Oxidation during *in vitro* digestion of meat

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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, August 2012

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Signature:
Dedication

To my great parents, my father Fugen Feng, my mother Xiujuan Zhang, who always love and support me, wherever and whatever I am.

To my grandmother, Meizhen Wu, a strong woman fighting with osphagus cancer.

To my best friend Xuan Wang and her coming baby.

To my special friend, Liang Yu who have encouraged me to apply this master program and always supports me.

To all the people working with nutrition and cancer.
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Table of content

COPYRIGHT .............................................................................................................. I
DEDICATION ........................................................................................................... II
ACKNOWLEDGEMENTS ......................................................................................... III
TABLE OF CONTENT ............................................................................................. IV
LIST OF FIGURES ..................................................................................................... VI
LIST OF TABLES ....................................................................................................... VII
LIST OF ABBREVIATIONS ....................................................................................... VIII
ABSTRACT ................................................................................................................ - 1 -

LITERATURE REVIEW ........................................................................................... - 3 -
1.1 INTRODUCTION ................................................................................................. - 3 -
1.2 COLORECTAL CANCER .................................................................................... - 4 -
   1.2.1. The occurrence of colorectal cancer ....................................................... - 4 -
   1.2.2 Hypotheses on the causal mechanisms ................................................... - 6 -
1.3 LIPID OXIDATION ............................................................................................... - 10 -
   1.3.1 Mechanisms of lipid oxidation ................................................................. - 10 -
   1.3.2 Lipid oxidation in in vivo ............................................................................ - 13 -
   1.3.3 Lipid oxidation and colorectal cancer ....................................................... - 13 -
1.4 IN VITRO DIGESTION ....................................................................................... - 14 -
   1.4.1 Digestion models ....................................................................................... - 14 -
   1.4.2 Oxidation in in vitro digestion model: interaction with gastrointestinal juices - 15 -
1.5 OBJECTIVES ..................................................................................................... - 17 -

2. MATERIAL AND METHODS ................................................................................. - 18 -
2.1 CHARACTERISTICS OF MEAT ....................................................................... - 18 -
   Dry matter, crude protein and crude fat content ............................................. - 18 -
2.2 THE IN VITRO DIGESTION OF MEAT .......................................................... - 18 -
2.3 LIPID OXIDATION ANALYSIS ....................................................................... - 21 -
2.4 STATISTICS ANALYSIS ................................................................................... - 22 -

3. RESULTS .............................................................................................................. - 23 -
3.1 CHARACTERIZATION OF MEAT ..................................................................... - 23 -
3.2 EXPERIMENT 1 .................................................................................................. - 24 -
3.3 EXPERIMENT 2 .................................................................................................. - 27 -

4. DISCUSSION ...................................................................................................... - 29 -
4.1 IN VITRO DIGESTION MODELS .................................................................... - 29 -
4.2 CHARACTERIZATION OF MEAT ..................................................................... - 30 -
4.3 EXPERIMENT 1 .................................................................................................. - 32 -
4.4 EXPERIMENT 2 .................................................................................................. - 36 -

5. CONCLUSION ...................................................................................................... - 39 -

REFERENCES ......................................................................................................... - 41 -
Annex 1. The pH of the meats and the digesta during incubation processes (Date: 2012-10-13). - 2 -

Annex 2. The pH of the meats and the digesta during incubation processes (Date: 2012-10-21). - 3 -

Annex 3. The pH of the meats and the digesta during incubation processes (Date: 2012-11-04). - 4 -
List of figures

Figure 1: Colorectal cancer incidence and red-meat consumption worldwide in men..- 5 -

Figure 2: Catalytic effect of haem on fat peroxidation and N-nitrosation, and their inhibition by dietary means. ..............................................................- 7 -

Figure 3: An outline mechanism of lipid peroxidation. .................................................- 12 -

Figure 4: Schematic representation of the in vitro digestion procedure.......................- 20 -
List of tables

Tabel 1: Constituents and concentrations of the various synthetic juices of the in vitro digestion........................................................................................................................................- 19 -

Tabel 2: Dry matter, protein and lipid content (mean ± standard deviation; g/100g meat), haem iron content (mean ± standard deviation; mg hematin/kg meat), and lipid oxidation products (mean ± standard deviation; nmol/g meat) of the raw meat samples of experiment 1 and 2...........................................................................................................................................- 24 -

Tabel 3: Hydroperoxides values (mean ± standard deviation; nmol/g meat) detected in chicken lean, chicken fat, beef lean, and beef fat during the different incubation steps (n=3)...........................................................................................................................................- 25 -

Tabel 4: TBARS values (mean ± standard deviation; nmol MDA/g meat) detected in chicken lean, chicken fat, beef lean, and beef fat during the different incubation steps (n=3)...........................................................................................................................................- 26 -

Tabel 5: The Hydroperoxides values (mean ± standard deviation; nmol/g meat) detected in beef meat with different vitamin C concentrations (mg/9 g meat) during the different incubation steps (n=3)...........................................................................................................................................- 27 -

Tabel 6: TBARS values (mean ± standard deviation; nmol MDA/g meat) detected in beef meat with different vitamin C concentrations (mg/9 g meat) during different incubation steps (n=3). ...........................................................................................................................................- 28 -
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HNE</td>
<td>4-hydroxynorenal</td>
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<tr>
<td>AH</td>
<td>Antioxidant</td>
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<td>ALEs</td>
<td>Advanced lipid oxidation endproducts</td>
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<td>ATNC</td>
<td>Apparent total nitroso-compounds</td>
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<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HCAs</td>
<td>Heterocyclic aimes</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NOCs</td>
<td>N-nitroso compounds</td>
</tr>
<tr>
<td>‘OH</td>
<td>Hydroxyl radicals</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<tr>
<td>R’</td>
<td>Radicals</td>
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<td>RH</td>
<td>Lipid molecule</td>
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<td>Proxyl radicals</td>
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<td>ROOH</td>
<td>Lipid hydroperoxides</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SAFA</td>
<td>Saturated fatty acid</td>
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<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
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<tr>
<td>TBARS</td>
<td>Thiobarbituric acid test</td>
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<tr>
<td>TMP</td>
<td>1,1,3,3-Tetramethoxy-propane</td>
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<td>WCRF</td>
<td>World Cancer Research Center</td>
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Abstract

Colorectal cancer may result partly from the digestion process of red meat which involves the presence of oxidized lipids. The aim of this study was to evaluate the lipid oxidation of four meat samples, varying in haem and fat content: beef lean (Belgium Blue young bull), beef fat (culled cow), chicken lean (chicken beast) and chicken fat (chicken thigh) under the condition of in vitro digestion; and the role of vitamin C with different concentrations in the oxidation process during the in vitro digestion of beef meat. The characterization of four types of meat were measured and the hydroperoxides (ROOH) and malondialdehyde (MDA) levels of the meats were evaluated during the simulated digestion process.

The TBARS values and the haem content of raw meat samples were significantly higher for beef samples than for chicken samples, while fat content, especially PUFA content, of chicken fat was significantly higher than that for both two types of beef meat and chicken lean. No hydorperoxides value was detected in the raw meat samples except for beef fat. For all meat samples, independence of fat and haem content, both the ROOH and MDA concentration increased substantially after 2h-incubation in the simulated stomach condition, compared with those found at the onset of the incubation. The TBARS values of four types of meat increased approximately 3- to 5-fold and the hydroperoxides values increased substantially, but no clear pattern could be found. A similar trend was obtained for different vitamin C concentrations added to beef fat samples. Elucidation of the antioxidant effect of different vitamin C concentrations on lipid oxidation of beef meat during the digestion process, demonstrated that vitamin C with the concentrations tested had no effect on the lipid oxidation of beef meat during the in vitro digestion processes.

The results indicated that beef muscles are more susceptible to lipid oxidation than chicken muscles. Thus, we confirmed the hypothesis that haem content of raw meats may increase lipid oxidation during digestion and found that the total haem content is probably more important than the fat content regarding the onset of oxidation. Moreover, we suggest that the consumption of red meat might increase lipid oxidation in the stomach and cytotoxic lipid peroxidation products might be later absorbed into the body. The addition of vitamin C to the beef meat with the concentrations tested in present study may not alter these outcomes.
However, further studies should be done to investigate whether other doses of vitamin C could act as an anti-oxidant during digestion process.

*Key words: meat, oxidation, in vitro digestion, colorectal cancer.*
Literature review

1.1 Introduction

There are numerous literature studies which support the hypothesis that an increased risk of cardiovascular diseases and several kinds of cancer are associated with the high consumption of red meat [1-6]. The correlation between cancer mortality and diet is remarkably strong at the international level. However, colorectal cancer (CRC) is more frequent in Western countries where red meat is frequently consumed, compared with less affluent countries where meat consumption is low [6]. The most sensitive cancer sub-site of CRC is distal colon. A Swedish Mammography cohort study demonstrated that high consumption of red meat was associated with a statistically significant more than 2-fold increased risk of distal colon cancer, whereas there was no apparent association with risks of proximal colon or rectal cancers [7].

In the last decades, scientists were searching for the reasons why red meat can contribute to CRC. There are numerous reasons and we are most interested to one of them: lipid oxidation. An in vivo study demonstrated that colorectal carcinogenesis was associated with serious oxidative stress [8], which refers to a remarkable imbalance status between the production of reactive oxygen species (ROS) by pro-oxidants and the removal of ROS by antioxidants [9]. ROS and the other lipid oxidation products are involved in cancer initiation and progression [10]. In another words, lipid oxidation has an association with CRC.

In order to reduce the incidence of CRC, the most efficient way might be reducing the consumption of red meat. Therefore, the Report of the World Cancer Research Fund (WCRF) makes the recommendation to limit the consumption of red meat and to avoid processed meat intake [11]. Based on this report, the French National Cancer Institute recommended to limit the intake of red meat to less than 500g per week and to also limit the intake of cured meats, especially high fat or very salty ones. Those who eat cured meat should choose it less often and reduce portion size [3]. However, if we follow these recommendations, can we really avoid CRC? And if we follow these recommendations, does it means that the meat industry will suffer great economic losses? As we known it is difficult to change the consumption behavior of people, therefore, nowadays, the meat industry is challenged to look for healthier alternatives.
1.2 Colorectal cancer

1.2.1. The occurrence of colorectal cancer

Colorectal cancer is the third most common disease in men and the second in women. High risk areas include North America, Europe, Australia and New Zealand. The lowest incidence is in Africa (except Southern Africa) and South-Central Asia (showed in Fig.1). In other words, the developed countries accounts for over 63% of the total global incidence, but the incidence is also increasing in previously low-risk areas [12]. Incidence and mortality rates are higher in men than in women, with the sex ratio 1.4: 1 [13]. In 2008 worldwide estimation, about 608,000 deaths are from CRC, accounting for 8% of the cases in the both sexes. It is the fourth most common cancer (after breast, lung and prostate) in the world [13]. Nowadays, the five-year survival rate of colorectal cancer is over 50% in both male and female CRC, while ten-year survival rates are only a little lower than those at five-year. Therefore, it is indicated that patients who survive five years are cured for this disease [14]. The five-year survival rate in CRC patients was much higher than 30 years ago. Improvements attribute to earlier diagnosis and better treatments, but there is still much space for further progress.

Although the exact causes of developing CRC are not known, there are many factors that increase the risk of developing such cancer. Acknowledged, age factor, inflammatory diseases of colon, and family history of CRC and many genetic syndromes, such as familial adenomatous polyposis and hereditary non-polyposis colorectal cancer are very important factors for developing CRC [16]. Nevertheless, many epidemiological studies demonstrated that a substantial number of dietary factors, and factors related to diet, possibly influence the risk of colorectal cancer [17-19]. Obesity, greater adult height, frequent eating, and diets high in sugar, total and saturated fat, eggs, processed and red meat, all increase the risk for the development of CRC, while diets, high in starch, non-starch polysaccharides, carotenoids, and polyphenols, which are all found in food of plant origin, are the protective factors of developing such cancer [20-22]. Other non-dietary factors, such as smoking tobacco, lack of physical activities and high alcohol consumption are also risk factors of developing CRC. In contrast, increasing physical activities is the convincing factor for decreasing the incidence. Possible protective factors for developing CRC are aspirin and other Nonsteroidal Anti-inflammatory Drugs [12].
Figure 1: Colorectal cancer incidence and red-meat consumption worldwide in men [15]

**a:** Countries with a high incidence of colon cancer (cases per 100,000 people) are indicated with blue (North America, Australia); countries with moderate levels in pink or red; and countries with low incidence in green (Asia, Africa). Colon cancer incidence is correlated with red-meat intake.

**b:** Countries that consume the most red meat, in g/day, are indicated in blue (North and South America, Australia); countries with moderate levels of consumption in pink or red; and countries with the lowest levels of red-meat intake in green (Africa, Asia).
1.2.2 Hypotheses on the causal mechanisms

There are several mechanisms that may explain the association between the risk of colorectal cancer (CRC) and the intake of red or processed meat. First of all, the mutagenic heterocyclic amines (HCAs) which are induced by cooking meat at a high temperature may play a role in CRC risk. Current evidence showed that the cancer risk had association with very well cooked meats, which had a high level of HCAs [23]. However, Koutros et al. [24] reported that there was no association between meat type or specific cooking method and cancer risk. Secondly, many literature studies suggested that haem in the form of haemin, a ferric form of haem, may explain the link between the risk of CRC and red meat intake [25]. Huang [26] reported a strong association between iron overtake and cancer risk in human. The third hypothesis is that CRC has an association between total dietary fat, including saturated fat, monounsaturated fat (MUFA) and polyunsaturated fat (PUFA), and the risk of CRC. Some studies have reported the positive association [27], while several studies have not observed any consistent epidemiologic evidence of the association between saturated fat or polyunsaturated fat (PUFA) intake and the risk of CRC [28-32]. Recent meta-analysis studies [33-35] demonstrated that there was a limited suggestive association between the increased risks of colorectal CRC with intake of food containing animal fat. Other hypotheses involve the high protein, cholesterol, salt content; lack of physical activities; high alcohol consumption, and cigarette smoking; obesity and diabetes etc.

However, none of those hypotheses seem to be able to explain the association between meat intake and CRC. Because these cohort studies are observation studies, they cannot fully avoid confounding factors, and thus a meta-analysis of cohort studies cannot demonstrate that a type of food is the cause of cancer. Only a direct experiment can prove that a cause links to an effect [3]. Therefore, for the sake of supporting the meat-cancer link, I will briefly review below the mechanistic hypotheses on the link between CRC and meat consumption.

Haem

Haem, the iron-carrier of red meat, is included in so-called haemoprotein, that is haemoglobin, myoglobin in cytochromes [36]. Many literature studies [36] speculated that haem would be a major player in CRC promotion, explaining why red meat, but not white meat is associated with cancer risk. This hypothesis was supported by three meta-analysis of epidemiological studies [37-39], which found that the high intake of haem was linked with the
higher risk of colon cancer. There were also many experimental evidences demonstrated that CRC may promote by haem. Sawa et al. [40] showed that dietary haemoglobin promote the production of lipid peroxyl radicals (ROO\(^{·}\)) and increases the incidence of nitrosomethylurea-induced colon cancer in rats fed polyunsaturated fat. Pierre et al. [41] fed three types of meat with different haem content (chicken, beef, and blood sausage) to rats treated with azoxymethane and fed a low-calcium diet. This study was the first time to show that dietary meat can promote colon carcinogenesis, and the effect depends on the haem concentration [36].

Huang [26] reported that there was the suggestive evidence that iron is a carcinogenic compound, especially Sesink et al. [42] found the evidence that haem increases cell proliferation in the mucosa. However, it may be more likely that haem plays a role of co-carcinogenesis. Corpet et al. [3] speculated that the mechanism implicated in the promotion of colorectal cancer by haem are the catalytic effect of haem on the formation of N- nitroso compound (NOC) and the formation of lipid oxidation end-products, as showed on Fig 2.

![Figure 2: Catalytic effect of haem on fat peroxidation and N-nitrosation, and their inhibition by dietary means [36]](image_url)

There are the Consequences for the development of colorectal cancer- Reprinted with modifications from Cancer Prevention Research [36]. Haem catalyzes nitrosation and fat peroxidation. End products are N- nitroso compounds (NOCs), malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE). These pathways explain, at least in part, the promoting effect of red and cured meat on CRC. The catalytic effects of haem can be inhibited by trapping haem with calcium carbonate or chlorophyll. The endogenous formation of NOCs is inhibited by vitamin C, vitamin E and specific polyphenols [3].
There were many evidences that haem catalyzes N-nitrosation [43-45]. Santarelli et al. [43] demonstrated that cured-meat promoted carcinogenesis in rats and this was associated with a high level of fecal apparent total nitroso-compounds (ATNC). This evidence was not only limited to cured meat, since a diet high in fresh meat (600g/d compared with 60g/d) induced a 3-fold increase in fecal nitroso-compounds [44]. Regarding the release of nitric oxide from the biological samples, Kuhnle et al. [45] gathered Fe-nitrosyl haem, S-nitrosothiols with N-nitroso-compounds, which were collectively referred to as ATNC. Many studiies suggested that ATNC may be an important genotoxin [46-47]. Furthermore, Lewin et al. [48] found that in volunteers, the red meat associating with endogenous NOC formation has been correlated with the formation of the N-nitroso-specific DNA adduct, which was called O⁶-carboxymethylguanine. The research form Gottschalg et al. [49] reported that the O⁶-carboxymethylguanine adducts in exfoliated colorectal cells which were related to CRC.

Haem also catalyzes the oxidation of polyunsaturated fats, which plays an important role in the process of lipid oxidation. The initial products of lipid oxidation are lipid hydroperoxides (ROOH), but they are very reactive. They are either reduced by glutathione peroxidase to unreactive fatty acid alcohols or they react with metals to produce a variety of reactive compounds such as epoxides and aldehydes. The major aldehyde molecules are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [50]. MDA is toxic and binds DNA, forming mutagenic adducts and 4-HNC performs a selected tumor promotion [50-52].

**N- nitroso compound**

As we known, salt, nitrites and nitrates are often used in meat production processes. But, WCRF reported that the dietary nitrites and nitrates were to be considered as human carcinogens, because they may be converted in the body to N-nitroso compounds (NOCs) that were well known as carcinogens [10]. The occurrence of NOCs could therefore explain the reason of nitrite-cured meat products favoring cancer [3]. NOCs are produced by the reaction of nitrite and nitrogen oxides with secondary amines and N-alkylamides [53]. *In vivo*, NOCs can be formed endogenously in the colon by reacting with the amines and amides produced during bacterial decarboxylation of amino acids. A number of facultative and anaerobic colonic bacteria can catalyze the formation of NOCs at optimum pH 7.5 [54, 55]. Therefore, N-nitrosation in the colon has been demonstrated that it was dependent on the presence of gut flora [56].
Some epidemiological studies demonstrated that NOCs are carcinogenic. For instance, a cohort study of CRC risk showed that there was a significant positive association between the intake of N-nitrosodimethylamine (cured meat and sausages are sources of it) and CRC [57]. The probability of NOCs formation was affected by other dietary components, including haem, which may catalyze the formation of NOCs from natural precursor in the gut [58]. Bingham et al. [59] investigated the effect of white and red meat on endogenous N-nitrosation formation with 18 healthy male volunteers which found that the ATNC showed a consistent dose response to red meat consumption. This study support the most probable hypothesis of CRC causal factor, which proposed that nitrite alone, is not the causal factor, but the combination of N-nitroso compounds and haem. This point of view was also mentioned in the haem part of my literature review.

**Animal fat consumption**

Acknowledged, red meat usually contains a large amount of haem primarily haemoglobin and myoglobin, together with fat [60]. The effect of simultaneous intake of fatty acids and haem were elucidated at molecular and in vivo levels [60]. It is well known that PUFAs are spontaneously oxidized in air, and they form ROOH [61]. There were studies done in a water-soluble model compound of ROOH (t-BuOOH) and was shown that the reaction between t-BuOOH and haem generated predominantly ROO’ [62, 63]. Therefore, Sawa et al. [60] elucidated the hypothesis that simultaneous intake of fatty acids and haem might cause generation of lipid ROO’ in intestinal tract and promote colonic carcinogenesis in in vivo model. Moreover, the bactericidal activity of ROO’ [62, 63] may indicate an additional cytotoxic effect on endothelial cells as well as damage to DNA, thereby enhancing carcinogenic potential by introduction of new cell proliferation as a repair process [64].

Another hypothesis had postulated that a high fat diet promoted the production of bile acids, which exerted a promoting effect on colonic carcinogenesis [65]. Epidemiological evidence has showed that populations with a high incidence of CRC and consuming a high fat and animal fat diet, excreted about twice the amount of secondary bile acids [66]. Another epidemiological study has demonstrated that the concentration of fecal bile acids was positively related to CRC incidence [67]. For experiments on animals [65], one mechanism by which the pro-CRC effects were possibly exerted was through the metabolism of secondary bile acids from primary bile acids by anaerobic bacteria in the large bowel. It has been shown
that these compounds from secondary bile acid may have tumor-promoting capacities in *in vivo* models [68, 65]. Therefore, based on the above studies, animal fat consumption performs an indirect role in the formation of carcinogens in the colon.

### 1.3 Lipid oxidation

#### 1.3.1 Mechanisms of lipid oxidation

Lipid oxidation in food is one of the major degradative processes for losses in food quality, such as flavor, color, and texture. The oxidation of unsaturated fatty acids results in significant generation of cytotoxic and genotoxic compounds [69, 70]. Moreover, during the process of lipid oxidation, there are many secondary by-products generated which could be not only carcinogenic compounds, but also be co-oxidants of vitamins, such as vitamin A, vitamin C and vitamin E, which impair the nutritional value of the foods [71, 72]. There are also many polymerized compounds, which might influence the color and flavor of meat and meat products.

The processes of lipid autoxidation in meat and meat product are described in terms of initiation, propagation and termination processes (Fig.2). Lipid oxidation is initiated when a hydrogen atom is removed from a methylene group in the hydrocarbon chain of a lipid molecule (RH), especially dietary PUFAs, such as linoleate (18:2n-6), linolenate (18:3n-3), and arachidonic acid (20:4n-6) [73]. The initiation step is also called hydrogen abstraction. The reaction is as follow:

\[ \text{RH} + \text{HO} \cdot \rightarrow \text{R} \cdot + \text{H}_2\text{O} \]

In the propagation phase, the resulting radical (R·) in aerobic cells undergoes a molecular rearrangement, followed by reaction with O₂ to give a peroxyl radical (ROO·) (Fig. 2) [74]. ROO· abstracts hydrogen from adjacent fatty acid side chains in a membrane, thereby propagating the chain reaction of lipid peroxidation. Hence a single initiation event can result in conversion of hundreds of fatty acid side chains into lipid hydroperoxides (ROOH) [90].
The reactions are as follow:

\[ R^* + O_2 \rightarrow ROO^* \] (2)

\[ ROO^* + ROO^* \rightarrow ROOH \] (3)

\[ ROOH \rightarrow RO^* + HO^- \] (4)

ROOH in the condition of metal irons or at high temperature, break down to free radicals. These free radicals, in the presence of oxygen, can form secondary oxidation products, which can decompose to low-molecular-weight breakdown products or condense to polymers [74]. One of the important products is MDA. MDA is in many instances, the most abundant individual aldehyde that results from lipid peroxidation in foods [72, 75].

Many biological studies demonstrated that haem, present in haemoglobin and myoglobin, plays an important role in lipid oxidation [36]. Reactive oxygen radicals, such as hydroxyl radicals (\( ^\cdot \text{OH} \)), reduce \( O_2 \) to superoxide radical (\( O_2^- \)), further produce \( H_2O_2 \) that can then react with \( Fe^{2+} \) to form another \( ^\cdot \text{OH} \). These \( ^\cdot \text{OH} \) radicals are highly reactive and can attack the hydrocarbon chain of a lipid molecule. Hence, haem plays a role in the formation of hydroperoxides and subsequently catalyzes lipid oxidation. This has led to the suggestion that a \( Fe^{2+}-Fe^{3+}-O^2 \) complex is the initiator of peroxidation (in the sense of abstracting the first hydrogen atom). Added metal irons may largely or entirely stimulate peroxidation by decomposing peroxides to radicals, which are capable of abstracting hydrogen and continuing the chain reaction [76]. The Fenton reactions are as follow:

\[ Fe^{2+} + O_2^- \rightarrow Fe^{3+} + O_2 \] (5)

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \] (6)

\[ Fe^{2+} + H_2O_2 \rightarrow HO^* + HO^- + Fe^{3+} \] (7)

Generally speaking, the terminal phase happens when radicals react with each other to form stable products. The termination depends on the length of the propagation chain, which depends on many factors, including the lipid/protein ratio in a membrane, the fatty acid composition, oxygen concentration and the presence within the membrane of chain-breaking antioxidants [72].
Terminal reactions are as follow:

\[ R' + R' \rightarrow RR \quad (8) \]

\[ R' + ROO' \rightarrow ROOR \quad (9) \]

\[ ROO' + ROO' \rightarrow ROOR + O_2 \quad (10) \]

The chain-breaking antioxidants (AH), such as e.g. vitamin C, carotenoids, and phenolic or polyphenolic compounds interrupt the chain reaction by providing hydrogen for abstraction by ROO’ [74]. The reaction is as follow:

\[ AH + ROO' \rightarrow ROOH + A' \quad (11) \]

Figure 3: An outline mechanism of lipid peroxidation [74]

Abstraction of hydrogen from a fatty acid with three double bonds is shown. A,B and C have conjugated diene double bond structure (double bond-single, bone double-bone) that absorb UV light at or around 234 nm.
1.3.2 Lipid oxidation in *in vivo*

Food products do not only oxidize during manufacturing and storage, but it is also possible that they oxidize after ingestion and during digestion [73]. The causes of this oxidation can be explained in two aspects. On one hand, when the stomach receives the masticated food, the stomach is exposed to an aerobic environment. On the other hand, muscle tissue contains free iron and myoglobin, both of them act as catalysts, which affect lipid peroxidation in the gastrointestinal tract. Meanwhile, the free radicals produced during the peroxidation reaction, oxidize vitamin E, β-carotene and vitamin C [73]. Once formed, ROOH may undergo reductive degradation which either diminishes or enhanced cytotoxic potential, depending on a variety of circumstances [77, 78]. The reaction occurring in the stomach plays a key role in the further research of reducing the toxic effect of oxidation. For instance, haem-proteins are known to decompose ROOH catalytically to various aldehydes, such as 4-hydroxy-2-nonenal (HNE), MDA, and other alkenals [79]. However, in the presence of high concentrations of polyphenol antioxidants, hydroperoxides are decomposed mostly to hydroxy compounds (ROH) and not to genotoxic and cytotoxic aldehydes [72, 80]. Acknowledged, these advanced lipid oxidation endproducts (ALEs) are cytotoxic and genotoxic compounds. Our gastrointestinal tract constantly expose to these ALEs, which affect cell membrane and other lipid-containing structures under condition of oxidative status [77].

1.3.3 Lipid oxidation and colorectal cancer

Reactive oxygen species, formed *in vivo* are powerful oxidizing agents, which are capable of damaging DNA and other biomolecules [81]. However, in order to protect the body tissue from the destructing by ROS, vital tissues are equipped with an intricate antioxidant defense system. Therefore the concept of Oxidative stress generates when there is a remarkable imbalance between the production of ROS and the removal ROS by antioxidants [82].

The intracolonic environment has abundant resources of ROS. They are from unsaturated lipids derived from dietary sources, the turnover of epithelial cells, or from synthesis by fecal microorganisms and/or epithelial cells [83, 84]. Furthermore, the conditions may also be suitable for producing ROS, especially near the surfaces of fecal masses, which contain the right amount of oxygen, the right temperature (30 C° to 60 C°), and superoxide
producing microorganisms, abundant residual iron form a high iron diet, bile pigments acting as Fenton-promotion iron chelators, organic compounds to form ROO’ etc. [84]. Intracolonic ROS formation might also explain the high incidence of cancer in colon and rectum, compared to other regions of the gastrointestinal tract [84].

When the colorectal cells expose to high concentrations of ROS, ROS may have a pro-cancer effect (promoting proliferation, invasiveness, angiogenesis, metastasis and suppressing apoptosis). [67]. It has been estimated that one human cell is exposed to approximately $1.5 \times 10^5$ oxidative hits a day from \( \cdot \)OH and other such reactive species [85]. The \( \cdot \)OH are known to react with all components of the DNA molecule and damage the structure of DNA [86]. The permanent modification of genetic material resulting from these “oxidative damage” represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, as is well established, in various cancer tissues free radical mediated DNA damage has occurred. Acknowledged, DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis [87, 88]. Moreover, oxidative damage has a result of chronic inflammatory states which has been well known, since the detrimental effects of ROS have been well established in the inflammation process [89]. The oxidative DNA damage may also use to explain why patients with ulcerative colitis are at increased risk for development of colorectal cancer.

1.4 **In vitro digestion**

1.4.1 Digestion models

*In vitro* digestion models are widely used to study the structural changes, digestibility/degradation, bio-accessibility, and sample stability under simulated gastrointestinal conditions [90], because it is more convenient, ethical superior, faster and less expensive than *in vivo* models [91]. The ideal *in vitro* digestion method would provide accurate results in a short time [92] and could thus serve as a tool for rapid screening foods ingredients or delivery systems with different compositions and structures [90]. However, in practice, any *in vitro* method inevitably fails to match the accuracy that can be achieved by *in vivo* study [92, 93]. This is because the results of *in vitro* digestion depend on many inherent factors, such as food composition, structure and amount [94].
The sample characteristics are the key factors that influence the *in vitro* digestion. Thus, *in vitro* digestion characteristics such as digestion time, enzyme contents or enzyme composition must be adjusted according to the food characteristics [90]. For example, if the concentration of the target substance (protein, lipid or carbohydrates) or the size of food particles is increased, the concentration of enzyme or digestion time should also be modified. For the incubation temperature, all the *in vitro* digestion models studied in the survey done by Hur et al. [90] was 37 °C, but variations in the amounts of enzyme employed were found.

Although a number of *in vitro* digestion models have been reported in the areas of food science, up to now few *in vitro* digestion models for meat have been carried out. In these *in vitro* digestion models for meat [95-99], the total incubation time is from 4 hours to 4.5 hours, the enzyme composition was varied with the research objectives. Majority of these *in vitro* digestion models did not take the large intestine into account, because the absorption of compounds mainly took place in the small intestine. More specifically, Brandon et al. [99] reported that only the bio-accessibility determined in the chyme of the small intestine was relevant for the risk assessment [99]. For lipid based sample, acknowledged, lipids cannot be fermented, thereby lipids are less influenced during passage the large intestine [90].

**1.4.2 Oxidation in *in vitro* digestion model: interaction with gastrointestinal juices**

During the processes of lipid oxidation in the gastrointestinal tract, the gastrointestinal juices might play important roles in the oxidation of food, as well. An early study reported that the gastrointestinal (GI) tract itself could be a major site of pro-oxidant and antioxidant actions [100]. Halliwell et al. [101] explained the reason that compounds that are only present in body fluids at µM levels may be present in the stomach and lumen of the intestines at much greater concentrations. Therefore, it is inevitable to take the GI environment into account, when we discuss the oxidation processes *in vitro* model. Kanner [102] proposed that lipid oxidation produced not only from the food itself, but may be also generated during digestion, especially in the gastric fluid, which contained absorbed oxygen and has a low pH. Thus, the concept of “the stomach act as a bioreactor” was elicited, which demonstrated that the stomach was a primary temporary location for food storage, and it enabled chemical and biochemical interactions between food constituents. In another words, the gastric fluid acts as a medium for further dietary lipid peroxidation and/or antioxidation [102].

Started with the primary step of digestive processes, *in vivo* condition, saliva is
stimulated as food is chewed. It moistens and lubricates the food debris into a coherent and slippery bolus that can be easily swallowed [103]. Thus, there is considerable amount of saliva mixed with the food and reached to the stomach. Saliva contained both pro-oxidation and anti-oxidation, especially, nitrite in some circumstance, increase the lipid peroxidation [104], and thiocyanate, peroxidase and uric acid reduce lipid oxidation [105].

Kanner et al. [105] reported that the pseudoperoxidase activity of lactoperoxidase was pH dependent, thus the low pH environment of stomach accelerated the decomposition of hydroperoxides by pseudoperoxidase, and also enhanced the lipid peroxidation process [102]. It also demonstrated that Lactoperoxidase acted similar to myoglobin, which decomposes lipid hydroperoxidase to free radicals e.g. LOO’, LO’ [105].

Gorelik et al. [105] also indicated that in the acidic condition, the antioxidant effect of the saliva specimen on lipid peroxidation of the heated muscle tissue correlated with the nitrite concentration of the saliva specimen. The higher the nitrite concentration of the saliva specimen is, the stronger the antioxidant effect of saliva specimen will be. [105]. In the stomach, all gastric juice mixed with food and drinks, therefore in the presence of reducing agents, such as ascorbic acid [106, 107]. The reduction of nitrite to nitric oxide (NO) is more favored than decompose NO’ and NO2’ [108]. Thus, Gorelik et al. [105] elucidated that the antioxidant effect of nitrite may be due to its conversion to NO, which decomposed oxygen activity species and modulate lipid oxidation [105].

The correlation between the antioxidant effect and the nitrite contents of saliva specimens was significant [105] and nitrate/nitrite in the diet was also originated from fruit and vegetables. Furthermore, studies found that the ascorbic acid and polyphenols in the fruit and vegetables can interact with iron haem and non-haem compounds from the red muscle in the stomach and increasd NO production [107, 109-110]. Therefore, high consumption of fruit and vegetables might increase the antioxidant effect, together with saliva in the stomach condition [106].

An early report [111] indicated that bile acids played an important role in the etiology of colon cancer, which was supported by epidemiological data, mutagenetic studies, signaling studies and investigations on the effect of bile salts in carcinogen treated rats [112]. One of the probable mechanisms was the secondary bile acids which were converted by primary bile acid by anaerobic bacteria in the large bowel, were colonic carcinogenesis [65]. The other probable
mechanism of bile acid was that it induced oxidation stress, which may also have an association with cancer [113-114]. One in vivo study [113] demonstrated that bile acid induced oxidative stress in gastrointestinal cancer, by causing DNA damage of one of the key components in the complex anti-oxidant defense system of eukaryotic cells. Another in vivo study [114] did the research to evaluate the induction of ROS by bile acids in low pH condition. This study found out that under the exposure of low pH and bile acids, there was an increase in oxidative DNA damage on oesophageal cell. Therefore, they elicited the hypothesis that chronic exposure to bile acids and low pH probably play a role in the progression to cancer [114]. However, to the best of my knowledge, no in vitro study investigating the association between bile acid and oxidation was found.

1.5 Objectives

The association between CRC and meat consumption is still a controversial issue. Based on the arguments in the literature review, we draw the conclusion that CRC might result partly from the digestion process of red meat and that involves the occurrence of oxidized lipids. Therefore, we aim to investigate the effect of different types of meat (fresh meat with different Fe and fat content) on oxidation processes during digestion. In addition, we also evaluate the role of antioxidants on the oxidation parameters. In order to get more insights into the oxidation processes during digestion, samples will evaluate from different incubation steps.
2. Material and methods

2.1 Characteristics of meat

**Dry matter, crude protein and crude fat content**

Dry matter, crude protein and crude fat content were analyzed on all meat samples according to the ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods, respectively. Analyses were carried out in duplicate and results are expressed as g/100g meat.

**Fatty acid composition**

The lipids from the different meat samples were extracted using chloroform/methanol (2/1; v/v) [115]. The fatty acids were methylated and analyzed by gas chromatography on a CP-Sil88 column for fatty acid methyl esters (100m × 0.25mm × 0.25mm), according to Raes et al. [116]. Peaks were identified based on their retention times, corresponding with standards. Nonadecanoic acid (C19:0) was used as an internal standard to quantify the individual and total fatty acid content. Analysis was performed in duplicate and the fatty acid profiles are expressed in mg fatty acid/100g meat.

**Total haem pigments**

The method of determine the haem content of meat was based on Homsey [117]. The total haem pigments were extracted from the meat samples using a mixture of aceton/H₂O/HCl 12M (40/2/1 w/w/w). During the whole procedure, the mixture was kept away from light to avoid oxidation of the haem pigments. After homogenisation, the mixture was left for one hour in dark to complete the extraction of the haem pigments. Subsequently, the mixture was filtered and the absorbance of the filtrate was measured at 640nm in a 1cm cuvet. The blank consisted of 80% aceton in water. The total haem content was calculated by multiplying the obtained absorbance value by 680 and expressed as mg hematin/kg meat sample. The analysis was done in duplicate.

2.2 The In vitro digestion of meat

In the *in vitro* digestion model, the digestion process of gastrointestinal tract in humans is executed in a simplified manner by simulating physiological conditions in humans,
such as the chemical composition of digestive fluids, pH, and residence time periods for each step. The model introduced by Versantvoort et al. [118] was used as a starting point. The model simulates the digestive processes (digestive process in mouth, stomach and small intestine) aerobically at 37 °C under fasted conditions [118]. However, in order to investigate the oxidation status during digestion, some changes in the procedure concerning the pro- and antioxidants present in the gastro-intestinal tract were made. For instance, iron, nitrite and ascorbic acid were additionally included in the digestive juices. All digestive juices were artificially prepared fresh every day. Especially, solutions used to prepare the digestive fluids, were prepared one day in advance (except for those containing enzymes) and stored overnight in the fridge. The constituents and concentration of digestive juices of the in vitro digestion model are shown in table 1.

**Tabel 1: Constituents and concentrations of the various synthetic juices of the in vitro digestion.**

<table>
<thead>
<tr>
<th>Constituents and concentrations</th>
<th>Saliva</th>
<th>Gastric Juice</th>
<th>Duodenal Juice</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic solution</td>
<td>0.2mM NaNO₂</td>
<td>0.04mM FeSO₄ · 7H₂O</td>
<td>Phosphate buffer, 100mM, pH 6.5</td>
<td>Phosphate buffer, 100mM, pH 6.5</td>
</tr>
<tr>
<td></td>
<td>1mM NH₄SCN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50mM NaHCO₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic solution</td>
<td>0.2mM uric acid</td>
<td>0.05mM ascorbic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05mM dehydroascorbic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2mM H₂O₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Add to mixture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic + inorganic solution</td>
<td>0.0025IU /ml lactoperoxidase</td>
<td>1% pepsin (g/100g protein)</td>
<td>25mM NaHCO₃</td>
<td>30g/L bile extract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% lipase (g/100 g lipid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
<td>2.0 (adjusted with HCl)</td>
<td>6.5</td>
<td>6.5</td>
</tr>
</tbody>
</table>

The schematic representation of the applied in vitro digestion model is presented in Figure 2. The digestion starts by adding 6ml saliva (pH 6.8) to 9g meat. The digesta was minced using a kitchen mixer for five minutes. After that, 12ml gastric juice (pH 2.0) is added,
and the digesta were shaken head-over-heels by hand. After incubation for one hour, the pH was adjusted to 2.0 using diluted HCl. Subsequently, the digesta were incubated for another hour. Finally, 12ml duodenal juice and 6ml bile were added simultaneously to the digesta, the pH was adjusted to 6.5 with diluted NaOH and the digesta were again shaken head-over-heels by hand. After that, the mixture was incubated for two hours. All the incubations were implemented in a shaking water bath of 37 °C. During the different incubation steps and at the end of the in vitro digestion process, the digesta were transferred into polyethylene flasks and stored in the freezer at -20°C until analysis.

Two experiments were conducted. For the first experiment, the effect of fat and haem content in the meat was investigated. Four different types of meat samples were used: beef lean (Belgian Blue young bull), beef fat (culled cow), chicken lean (chicken breast) and chicken fat (chicken thigh). The aim of the second experiment was to study the effect of antioxidants on the oxidative processes during the in vitro digestion. Ascorbic acid was used in four different concentrations: 0, 2.5, 5, and 10mg/ml. Exactly 1mL of the ascorbic acids solutions was added to 9g of meat at the same moment the saliva was added. These concentrations mimic the amounts of ascorbic acid present in respectively 0, 50, 100 or 200ml of orange juice taken during a meal with 90g of meat (taking into account that orange juice contains 50mg/100ml vitamin C) [119].
2.3 Lipid oxidation analysis

**MDA analysis**

This method of detecting (4,4'-Methylenedianiline) MDA in meat and digesta is based on Grotto et al. [120]. The reaction of MDA with thiobarbituric acid (TBA) forms thiobarbituric acid reactive substances (TBARS), which can be measured spectrophotometrically at 532nm. Furthermore, in order to improve the specificity of MDA, an alkaline hydrolysis step to release the bound MDA and a sample extraction step with n-butanol were added.

1,1,3,3-Tetramethoxy-propane (TMP) was used to prepare standard solutions. After water dilution, a 10000mM TMP stock solution was made. In order to prepare the TMP working solution, 100mM TMP solution was diluted in water, obtaining concentration from 0 to 30nmol/ml. Exact 450mg meat and digesta samples from all in vitro digestion steps (T0 to T5) were weighed into glass tubes. Then, 300μl NaOH (1.5M) was added to the digesta, meat sample or standard solution, each tube was vortexed and incubated at 60 °C in a water bath for 30 min. Subsequently, after cooling down the samples, 750μl of 6% H₃PO₄ and 750μl of 0.8% TBA were added. Then, the mixtures were vortexed and heated at 90°C in a warm water bath for 45 min. After that, the mixtures were cooled down again and 300μl of 10% SDS was added to the mixtures. The TBARS were extracted with 3ml of n-butanol, which was carried out by vortexing the mixtures for one minute and centrifuging them at 2000rpm for five minutes. Finally, the upper layer was taken off with a pasteur pipette and injected into the cuvet, and the absorbance was measured immediately with the spectrophotometer at 532nm. The analysis was done in duplicate.

**Hydroperoxides**

The method to determine lipid hydroperoxides is a spectrophotometric method, which was based on the procedure of Osrdal et al. [121]. Standard solutions and the Fe²⁺/thiocyanate solution were prepared before starting the analyses. In order to quantify lipid hydroperoxides, 88% cumene hydroperoxide was used to prepare standard solutions. After diluted by chloroform, standard solutions were obtained with concentration varying from 0 to 60μM. The preparation of Fe²⁺/thiocyanate solution consisted of three solutions. Solution I was prepared by two steps. Firstly, 0.4g BaCl₂·2H₂O in 25ml distilled water was mixed with 0.5g
FeSO₄·7H₂O in 50ml distilled water. Secondly, the mixed solution was filtered, and the filtrate was the solution I. The solution II was prepared by dissolving 3g NH₄SCN in 10ml distilled water. The solution III was prepared by mixing 50ml chloroform with 50ml methanol. The final Fe²⁺/thiocyanate solution was prepared by mixing 250µl solution I and 250µl solution II and diluted to 100ml with solution III.

2.5ml of sample and standard solution were mixed with 2.5ml methanol and vortexed. Then, 5ml chloroform was added to each tube and vortexed again for thirty seconds. Subsequently, all of samples and standard solutions were centrifuged for 10 minutes at 2500rmp. After that, the lower chloroform layer of each tube was transferred to another test tube by using a pasteur pipette. In the next step, 2ml of standard solution and chloroform extracted sample was transferred from each tube to a new test tube and mixed with 2ml of Fe²⁺/thiocyanate solution. The mixtures were vortexed and left for five minutes at the room temperature, and the absorbance was measured by spectrophotometer at 500nm. The analysis was done in duplicate.

2.4 Statistics analysis

Samples were done in duplicate and the incubation experiment was repeated three times. The statistical significance of differences among samples were tested with one-way ANOVA, Duncan test a at 5% level of significance, considering ‘incubation step’ and ‘meat type’ (experiment 1) or ‘vitamin C concentration’ (experiment 2) as independent values. For one-way ANOVA and Post Hoc test which was used to test the effect of meat types and incubation steps, only experimental units with more than one observations was considered to be tested. All the statistical analyses were carried out by SPSS 19.0 for windows.
3. Results

3.1 Characterization of meat

The results regarding the characterization of the meat samples are presented in Table 1. In experiment 1, no significant differences in the amounts of dry matter content were observed among the four types of meat, while there were significant differences in the amount of protein content, except for values between chicken lean and beef lean. The highest values were found in the beef lean and chicken lean samples, followed by beef fat and chicken fat.

There were significant differences in the lipid profile among the tested types of meat. The chicken fat had significantly the highest levels of total fat content, total saturated fatty acid, MUFA and PUFA including n-3 fatty acid and n-6 fatty acid, followed by beef fat, chicken lean and beef lean. Specifically, the amount of fat content and PUFA content in the chicken fat sample were respectively about 12- and 7-fold higher than that in beef lean sample. The beef fat sample had significantly higher levels of fatty acids and total fat content compared to the beef lean sample. Especially, the values of total fat content and PUFA in beef fat sample were respectively 7- and 1.5-fold higher than those in beef lean sample.

With regard to the haem content, there are significant differences between beef fat and beef lean, while for the chicken samples no significant differences were found between chicken lean and chicken fat. Results demonstrated that beef fat had the significantly highest level of haem, which was 8 and 15- fold higher than that of chicken fat and chicken lean respectively, and 3-fold higher than that of beef lean.

The results of the lipid oxidation products among four types of meat were dramatically different. The only detected value of hydroperoxides was in beef fat, while among other three types of meat, no hydroperoxides could be detected. In contrast, the TBARS values could be detected in all the tested types of meat. The beef fat sample had significantly the highest TBARS value followed by beef lean, chicken fat and chicken lean. No significant differences were observed between beef lean and beef fat, between chicken lean and chicken fat or between beef lean and chicken fat.
### Tabel 2: Dry matter, protein and lipid content (mean ± standard deviation; g/100g meat), haem iron content (mean ± standard deviation; mg hematin/kg meat), and lipid oxidation products (mean ± standard deviation; nmol/g meat) of the raw meat samples of experiment 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Chicken Lean</td>
<td>Chicken Fat</td>
<td>Beef Lean</td>
<td>Beef Fat</td>
<td>p value</td>
<td>Beef Fat</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Dry matter (n=2)</td>
<td>25.3±0.4</td>
<td>28.2±2.0</td>
<td>25.7±0.1</td>
<td>27.9±0.4</td>
<td>0.097</td>
<td>32.0±0.1</td>
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</tr>
<tr>
<td>Protein (n=2)</td>
<td>22.4±0.5a</td>
<td>17.5±0.7c</td>
<td>23.0±0.2a</td>
<td>20.4±1.1b</td>
<td>0.004</td>
<td>19.4±0.5</td>
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<tr>
<td>Fat (n=2)</td>
<td>1.12±0.18c</td>
<td>8.88±0.93c</td>
<td>0.722±0.296c</td>
<td>5.22±0.90b</td>
<td>0.001</td>
<td>11.8±0.0</td>
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<tr>
<td>SFA (n=2)</td>
<td>0.363±0.004c</td>
<td>3.01±0.03a</td>
<td>0.187±0.002d</td>
<td>2.31±0.01b</td>
<td>&lt;0.001</td>
<td>4.96±0.19</td>
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<tr>
<td>MUFA (n=2)</td>
<td>0.335±0.003c</td>
<td>3.18±0.03a</td>
<td>0.144±0.002d</td>
<td>2.37±0.04b</td>
<td>&lt;0.001</td>
<td>4.44±0.19</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUFA (n=2)</td>
<td>0.253±0.000b</td>
<td>1.39±0.01a</td>
<td>0.195±0.004c</td>
<td>0.268±0.008b</td>
<td>&lt;0.001</td>
<td>0.265±0.009</td>
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</tr>
<tr>
<td>n-3 (n=2)</td>
<td>0.029±0.001c</td>
<td>0.126±0.001a</td>
<td>0.018±0.000d</td>
<td>0.077±0.000b</td>
<td>&lt;0.001</td>
<td>0.066±0.001</td>
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<td></td>
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</tr>
<tr>
<td>n-6 (n=2)</td>
<td>0.225±0.001b</td>
<td>1.26±0.01a</td>
<td>0.177±0.004c</td>
<td>0.191±0.008c</td>
<td>&lt;0.001</td>
<td>0.199±0.008</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Haem iron (n=2)</td>
<td>21.1±2.9c</td>
<td>38.8±1.0c</td>
<td>97.6±10.1b</td>
<td>324.0±21.6a</td>
<td>&lt;0.001</td>
<td>291.0±7.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroperoxides (n=1)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.15</td>
<td>-</td>
<td>9.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS (n=3)</td>
<td>9.13±2.73c</td>
<td>16.1±3.1bc</td>
<td>24.0±4.3ab</td>
<td>30.1±9.5a</td>
<td>0.009</td>
<td>22.8±0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d., not detected

For experiment 1, different letters (a–d) in the same row express significant differences among meat types (P <0.05).

### 3.2 Experiment 1

The results of the hydroperoxides in the four types of meat during five incubation steps of simulated gastrointestinal processes are summarized in Table 2. Within each incubation step, significant differences among types of meat were only found at the end point of simulated stomach (T3). The beef fat sample had significantly the highest values of hydroperoxides, followed by chicken fat, beef lean and chicken lean. No significant differences were observed between beef lean and chicken lean, or between chicken fat and beef lean. On the contrary, no significant differences were found among the different meat types for incubation steps T1 and T2. Incubation step T4 and T5 were lacking data to do the statistical test.
Further, within each meat type, significant differences among the incubation steps were observed in the chicken fat sample, which demonstrated that after incubation for two hours in mimicked saliva and gastric juice (T3) the value of hydroperoxides reach to the highest point, while no significant differences were found between T1 and T2. Also for beef fat a significantly higher hydroperoxide value was found at T3 compared to T2. But no significant differences among the incubation steps were found for the other types of meat.

Table 3: Hydroperoxides values (mean ± standard deviation; nmol/g meat) detected in chicken lean, chicken fat, beef lean, and beef fat during the different incubation steps (n=3).

<table>
<thead>
<tr>
<th></th>
<th>Chicken Lean</th>
<th>Chicken Fat</th>
<th>Beef Lean</th>
<th>Beef Fat</th>
<th>P (meat type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (meat+saliva)</td>
<td>1.98*</td>
<td>1.83±0.49</td>
<td>2.52±1.49</td>
<td>6.12*</td>
<td>0.597</td>
</tr>
<tr>
<td>T2 (T1+gastric juice)</td>
<td>22.7±9.0</td>
<td>13.3±5.6B</td>
<td>24.4±5.2</td>
<td>7.99±6.03B</td>
<td>0.128</td>
</tr>
<tr>
<td>T3 (T2 after 2h-incubation)</td>
<td>10.1±9.1c</td>
<td>41.9±15.2b,A</td>
<td>24.1±13.7c</td>
<td>95.1±0.6A</td>
<td>0.001</td>
</tr>
<tr>
<td>T4 (T3+duodenal juice&amp;bile)</td>
<td>n.d.</td>
<td>0.446*</td>
<td>n.d.</td>
<td>4.02*</td>
<td>-</td>
</tr>
<tr>
<td>T5 (T4 after 2h-incubation)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>P (incubation step)</td>
<td>0.163</td>
<td>0.016</td>
<td>0.129</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

n.d., not detected
a-c effect of type of meat: values with a different letter within a row of the same incubation step are significantly different (P < 0.05).
A-B effect of incubation step: values with a different letter within a column of the same type of meat are significantly different (P < 0.05).
* Value excluded from the statistical analysis, as only one detectable value was measured during the three repetitions.

Similar to the hydroperoxide values, the results of TBARS values for experiment 1 are presented in Table 3. Within each incubation step, significant differences among the meat types were found. After adding saliva to the meat (T1), significantly higher TBARS values were observed for both beef meat samples compared to both chicken samples, but no significant differences were found between beef fat and beef lean or between chicken fat and chicken lean. After adding gastric juice (T2), beef fat still had significantly higher values compared to both chicken samples, but no significant differences were found between beef fat and beef lean, or between beef lean and both chicken meat samples. Through the two-hour incubation in saliva and gastric juice (T3), a significantly lower TBARS value was found in chicken lean, which was 2.5- to 3-fold lower than the other meat samples, while no significant differences were found among the other types of meat. At the beginning of adding bile and duodenal juice to the digesta (T4), significant differences were found between chicken lean sample and both beef meat samples, with approximate 2-fold higher TBARS values in the both beef samples, compared with that in chicken lean sample. No significant
difference was observed between chicken lean and chicken fat or between chicken fat and both two types of beef meat. After finishing the two-hour incubation in simulated gastrointestinal juice (T5), the differences between the meat types were similar as in T4.

Within each meat types, significant differences among the different incubation steps were found. With regard to chicken lean, significantly lower TBARS values were found at T1 compared to the other incubation steps, except for T3, and no significant differences between T2, T3, T4 and T5 were found. During the incubation, the TBARS values of chicken lean increased more than 3-fold from T1 and T5. For chicken fat, significantly lower TBARS values were also found at T1 compared to the other steps, while no significant differences were found between T3, T4, and T5. The TBARS value at T2 was 2-fold higher than T1 and about 1.5-fold lower than T3 and T4 and no significant differences were found between T2 and T5. During the incubation, the TBARS values firstly increase approximately 3-fold from T1 to T4, then decrease slightly, but not significantly. With respect to beef lean, no significant differences were observed between T1 and T2, or between T2, T3, T4 and T5. There was a 2-fold increase in TBARS values starting from the significant lowest incubation step T1 and ending in T4 and after that the TBARS values decreased slightly again, but not significantly, at T5. Regarding to beef fat, significantly lower TBARS values were found in T1, and no significant differences were observed between T2, T3, T4 and T5. During the incubation, the change in TBARS values was similar to chicken fat and beef lean, which increased more than 2.5-fold from T1 to T4, then decreased moderately, although not significantly, at T5.

Tabel 4: TBARS values (mean ± standard deviation; nmol MDA/g meat) detected in chicken lean, chicken fat, beef lean, and beef fat during the different incubation steps (n=3).

<table>
<thead>
<tr>
<th></th>
<th>Chicken Lean</th>
<th>Chicken Fat</th>
<th>Beef Lean</th>
<th>Beef Fat</th>
<th>P (meat type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (meat+saliva)</td>
<td>14.8±4.9(^b), B</td>
<td>24.7±4.1(^b), C</td>
<td>46.8±11.0(^b)</td>
<td>58.2±4.9(^a), B</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>T2 (T1+gastric juice)</td>
<td>47.7±21.6(^b), A</td>
<td>50.5±13.3(^b), B</td>
<td>72.6±3.8(^ab), AB</td>
<td>92.9±20.9(^a), A</td>
<td>0.032</td>
</tr>
<tr>
<td>T3 (T2 after 2h-incubation)</td>
<td>31.1±3.7(^b), AB</td>
<td>75.7±10.5(^a), A</td>
<td>90.9±9.4(^a), A</td>
<td>86.8±11.28(^a), A</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T4 (T3+duodenal juice&amp;bile)</td>
<td>47.9±11.1(^b), A</td>
<td>73.1±9.9(^ab), A</td>
<td>95.4±22.2(^a), A</td>
<td>96.4±15.5(^a), A</td>
<td>0.015</td>
</tr>
<tr>
<td>T5 (T4 after 2h-incubation)</td>
<td>48.3±9.6(^b), A</td>
<td>66.2±5.9(^ab), AB</td>
<td>88.4±22.0(^a), A</td>
<td>89.3±11.0(^a), A</td>
<td>0.016</td>
</tr>
<tr>
<td>P (incubation step)</td>
<td>0.022</td>
<td>&lt; 0.001</td>
<td>0.019</td>
<td>0.043</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a-b}\) effect of meat type: values with a different letter within a raw of the same incubation step are significantly different (P < 0.05).

\(^{A-C}\) effect of incubation step: values with a different letters within a column of the same type of meat are significantly different (P < 0.05).
3.3 Experiment 2

The effects of different concentrations of vitamin C on the hydroperoxides values of the beef sample during different incubation steps are presented in Table 4. Within each incubation step, no effect of vitamin C on hydroperoxides values was found, based on the absence of a significant difference between each concentration of Vitamin C and the control treatment.

Within each Vitamin C concentration group, significant differences were only found in the group with 2.5 mg/9 g meat vitamin C. Significantly lower hydroperoxides value were found at T4, compared to T2 and T3 and no significant differences were found between T1, T2 and T3. During the incubation steps, the hydroperoxides values decreased approximately 2-fold from T1 to T4. The highest hydroperoxide value was found at T3. A similar amount of hydroperoxides was measured at T5 compared to T4, but as only one detectable value was found, no further statistics were done. In the other vitamin C concentration groups, incubation steps did not affect the hydroperoxides values, since no significant difference was observed among each incubation steps.

Table 5: The Hydroperoxides values (mean ± standard deviation; nmol/g meat) detected in beef meat with different vitamin C concentrations (mg/9 g meat) during the different incubation steps (n=3).

<table>
<thead>
<tr>
<th>Vitamin C concentration (mg/9 g meat)</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>P (samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (meat+saliva)</td>
<td>6.66±1.56</td>
<td>6.35±3.46</td>
<td>3.95±0.59</td>
<td>4.07±1.76</td>
<td>0.310</td>
</tr>
<tr>
<td>T2 (T1+gastric juice)</td>
<td>10.9±9.0</td>
<td>10.8±5.6</td>
<td>16.5±6.1</td>
<td>4.84±1.14</td>
<td>0.224</td>
</tr>
<tr>
<td>T3 (T2 after 2h-incubation)</td>
<td>16.2±2.6</td>
<td>12.4±3.2</td>
<td>8.51±8.03</td>
<td>9.92±6.03</td>
<td>0.388</td>
</tr>
<tr>
<td>T4 (T3+duodenal juice&amp;bile)</td>
<td>10.4±4.1</td>
<td>2.90±0.51</td>
<td>5.27±1.95</td>
<td>6.84±4.38</td>
<td>0.095</td>
</tr>
<tr>
<td>T5 (T4 after 2h-incubation)</td>
<td>n.d.</td>
<td>2.42*</td>
<td>n.d.</td>
<td>5.93±0.09</td>
<td>0.021</td>
</tr>
<tr>
<td>P (incubation step)</td>
<td>0.243</td>
<td>0.047</td>
<td>0.066</td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

n.d., not detect

A-B effect of incubation step: values with a different letter in a column of the same treatment of Vitamin C concentration are significantly different (P < 0.05)

*Value excluded from the statical analysis, as only one detectable value was measured during the three repetitions.

Similar to the hydroperoxides values, the results of TBARS values for experiment 2 are presented in Table 5. Within each incubation step, the results were similar to those of the hydroperoxides: no effect of vitamin C on TBARS values was observed, since no significant
differences were found between each vitamin C concentration and the control group without vitamin C.

However, within each vitamin C group, significant differences between the incubation steps were found in the control group and two vitamin C treatments with 2.5 and 10 mg/9 g meat. For the control group, significant lower TBARS values were found in T1 and T3 compared to T4 and T5 and no significant differences were found between T1, T2 and T3, or between T2, T4 and T5. During the incubation, the TBARS values increased more than 3-fold from T1 to T5. With respect to the concentration of 2.5mg/9g vitamin C, the lowest TBARS value was found at T1, which was significantly different from the other incubations steps except for T3. No significant differences were found between T2 and T3, or between T2, T4 and T5. After the incubation, the TBARS values increased about 3-fold from T1 to T5. Regarding to the concentration of 10 mg/9 g meat vitamin C, significant lower TBARS values were observed at T1 and T3 compared to all other incubation steps, while no significant differences were observed between T2, T4 and T5. During the incubation processes, the TBARS values rised about 4-fold from T1 to T5. In the 5 mg/9 g meat vitamin C treatment group, the different incubation steps did not influence the TBARS values, as no significant difference was found between each incubation step.

**Table 6: TBARS values (mean ± standard deviation; nmol MDA/g meat) detected in beef meat with different vitamin C concentrations (mg/9 g meat) during different incubation steps (n=3).**

<table>
<thead>
<tr>
<th>Vitamin C concentration</th>
<th>0</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>P (samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (meat+saliva)</td>
<td>28.2±4.3H</td>
<td>29.1±8.2A</td>
<td>46.5±20.4</td>
<td>26.6±3.8H</td>
<td>0.190</td>
</tr>
<tr>
<td>T2 (T1+gastric juice)</td>
<td>57.8±6.1AB</td>
<td>75.7±33.3AB</td>
<td>96.1±5.4</td>
<td>115±55.8A</td>
<td>0.240</td>
</tr>
<tr>
<td>T3 (T2 after 2h-incubation)</td>
<td>51.8±2.9B</td>
<td>48.0±12.0BC</td>
<td>71.9±31.3</td>
<td>41.1±8.6B</td>
<td>0.234</td>
</tr>
<tr>
<td>T4 (T3+duodenal juice&amp;bile)</td>
<td>92.9±31.4A</td>
<td>104.0±6.3A</td>
<td>76.3±21.2</td>
<td>113±15.7A</td>
<td>0.240</td>
</tr>
<tr>
<td>T5 (T4 after 2h-incubation)</td>
<td>92.6±32.9A</td>
<td>87.3±11.6A</td>
<td>86.1±3.5</td>
<td>106.0±25.9A</td>
<td>0.688</td>
</tr>
<tr>
<td>P (incubation step)</td>
<td>0.013</td>
<td>0.002</td>
<td>0.087</td>
<td>0.007</td>
<td></td>
</tr>
</tbody>
</table>

A-C effect of incubation step: values with a different letter in a column of the same treatment of Vitamin C concentration are significantly different (P < 0.05).
4. Discussion

The accumulation of lipid peroxidation products in the body is known to present risks to human health [52]. In the present study, in order to determine the relationship between colorectal cancer and meat oxidation, we evaluated the lipid oxidation processes from raw meat during different digestion steps by using *in vitro* digestion models. Moreover, for seeking the primary prevention of colorectal cancer by dietary modification, we evaluated the putative beneficial role of vitamin C in preventing lipid oxidation in raw beef meat by using *in vitro* digestion models.

4.1 In vitro digestion models

*In vitro* digestion models are widely used to study the structural changes, digestibility, and release of food components. Compared with previous studies [95-99], the parameters of our *in vitro* digestion model reached agreement with these literatures, for instance, the incubation temperature, the pH and the incubation time. In the present study, we designed the simulated digestion model by using the commercial digestive enzymes of porcine or bovine origin, which reach the agreement with many previous studies [122-126].

However, a recent review of *in vitro* digestion models by Hur et al. [94] demonstrated the vast variability of digestive fluids used and emphasized the importance of using physiological relevant levels of enzymes and other minor components. Ullebergy et al. [127] also reported that human digestive juices contain enzymes and cofactors in a physiological combination and may therefore represent an advantage over artificial solutions composed of purified commercial enzymes. Their study evaluated the enzymatic activities and stability of gastric and duodenal juices aspirated form 20 fasting volunteers [127]. Among all the results drawn from that study, we paid attention to the enzymatic activity of two enzymes that are related to our study: lipase and bile acid. The study reported that the enzymatic activity of lipase was 951.0 U/ml and that of bile acids was 4.5 mM. In our design of *in vitro* model, the enzymatic activity of lipase was 250 U/mg protein (measured by olive oil 30 min), but as the measuring method and conditions were quite different, no comparisons can be elicited between present study and the study from Ulleberg et al. [127]. Regarding the composition of bile in our trail, the commercial bile salts we used was from the porcine. The product contains glycine and taurine conjugates of hydeoxycholic acid and other bile salts, but the proportion
of each component is not mentioned, because it is an extract. Therefore, no comparison could be made. Also in order to make a correct comparison, the physiological differences between animal and human should be taken into account.

Our study is designed to investigate lipid oxidation during digestion and several pro- and antioxidants were added (e.g. H$_2$O$_2$, uric acid and lactoperoxides etc.), supplementary to what is used in Versantsvoort et al. [99], in order to mimic the oxidative/antioxidative status of the digestive fluids. In addition, to make sure all the lipid in the meat sample was totally digested, adequate lipase and bile acid should be added [94]. Furthermore, in our study, comparing the original status of the meat and the status of the digesta after incubation, the TBARS values of both two types of beef meat increased 3- to 3.7-fold. This increase was similar to the results described by Hur et al. [128], which showed that the TBARS value of the beef patty sample increased approximate 4-fold compared with those two statuses. Based on these findings, we speculate that our in vitro digestion model is suitable for our study. After all, all physiological mechanisms operated in vivo cannot be transposed to any in vitro conditions.

4.2 Characterization of meat

According to the results presented by Table 1, the conclusion can be drawn that the susceptibility to lipid oxidation was meat type dependent. Tang et al. [129] drew the conclusion that the susceptibility to lipid oxidation for different meat species of untreated cooked patties was closely related to fat levels, unsaturated fatty acid and iron content. This conclusion indicates that the higher of fat, polyunsaturated fatty acid and iron contents each type of meat contains, the higher the level of lipid oxidation products will be found. Acknowledged, fatty acid composition of muscle affects the oxidation stability, especially those with several double bonds (polyunsaturated fatty acids). Previous literature [36] demonstrated that haem acts as a catalyst in the process of lipid oxidation. Haem may exhibit the Fenton reaction [76] and produce $\cdot$OH, which contributes to the propagation phase of lipid oxidation. However, to what extent lipid oxidation potential differs among different types of raw meat, maybe due to qualitative and quantitative differences in haem or fatty acids, has not been established [130].

Although, recently nutritionists have paid lots of attention to the PUFA (mainly n-3 fatty acids and n-6 fatty acids) for their health aspects of preventing coronary disease and
cancers, especially n-3 fatty acids [131], meat quality studies [132] reported that the more PUFA the meat contains, the more lipid oxidation occurred in the meat. This argument indicates that as PUFA is easy to be oxidized, the more PUFA content the meat contains, the more hydroperoxides will be presented in the raw meat. In our experiment, within each type of meat, the PUFA content, especially the n-3 fatty acids, was quite low. Further, the chicken meat sample had the highest PUFA content and n-3 fatty acid content, while the TBARS values of chicken meat sample were not the highest among the four types of meat. Thus we can draw the conclusion that in our experiment, the PUFA content might not play a major role in the meat oxidation, but that other factors, such as fat content and haem, are possibly more important.

Acknowledged, during the meat oxidation processes, haem acting as a catalyst plays an important role in lipid oxidation [3]. For our study, comparing the results of chicken fat and beef lean samples, the amount of fat content for chicken fat sample was 12-fold higher than that for beef lean sample, and the amount of haem was 2.5-fold lower. However, the TBARS value of beef lean sample was on average 1.5-fold higher than that of chicken fat sample, showing that haem content probably influenced the oxidation processes more than the fat content. Still, the beef fat sample that had a relatively high fat content and the highest haem level among all meat types, obtained significantly the highest TBARS values among four meat types, indicating that both fat and haem content are of importance. Further, according to our results, the order of oxidation susceptibility can be beef sample > chicken sample, evidenced by the level of TBARS values after digestion (Table 1), which agreed with the Rhee et al. [130] which reported that oxidative stability of meat samples in retail decrease in the order beef > pork > chicken. However, Wilson et al. [133] reported that the meat susceptibility to lipid oxidation decreased in the order chicken > beef > pork. This may attribute to two reasons. On one hand, the pre- and post-slaughter condition differences in experiment variables between studies. On the other hand, polyunsaturated fatty acid, haem content and anatomical location of muscles or tissue site were probably different between different meat samples [131, 134].

Based on the above arguments, we thus speculate that the raw red meats should be more susceptible to lipid oxidation than raw chicken due to main differences in haem content. Rhee et al. [130] reported similar speculation which demonstrated that haem content may determine lipid oxidation potential of raw meats. Thus, this point of view might partly relates to the study of Corpet [3] which made the hypothesis that haem-iron would be a major player
in cancer promotion, explaining why red meat, but not white meat is associated with cancer risk.

Furthermore, the results presented that the beef fat sample that had the relative high PUFA content and haem content obtained the highest TBARS values in original raw meat and at every incubation step of the *in vitro* digestion model. The result supported the hypothesis that simultaneous intake of fatty acids and haem might cause generation of lipid peroxyl radicals (ROO’) in the intestinal tract [40]. Red meat usually contains a large amount of haem, primarily in haemoglobin, and myoglobin, together with fat. Therefore, it may indicate that the more consumption of red meat, the more lipid peroxyl radicals (‘OH) will be in the intestinal tract and promote colonic carcinogenesis, which agreed with many cohort epidemiology researches [28-30] and experimental data that suggested that diets high in fat and red meat are risk factors, contributing to the development of several kinds of cancer and atherogenesis [52]. Regarding to the results of two types of beef sample, both the fat content and haem content in beef fat were higher than that in beef lean. We expected that beef fat would have the higher levels of TBARS values than beef lean, but it is not the case. There were no significant differences in susceptibility to lipid oxidation, evidenced by the TBARS values of both samples at the original status and at every incubation steps. Therefore we hypothesize that other factors also influenced the lipid oxidation in this case, such as for instance the difference of antioxidant content between two types of beef meat, due to varying feed composition.

4.3 Experiment 1

Irrespective of the types of meat, high hydroperoxides values were found after incubation for 2 hours in the simulated gastric fluid, with some variations. For instance, the values at the incubation step of simulated stomach condition, the amounts of hydroperoxides of beef fat sample increased more than 15-fold, compared with that at the incubation step of the saliva digestion condition. The probable reasons of this phenomenon might attribute to two aspects. Firstly, according to the studies of Riley et al. [135] and Sheard et al. [136], minced and comminuted meat products developed higher levels of oxidation because of the facilitated interactions of free fatty acids and oxygen in the presences of pro-oxidants such as iron that are released from muscle cells. As at the onset of our trail, we used a mixer to mimic the mechanism of chewing processes, thus after the mimicked chewing processes, our meat
samples might behave the same way as minced and comminuted meat products and more iron might be released from muscle cells, which may partly contribute to the increase of hydroperoxides at later incubation steps. Secondly, previous researches elucidated the possibility that human stomach fluid has low pH [137] and dissolved oxygen following a meal is a suitable medium for further lipid peroxidation [102]. Kanner et al. [102] did the research on ground turkey muscle, which showed that the incubation of heated ground red turkey muscle in simulated gastric fluid for 2 h increased the lipid peroxidation level by about 4.7-fold, compared to the values at the onset of incubation. This research also explained that the acidic pH of the gastric fluid significantly amplified the peroxidation catalyzed by dietary endogenous catalysts, found in muscle tissue such as myoglobin and “free” iron molecules which reach the agreement with other two studies, who found that the consumption of hydroperoxides and hydrogen peroxide catalyzed by myoglobin was strongly pH dependent [138, 139]. Kanner et al. [102] also reported that human gastric fluid may be an excellent medium for enhancing the oxidation of lipids and other dietary constituents, which enhanced hydroperoxides accumulation of heated red turkey muscle by 6- fold after 3h-incubation. However in our trail, during the 2h-incubation in simulated stomach condition, the level of lipid hydroperoxides increased from 5 fold up to 23-fold. The variation might be due to the design of the trail, as H$_2$O$_2$ was added to the gastric juice and the different meat types used. In addition, large standard deviations were found for present results. The method we used to analyze the hydroperoxides value is a new method adapted from a protocol to analyze hydroperoxides in milk, but further research is needed to optimize the method.

Based on the above arguments, the substantial increase of hydroperoxides values in the stomach, in present study could attribute to the interaction results of increased release of iron because of the simulated chewing processes and the low pH value in simulated stomach condition. However, haem or iron acts as catalyst only in systems containing performed hydrogen peroxide or lipid hydroperoxides [140]. Therefore, once the concentrations of the hydroperoxides drop to zero, the haem catalyst system remain stable, which means that the system reaches a balance, and the propagation phase of lipid oxidation stops. This might explain when hydroperoxides values drop to zero, in our trail, no further hydroperoxides was generated. Regarding to the reason why the hydroperoxides value decreased notably at the last two steps of in vitro digestion model, there was an early study done by Gray et al. [141] which showed that the primary products rapidly decomposed to stable secondary products [141].
Therefore, when measuring the lipid oxidation in meat and meat products, it is more appropriate to measure the secondary products as an index of lipid oxidation [141].

Regarding the effect of gastrointestinal juices on lipid oxidation, gastrointestinal juice played various roles in lipid peroxidation processes. Moore et al. [142] reported that there was a significant function of saliva, as saliva can act as a redox system. The saliva specimen contains various molecules that have antioxidant capacity such as uric acid and peroxidase enzymes, but other molecules such as nitrite can act either as an anti-oxidant or pro-oxidant depending on the circumstance[104]. This study reported that uric acid in saliva, contributed 70% of the total salivary antioxidant capacity [142]. In addition, Terao et al. [143] demonstrated that human saliva has antioxidant effects on lipid peroxidation of liposomal phospholipids and fish meat, and that it can reduce fatty acid hydroperoxides. However, according to the data of our study, the saliva exhibited a pro-oxidant role. The result showed that after adding the saliva, the TBARS values of all the meat types increased, from 1.5 to 2-fold. As various compounds with either anti- or pro-oxidant capacity were present in the saliva and meat mixture, it could be possible that in this specific case, more pro-oxidants were active.

Reeder et al. [138] demonstrated that the lactoperoxidase in the saliva initiated lipid peroxidation of linoleic acid in the presence of halides at acidic condition. This is also supported by another study which found that the pseudoperoxidase activity of lactoperoxidase was pH dependent [105]. Further, the study demonstrated that when the pH of simulated saliva is 7.0 the peroxidation effect was very weak, but lowering the pH of simulated saliva to 3.0 accelerated the decomposition of hydroperoxides and enhanced the lipid peroxides by pseudoperoxidase, and also enhanced the lipid peroxidation process [105], which indicated that at stomach condition, lactoperoxidase acted as the role of pro-oxidant. This is the case of our study, with higher hydroperoxides in the stomach compared to the mouth. Gorelik et al. [105] elucidated that in a system of simulated gastric juice containing 1.25% human saliva for 2 hours, the LOOH values of heated ground turkey muscle tissue increased 2.5-fold and the TBARS values increased 2.7-fold, compared to the onset of the experiment. In our experiment, the concentration of saliva was 22.2% (v/v), the LOOH values of both two types of beef meat increased 9.5- to 15.5-fold and the TBARS values of them increased 3- to 3.7- fold after 2-incubation in the acidic condition. Although the LOOH values increased much more compared with previous study, the increase in TBARS values in the beef meat was quite close.
to the previous study. However, Gorelik et al. [105] did not mention how they calculated the concentration of the saliva and the molarity weight of saliva, thus it is difficult to compare our saliva concentration with theirs. Furthermore, Gorelik et al. [105] also hypothesized that lactoperoxidase, being a haem protein, acted similar to myoglobin, which can perform oxidation catalytically, non-enzymatically and decomposes lipid hydroperoxides to free radicals [144, 145]. Moreover, Gorelik et al. [105] reported that in stomach condition, the effect of a saliva specimen on lipid peroxidation was correlated with the concentration of nitrite in the specimen, but not with that of other saliva components. As our result supported more the pro-oxidation role of saliva than the anti-oxidation role, we doubt whether the concentration of lactoperoxidases in our trial is over-added. Compared with the study of Gorelik et al. [105], the measuring methods of lactoperoxidase activity were different, and the conditions of the two trails were different, thus no comparison can be elicited.

For the effect of duodenal juice and bile acid, in our simulated duodenal juice, commercial pancreatin was the major functioning component in our model. However, I could not find any study related to the pancreatin and lipid oxidation in intestinal condition. Whereas, some in vivo studies [146, 147] reported that bile acid mimic the oxidative stress and hydrophobic bile acid mediates oxidative stress in gastrointestinal cancer cells, especially in low pH condition. These findings might explain the slight increase of TBARS values after adding simulated duodenal juice and bile acid. Then, after 2h-incubation in the simulated gastrointestinal juice, the TBARS values also experienced a slight decrease. This phenomenon might happen when MDA react with other molecules in the in vitro digestion model.

In conclusion, the low pH and aerobic situation in the stomach and the pro-oxidation role of saliva contributed to the remarkable increase of hydroperoxides values and TBARS values after 2h-incubation in the simulated stomach condition. Therefore Kanner et al. [102] stated that the stomach acts as a “bioreactor” and the gastric fluid as a medium for further dietary lipid peroxidation and/or antioxidation. In addition, lipid oxidation products that are formed in the stomach could be harmful during the further digestion tract. Therefore, preventing oxidation in the stomach could have beneficial effects, as well as in other stages of digestion.
4.4 Experiment 2

It is well known that vitamin C is an important dietary antioxidant, which has the capacity to decrease the adverse effects of reactive species such as ROS and nitrogen reactive species (NOS) that can cause oxidative damage to macromolecules such as lipid, DNA and protein [148]. Dietary recommendations for healthy eating include the consumption of fruit juice [149] whose beneficial health effects are ascribed partly to vitamin C. There are several researches on the plant-derived antioxidant effect on meat oxidation using the in vitro digestion model. However, most of these researches focused on the tea catechins and polyphenols [150-152]. Whether the ascorbic acid has an antioxidant effect during the in vitro digestion of meat has been little studied. In our study, we simulated people having beef meat and orange juice in one meal, and evaluated the effect of vitamin C on lipid oxidation. We calculated 2.5, 5 and 10 mg ascorbic acid per 9 gram meat, which equals to 0.0015, 0.003 and 0.006 mmol per gram meat. The amount of antioxidant (ascorbic acid) we added compared well with Gorelik et al. [153] who added 0.003 mmol polyphenols per gram of meat. Although gastric juice also contained ascorbic acid (AA) and dehydroascorbic acid (DHAA), there won’t be a problem of an interfering factor. In our study, the concentration of AA+DHAA in the gastric juice was 0.0012 mmol per 9 gram of meat, while the minimum concentration of AA (2.5 mg AA) we added was 0.014 nmol AA per 9 gram of meat, which was more than 10-times higher than that in gastric juice. Therefore, the AA and DHAA in the gastric juice most probably did not interfere with the added ascorbic acid amount.

Both the hydroperoxides and TBARS values showed a similar behavior during the different incubation steps compared to experiment 1. According to our results, both hydroperoxides values and TBARS values demonstrated that there was no significant effect of vitamin C with the tested concentrations on either the antioxidant effect or pro-oxidant effect during the in vitro digestion model of beef meat. Regarding to the results, we will discuss two aspects as follow:

Firstly, for the experimental design, we simulated that people drink 50, 100, 200 ml orange juice (containing 25, 50 and 100 mg ascorbic acid respectively) with 90 gram meat, but in reality people usually drink more than 200 ml orange juice during one meal. Therefore, it is possible that a higher vitamin C concentration might exhibit an antioxidant effect on the
digestion process in beef meat. Consequently, we still have to optimize our experiment design in the future.

Secondly, ascorbic acid can act both as pro-oxidant or antioxidant [154]. Halliwell et al. [149] reported that vitamin C is an important dietary antioxidant, which significantly decreased the adverse effect of reactive species such as ROS and NOS. In addition, ascorbic acid can regenerate other antioxidants such as β-carotene, α-tocopheroxy radical cation from their radical species, which acts as a role of co-antioxidant. However, in vivo studies of oxidative DNA damage [155] reported that vitamin C acts as an antioxidant unless added or endogenous metal iron was present. Acknowledged, the pro-oxidant role of vitamin C is based on the fenton reaction. Therefore, the pro-oxidant role of vitamin C has an association with iron.

Moreover, a report demonstrated that in vivo large doses of exogenous iron and ascorbic acid (the ratio (iron mg: ascorbic acid mg): 2.7) promote the release of iron from iron binding proteins and also enhanced in vitro lipid peroxidation in serum of guinea pigs [156]. This finding supported the hypothesis that high intake of iron along with ascorbic acid could increase in vivo lipid peroxidation of LDL [157]. Compared to the iron/ascorbic acid ratio from the study Chen et al. [43], the haemhaem content in our experiment was approximate 2.62 mg/9 g meat, and the ascorbic acid was 2.5-10 mg/9 g meat. Thus, the ratio would be 0.26-1, which was less than the ratio (2.7) in previous study. In contrast, another study [158] demonstrated that in iron overloaded plasma, ascorbic acid acted as an antioxidant which prevented oxidative damage to lipids in vivo. However, it is still difficult to determine the effect on vitamin C on lipid oxidation.

In addition, some studies that specifically addressed the interaction of vitamin C with iron in physiological fluid and in vivo found either no effect of vitamin C or decreased oxidative damage [155, 159-160]. Furthermore, there was a human consumption studies demonstrated that giving vitamin C supplement either 60 mg/day or 6000 mg/day had no effect on oxidation DNA damage [161]. The last two conclusions in the literature agreed with our results.

Considering the results of previous mentioned studies and the hypothesis of the stomach acting as a bioreactor, we may conclude that the reason why no dose dependent effect of vitamin C on the beef meat oxidation in vitro model was found is that the final effect was
depend on the cross-effect of pro-oxidant and antioxidant roles of vitamin C together with the pro-oxidant and antioxidant of gastrointestinal juices in the simulated gastrointestinal condition.
5. Conclusion

The effect of meat consumption on cancer risk is a controversial issue. However, many recent epidemiological studies proved that there was a link between red meat and increased risk of colorectal cancer. That is why experimental studies are still needed to confirm this link. Acknowledged, colorectal carcinogenesis is associated with serious oxidative stress and ROS which are induced by oxidative stress can induce carcinogenesis via DNA injury. Therefore, our study was designed to evaluate the oxidative stability of two types of chicken meat and beef meat, and the potential effect of vitamin C on the oxidation of beef meat during the simulated digestion processes.

The essences of our finding from this study are as follow. First of all, raw red meat was more susceptible to oxidation than raw white meat, which might elicit the hypothesis that haem content determines the lipid oxidation potential of raw meat. In addition, we also speculated that consumption of fatty meat might cause generation of abundant lipid peroxyl radicals, which might promote chronic carcinogenesis to the gastrointestinal tract. However, from our results, it was found that the fat content had less influence on the lipid oxidation processes compared to the haem content. Secondly, the \textit{in vitro} model we designed was optimized for simulating the physiological digestion processes in relation with oxidation processes, and abundant lipid oxidative products originating from meat were produced in the stomach condition, because of the interaction of more released iron form meat cells attributed to chewing processes, acidic pH environment created by the gastric fluid, and together with catalyst role of lactoperoxides from saliva in acidic condition. Consequently, lots of these lipid oxidation products will still exist in the small intestine. Last but not the least, vitamin C with the tested concentrations, has neither pro-oxidant effect nor anti-oxidant effect on the lipid oxidation of beef meat during the digestion process. However, it is difficult to determine a dose of vitamin C which can exhibit the anti-oxidant or pro-oxidant effect during red meat digestion process, since it is determined by the cross-effect of anti-oxidant and pro-oxidant properties of vitamin C and the gastrointestinal conditions, which play a role of bioreactor with both antioxidant and pro-oxidant compounds present.

Further studies should make effort to optimize the \textit{in vitro} digestion model, like including more chemicals to the digestive fluids to compensate the function of enzymes and
cofactors in a physiological combination and assessing extent of lipolysis and proteolysis to make sure that the meat is well digested in the simulated model. In addition, in order to evaluate the putative beneficial role of vitamin C, higher vitamin C concentration should be added to the *in vitro* digestion models for meat. Furthermore, cooked meat and processed meat should also be investigated the oxidation process in the *in vitro* digestion model.
References


including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. Animal Feed Science and Technology 2005, 123-124: 421-444.


[133] B.R. Wilson, A.M. Pearson, and F.B. Shorland. Effect of total lipids and phospholipids on warmed-over flavor in red and white muscle from several species as measured by thiobarbituric acid analysis. *Journal of Agricultural and Food Chemistry* 1976, **24**: 7-11.


Annexes
Annex 1. The pH of the meats and the digesta during incubation processes (Date: 2012-10-13)

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## Annex 2. The pH of the meats and the digesta during incubation processes (Date: 2012-10-21)

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Note: The table entries represent the pH levels at different stages of incubation for each type of meat.
Annex 3. The pH of the meats and the digesta during incubation processes (Date: 2012-11-04)

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