEATNOX, which aims at answering the question if the consumption of red meat and processed meat increases the risk of colon cancer. To help answering that question, we have addressed the cytotoxic and genotoxic effects of simulated meat digests at the level of the individual cells, by optimizing and utilizing a number of in vitro cell-based assays. Using HT29 colon cancer cell lines as models, we analyzed coarse effects on cell morphology, cell death as well as more subtle effects on oxidative stress, nuclear morphology changes and DNA damage. Different types of meat such as pork, chicken and beef were compared, since they differ in chemical composition (e.g. heme iron), while other parameters such as cooking temperature and fat content were kept constant (5% fat, cooked meat to a core temperature of 65°C). In addition, digest samples from two different compartments, duodenum and colon, were compared to analyze effects of microbial fermentation.

According to our knowledge, this study is the first experiment examining adverse effects of meat in vitro digests on cell lines with various cell-based analyses. Previously, Lux et al. (2012) studied the chemopreventive effects of nut in vitro digestion and fermentation products on HT29 colon cancer cell lines (Lux et al., 2012). In this study, cell-based analyses were performed such as fluorometric determination of cell growth and quantification of DNA damage using the COMET assay. Being the study with closest resemblance to our setup, we have adopted their digests preparation procedure. In preliminary experiments we did verify whether it was necessary to include the centrifugation step, but given that non-centrifuged blanco digests (i.e. without meat) already caused adverse effects on cell morphology, we decided to keep the preparation procedure in its entirety.

In our initial cell morphology experiments, we also compared different incubation periods (1h, 2h, 6h, 8h, 10h and 24h), but only found an overt effect at 24h incubation periods. At this time point, a clear change in cellular morphology and a clear impact on cell size and granularity of the digests on HT29 cells was observed. The effects were concentration-dependent, with 10% meat digests causing the most pronounced change in cell morphology, including darkening at the perinuclear region in approximately 90% of cells, cell rounding and compaction, combined with reduction of cell culture density.
The morphological changes induced by duodenum or colon samples were comparable, although the darkening was already observed more readily at lower concentrations (5%) for the duodenum samples. According to the chemical composition data of the digests (cfr. Appendix 1), the concentration of malondialdehyde (MDA) was the highest in duodenum digests from beef, followed by pork and chicken. MDA is a lipid peroxidation product from PUFAs that is known for its ability to induce sequence-dependent frameshift mutations, base-pair substitutions in cells, and create interstrand cross-links in DNA which have potent adverse biological effects (Del Rio et al., 2005).

The darkening at the perinuclear region observed in cells treated with meat digests may be due to heme accumulation in ER and/or ER stress. The ER is an organelle responsible for folding and modification of protein destined for the secretory pathway and endosomal compartment. The ER is highly susceptible to perturbation by various insults (e.g. genetic mutations, aging, oxidative stress, etc.) that lead to protein misfolding and accumulation in ER (Apostolou et al., 2008). In addition, the enzyme for heme degradation, heme oxidase (HO), is localized in the ER (Kim et al., 2011). Therefore, cells treated with high concentration of meat digests (10%), having the highest concentration of heme, are likely to accumulate heme in ER to be degraded, which may cause the darkening.

To find out whether morphological differences were also reflected in changes in cell viability, we used a PrestoBlue® assay. During the optimization of this assay, it came to our attention that the digests themselves had a confounding effect on the fluorescence signals, complicating the interpretation of the results. Heme-protein (hemoglobin, myoglobin and cytochrome c) as one of digests constituents has autofluorescent activity as mentioned by Hirsch (2002), due to fluorescence emission from Tyr and Trp residues, which can be detected by spectroscopy. This explained the higher fluorescence reading in both cells treated with digests and without cells.

By introducing a number of controls in the PrestoBlue® assay, we were able to extract a robust metric that was normalized for potential interferential effects. With this metric, we showed that meat digests have a concentration-dependent growth-inhibiting/cytotoxic effect at concentrations >5%, pork and beef digests from colon having the most adverse effects, but we failed to show an effect of nitrite-curing.

Three possible causes for the enhanced toxicity of colon digests of pork and beef are heme iron, lipid oxidation and bacterial fermentation.
1. Both pork and beef are red meat with a high content of heme iron (Rhee and Ziprin, 1987). In fact, it is called red meat because of the high concentration of myoglobin that renders the muscle red in color, and it is this myoglobin, together with hemoglobin and cytochrome that has heme as their prosthetic group. In the colon cells, heme is absorbed and the metabolic degradation of heme iron (by \textit{i.a.} heme oxygenase and microsomal cytochrome P450 reductase) is considered to increase the production of ROS in the cell, which in turn may cause cellular toxicity (Bastide et al., 2011; Ishikawa et al., 2010). This pathway is considered to be a major mechanism of heme-induced cellular cytotoxicity (Ishikawa et al., 2010). According to chemical composition results of undigested meat (from the MEATNOX project, cfr. Appendix 1), beef has the highest amount of heme, followed by pork and chicken. However, the cytotoxic effect of pork and beef digests at 10% concentration did not differ significantly. This may be caused by interactions with other components of the digests that still need to be determined.

2. Bacterial fermentation of meat, which is high in protein, results in the formation of several toxic compounds: phenols, p-cresol, ammonia, amines, amides and methane (Cross and Sinha, 2004). However, the evidences of those compounds related to cellular toxicity in human large intestine are limited. One study revealed that low concentrations of ammonia, at level of 5-10 mM can alter cellular morphology and reduce cell life span (Viseti, 1978). Furthermore, most of these compounds are highly volatile and it cannot be assured whether it was still present in the digests we use to treat the cells.

3. Poly-unsaturated fatty acids (PUFA) represent a third source of cytotoxic components. These long chain fatty acids are very susceptible to oxidation, thus having the potential to produce reactive lipid peroxidation products such as lipid peroxyl radical, which can interact with various biological components in cells such as DNA, protein and lipid. According to Rhee and Ziprin (1987), chicken meat has the highest PUFA content, followed by pork and beef (Enser et al., 1996). However, in this experiment we used very lean meat and standardized the fat level to 5% with subcutaneous pork fat. Therefore, PUFAs might not be a major cause to explain cellular toxicity. Moreover, based on our results, which showed that chicken digests were least toxic, it seems reasonable to assume that this component is not the dominant factor. It is possible that the
interaction between heme and PUFAs may explain the higher cytotoxicity of beef digests and especially pork digests in HT29 colon cancer cell lines. •OH, a major heme metabolite product, is very reactive and can oxidize lipid, to produce lipid peroxyl radical.

We also found that pork digests from colon sample caused higher cytotoxicity compared to duodenum samples. This may be explained by the longer incubation in colon sample (72 hours fermentation) leading to longer exposure of meat digests to oxidation, or the effect of the microbial fermentation and its byproducts. However, this association still needs to be confirmed.

Next to causing cellular toxicity, heme and protein fermentation products from meat are also assumed to play a big role in carcinogenesis. Sesink et al. (2000) demonstrated that heme is associated with both the initiation and promotion process of carcinogenesis in rat colonocytes, while Glei et al. (2002) concluded that iron that was taken up by human colon cells participates in the induction of oxidative DNA damage, by catalysing ROS formation on HT29 colon cancer cell lines (Glei et al., 2002; Ishikawa et al., 2010). Our complementary experiments on lipid peroxidation, nuclear morphology and DNA damage did not corroborate this hypothesis. However, this may also be attributed to experimental flaws or lack of sensitivity (cfr. next paragraphs).

Nuclear morphology

In accordance with our PrestoBlue® results, 10% meat digests clearly affected cell culture density, as evidenced by an increased number of individual, rounded cells vs. the typical patches or cell conglomerates in control cultures. In addition, we noted a change in cellular morphology with the highest concentrations, indicative of increased apoptosis. Willingham (1999) noted that cellular morphology changes in cells which undergo apoptosis are: 1) a loss of adhesion to subtratum, resulting in cell rounding; 2) shrinkage of the cells and 3) cell blebbing. The first two morphological changes mentioned match with our cell morphology results. To obtain a more resolved view, we also monitored nuclear morphology. Typical nuclear changes found in pre-apoptotic cells include increased chromatin condensation and nuclear deformation. By visualizing chromatin using a DNA intercalating dye (DAPI), we found a significant increase of aberrant nuclei in cell cultures treated with 10% pork and chicken digests from colon samples.
Despite a few inconsistencies between two independent experiments, the general trend is maintained and the data support our earlier findings on cellular morphology and viability. One of the inconsistencies concerns the DAPI intensity, which did not show significant differences between samples. Possible explanations for this discrepancy could be of experimental or biological nature. From a technical perspective, variations in staining procedure (e.g. air bubbles or concentration effects) or microscopy settings are potential causes although care was taken to sample multiple regions with identical settings. Human errors can of course never be excluded, such as the use of different ND filters or exposure time for image acquisition, or mistakes when handling the cells that cause additional stress. From a biological perspective, differences in cell growth may explain the lack of consistency: cultures with high density show differential staining and are also more difficult to analyze automatically. In addition, a rise in apoptotic cells (with highly condensed and thus more intense chromatin) may be compensated by a simultaneous drop in mitotic cells (which also possess highly condensed chromatin), thus canceling potential intensity changes. While optimization may be recommended it remains difficult to exclude one or the other causative factor for this dataset.

**Lipid peroxidation**

In an attempt to reveal a potential causative factor of cytotoxicity, we investigated lipid peroxidation in living HT29 cells that were treated with the meat digests. Unfortunately, we failed to show any difference from control. Several remarks can be made here to nuance the relevance of this assay. First, the dynamic range of the assay was fairly low, with the positive control, cumene, only inducing a change in measured fluorescence ratio of ~1.5. This may indicate that the sensitivity is too low to measure more subtle effects and may call for further optimization of this assay. Another consideration is that the assay measures lipid peroxidation in live cells and not the presence of lipid peroxidation products. This assay only detects fluorescent lipid as a result of lipid peroxidation in cells, and cannot detect lipid peroxidation products formed during digestion. The molecular triggers for lipid peroxidation may have too short a life time in the digests to still pick them up in our assay. The only solution for this would be to monitor the effect immediately after digestion or to switch to a more direct approach (e.g. ROS measurement in cell). Finally, the presence of anti-oxidants such as ascorbic acid in the digests formulation (cfr. Table 2, Materials and Methods section) may have prevented the oxidation reactions. But in the later case, this also
means that lipid peroxides would have marginal effect in the cytotoxic effect we observed, which may be expected given the PUFAs data (cfr. Previous paragraph).

**DNA damage**

One possible route to apoptosis is the occurrence of irreparable or severe DNA damage. We quantified DSBs, the most detrimental form of DNA damage, by measuring $\gamma$-H2AX, a histone protein that becomes phosphorylated upon infliction of DNA double strand breaks. Also here, results were inconclusive.

Since the $\gamma$-H2AX immunofluorescence staining has been optimized and was proven successful in quantifying double strand breaks DNA damage caused by various genotoxic agents such as ionizing and X irradiation in human cell (Beck et al., 2012; De Vos et al., 2010), we believe this experiment should be repeated with more replicates and more standardized operational procedures. Firstly, the patch-like nature of the cell culture and variations in cell density may have complicated proper analysis. This can be corrected by improving cell counting and seeding at lower density. Secondly, optimal conditions in cell cultures should be maintained (e.g. 5% CO2 and 37°C) to prevent additional stress during incubation that might be the case for cells treated with 10% blanco colon digests and also negative control, which have very strong and saturated signal that might have masked the actual signals in the treatment group. Thirdly, image acquisition needs to be done with identical settings, taking along the proper controls (flat fields, untreated sample, negative controls) with every run and preceded by a quick screen to check the variations in signal intensities between different conditions.
5. CONCLUSION

Epidemiological studies show a clear and significant relation between increased red meat and processed meat consumption and colon cancer incidence. However, these correlations need backup from experimental studies that shed a light on the exact working mechanisms. Therefore, our study was designed to assess the adverse effects of \textit{in vitro} meat digestion products by integrating the \textit{in vitro} digestion process of meat with a multi-parametric cyto- and genotoxicity analysis in HT29 colon cancer cell lines.

The major conclusions of our study are the following:

- First of all, all meat digests affect cellular morphology of HT29 colon cancer cell lines, most significantly at 10% concentration with darkening at perinuclear region of the cells, more rounded and individual cells and reduced cell density.
- Secondly, cell viability assay confirmed that 10% meat digests (beef, chicken and pork) from both colon and duodenum samples and 5% pork digests with (0 ppm) and without (120 ppm) nitrite from colon samples reduce cell density significantly, thus having a growth-inhibiting and/or cytotoxic effect. This may be due to presence of heme, especially in beef and pork, and also interaction between heme and oxidation of PUFAs.
- Thirdly, there is almost no difference between normal and nitrite-cured pork.
- Fourthly, inconsistent results in nuclear morphology and $\gamma$-H2AX immunofluorescence analysis call for improved standardization and optimization of cell seeding and staining procedure.

For future follow-up, there are a number of comments and suggestions to be made.

A major comment with respect to the current investigation could be the fact that we have used HT29 cancerous cell lines, which is arguably not ideal cell line for this type of investigation due to its genetic instability. However, Forgue-Lafitte et al. (1989) reported that HT29 cell lines have retained certain characteristics of the normal tissue, such as human receptors (e.g. vasoactive intestinal peptide (VIP), or receptors for insulin, EGF) and under various culture conditions, HT29 cells can undergo differentiation to mucus-secreting and/or absorptive cells (Rousset, 1986). Several \textit{in vitro} genotoxicity assays also used HT29 colon cancer cell lines for investigating the
effects of various meat-related substances such as heme and lipid peroxyl radical (Glei et al., 2006), and \textit{in vitro} digestion products of nut (Lux et al., 2012).

With optimized procedures, the assays should also be repeated with using individual bio-active compounds (heme, lipid oxidation products...) to understand their individual contributions and potential synergistic/antagonistic effects. Ideally this should be done in a high-throughput format.

Next to optimization of the previously mentioned procedures, which are quite general, we also suggest for further studies to focus on more specific end-points in colonocytes such as NOCs-specific DNA adducts, mutations in K-Ras and p53, but also to have a look at more indirect measures of oxidative stress, such as changes in ROS-related gene expression patterns or protein turnover.

Other possible additions to future experiments include tuning the concentration range of meat digests and transit time in colon to correspond to those of the human organism. This study adopted concentration of digests from a previous study by Lux et al. (2012) and used 24 h incubation from initial experiment. It may be more appropriate to adapt the concentration of the digests to reflect the actual meat consumption in human, and to consider transit time in human colon, which is about 39 ± 5 hours (Arhan et al., 1981).

In conclusion, we have established an \textit{in vitro} cell-based analysis workflow for gauging potential adverse effects of meat digests. This approach has returned valuable results on the cytotoxicity of different meat digests and with further optimization, it will produce more insights in the fundamental mechanisms underlying this toxicity.
REFERENCES


Weisburger, J.H., 1999. Carcinogenicity and mutagenicity testing, then and now. Mutat Res-Rev Mutat 437, 105-112.


Appendixes
**Appendix 1.** Meat characteristics of chicken (*m. Pectoralis profundus*), pork (*m. Longissimus dorsi*) and beef (*m. Biceps femoris*) corrected to approximately 5% fat by adding subcutaneous pork fat

<table>
<thead>
<tr>
<th>General</th>
<th>Unit</th>
<th>Chicken</th>
<th>Pork</th>
<th>Beef</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µ ± SD</td>
<td>µ ± SD</td>
<td>µ ± SD</td>
</tr>
<tr>
<td>Dry matter</td>
<td>%</td>
<td>30,7 ± 0,5</td>
<td>31,6 ± 0,3</td>
<td>30,3 ± 0,2</td>
</tr>
<tr>
<td>Protein</td>
<td>%</td>
<td>23,4 ± 0,6</td>
<td>21,9 ± 1,8</td>
<td>21,4 ± 0,6</td>
</tr>
<tr>
<td>Fat</td>
<td>%</td>
<td>4,4 ± 0,5</td>
<td>5,8 ± 0,2</td>
<td>5 ± 0,0</td>
</tr>
<tr>
<td>ALA</td>
<td>mg/kg</td>
<td>36,8 ± 1,8</td>
<td>34,7 ± 1,5</td>
<td>41,7 ± 1,4</td>
</tr>
<tr>
<td>VLFA n-3</td>
<td>mg/kg</td>
<td>23,1 ± 0,4</td>
<td>14,8 ± 1,4</td>
<td>43,1 ± 0,2</td>
</tr>
<tr>
<td>LA</td>
<td>mg/kg</td>
<td>654,8 ± 42,3</td>
<td>655,0 ± 12,5</td>
<td>604,8 ± 0,2</td>
</tr>
<tr>
<td>VLFA n-6</td>
<td>mg/kg</td>
<td>54,5 ± 1,1</td>
<td>59,7 ± 3,2</td>
<td>67,2 ± 1,1</td>
</tr>
<tr>
<td>SFA</td>
<td>mg/kg</td>
<td>1747,3 ± 96,3</td>
<td>1925 ± 43,8</td>
<td>1743,9 ± 9,7</td>
</tr>
<tr>
<td>MUFA</td>
<td>mg/kg</td>
<td>1911,6 ± 121,1</td>
<td>2139 ± 47,3</td>
<td>1881,9 ± 0,9</td>
</tr>
<tr>
<td>PUFA</td>
<td>mg/kg</td>
<td>820,5 ± 42,7</td>
<td>812 ± 19,1</td>
<td>800,6 ± 5,7</td>
</tr>
<tr>
<td>Fresh samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual nitrite</td>
<td>mg/kg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hematin</td>
<td>mg/kg</td>
<td>16,0 (n=1)</td>
<td>28,6 ± 0,0</td>
<td>150,6 ± 5,3</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>mg/kg</td>
<td>4,3 ± 0,2</td>
<td>5,5 ± 0,3</td>
<td>13,8 ± 1,9</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6,2 ± 0,0</td>
<td>5,6 ± 0,0</td>
<td>5,7 ± 0,0</td>
</tr>
<tr>
<td>Cured samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual nitrite</td>
<td>mg/kg</td>
<td>84,5</td>
<td>36,4 ± 0,2</td>
<td>27 ± 1,2</td>
</tr>
<tr>
<td>Hematin</td>
<td>mg/kg</td>
<td>16,5 (n=1)</td>
<td>34,7 ± 1,0</td>
<td>152,7 ± 11,1</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>mg/kg</td>
<td>4,1 ± 0,3</td>
<td>4,9 ± 0,3</td>
<td>14,4 ± 0,2</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6,0 ± 0,0</td>
<td>5,5 ± 0,0</td>
<td>5,5 ± 0,0</td>
</tr>
</tbody>
</table>

ALA = α-linolenic acid (C18:3, n-3); VLFA n-3 = very long fatty acids n-3 (C20:5, n-3; C22:5, n-3; C22-6, n-3); LA = linoleic acid (C18:2, n-6); VLFA n-6 = very long fatty acids n-6 (C20:4, n-6; C22:4, n-6; C22:5, n-6), SFA = saturates fatty acids; MUFA = mono unsaturated fatty acids; PUFA = poly unsaturated fatty acids; FM = fresh matter; µ= mean; SD = standard deviation
## Appendix 2. List of meat digests and its important chemical composition (MDA, PCC and protein)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trial</th>
<th>Sample Nr.</th>
<th>Species</th>
<th>Fat</th>
<th>Nitrite (mg/kg)</th>
<th>Cooking</th>
<th>Microbiota</th>
<th>Stage</th>
<th>RCM (CFU/ml)</th>
<th>MDA (nmol/mL digest)</th>
<th>PCC (nmol DNPH/mL digest)</th>
<th>Protein (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken, 5% fat, 0 ppm nitrite, cook</td>
<td>1</td>
<td>49</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>11.8</td>
<td>8.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Chicken, 5% fat, 0 ppm nitrite, cook</td>
<td>1</td>
<td>50</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>11.9</td>
<td>5.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Chicken, 5% fat, 0 ppm nitrite, cook</td>
<td>1</td>
<td>51</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>6.9</td>
<td>8.3</td>
<td>4.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Chicken, 5% fat, 0 ppm nitrite, cook</td>
<td>1</td>
<td>52</td>
<td>1</td>
<td>5</td>
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<td>1</td>
<td>2</td>
<td>7.2</td>
<td>7.6</td>
<td>4.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Pork, 5% fat, 0 ppm nitrite, cook</td>
<td>1</td>
<td>53</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>16.9</td>
<td>6.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Pork, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>54</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>17.4</td>
<td>8.7</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Pork, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>55</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>7.3</td>
<td>11.0</td>
<td>3.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Pork, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>56</td>
<td>2</td>
<td>5</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>7.5</td>
<td>11.1</td>
<td>3.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Beef, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>57</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>29.1</td>
<td>12.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Beef, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>58</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>28.6</td>
<td>14.3</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Beef, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>59</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>7.0</td>
<td>19.7</td>
<td>5.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Beef, 5% vet, 0 ppm nitrite, cook</td>
<td>1</td>
<td>60</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>6.9</td>
<td>20.0</td>
<td>5.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Pork, 5% vet, 120 ppm nitrite, cook</td>
<td>1</td>
<td>65</td>
<td>2</td>
<td>5</td>
<td>120</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>10.5</td>
<td>3.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Pork, 5% vet, 120 ppm nitrite, cook</td>
<td>1</td>
<td>66</td>
<td>2</td>
<td>5</td>
<td>120</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>11.1</td>
<td>4.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Pork, 5% vet, 120 ppm nitrite, cook</td>
<td>1</td>
<td>67</td>
<td>2</td>
<td>5</td>
<td>120</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>7.0</td>
<td>6.0</td>
<td>3.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Pork, 5% vet, 120 ppm nitrite, cook</td>
<td>1</td>
<td>68</td>
<td>2</td>
<td>5</td>
<td>120</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>7.1</td>
<td>6.0</td>
<td>3.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>