FACULTY OF BIOSCIENCE ENGINEERING

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Formation of oxidation products and alterations in the gut metabolome and microbiome upon digestion of red and processed meat

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Let food be thy medicine

and medicine be thy food

- HIPPOCRATES -

Dutch translation of the title:

Vorming van oxidatieproducten en veranderingen in het darm metaboloom en microbioom als een gevolg van de vertering van rood en verwerkt vlees

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxy-2-nonenal
AAA	α-amino adipic acid
AAPH	2,2'-azobis-(2-amidinopropane)dihydrochloride
AAS	α-amino adipic semialdehyde
Acyl-CoA	coenzyme A with acylgroup
ANOVA	analysis of variance
CACT	carnitine/acylcarnitine translocase
CoASH	coenzyme A without acylgroup
CPT	carnitine palmitoyl-transferase
CrAT	carnitine O-acetyltransferase
CV	coefficient of variation
EDTA	ethylenediaminetetraacetic acid
FAME	fatty acid methyl esters
FAO	Food and Agricultural Organization
FIA	flow injection analysis
FW	fresh weight
DM	dry matter
DMEM	dulbecco's modified eagle's medium
DNPH	2,4-dinitrophenylhydrazine
DODE	dioxododecenoic acid
GC	gas chromatography
GGS	γ-glutamic semialdehyde
GSH	glutathione (reduced)
GSSG	glutathione disulfide (oxidized glutathione)
GSH-Px	glutathione peroxidase
HILIC	hydrophilic interaction liquid chromatography
HMDB	human metabolome database
HODE	hydroxy-octadecadienoic acid
HPLC	high performance liquid chromatography
HPODE	hydroperoxyl-octadecadienoic acid
IARC	International Agency for Research on Cancer
IEC	ion-exchange chromatography;
JECFA	joint FAO/WHO expert committee on food additives
KEGG	kyoto encyclopedia of genes and genomes
LC	liquid chromatography
LDA	linear discriminant analysis
LOD	Limit of detection
LOQ	Limit of quantification
LEfSe	linear discriminant analysis effect size
MeOH	methanol
MUFA	monounsaturated fatty acids
MS	mass spectrometry
MTT	3-[4,5-dimethylthiazol-2-yl]-22,3-diphenyl tetrazolium bromide

NADPH	nicotinamide adenine dinucleotide (reduced)
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NMDS	non-metric multidimensional scaling
NMR	nuclear magnetic resonance spectroscopy
NOC	N-nitroso compound
(O)PLS-DA	(orthogonal) projection to latent structures discriminant analysis
ORAC	oxygen radical absorbance capacity
OTU	operational taxonomic unit
oxoODE	oxo-octadecadienoic acid
PCA	principal component analysis
PCC	protein carbonyl components
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acids
QC	quality control
qTOF	quadrupole time-of-flight
RMSEE	root mean squared error of estimation
ROS	reactive oxygen species
SD	standard deviation
SFA	saturated fatty acids
SPME	solid phase micro-extraction
SRB	sulforhodamine B
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TMA	trimethylamine
TMAO	trimethylamine-N-oxide
TMP	1,1,3,3-tetramethoxypropane
TriHOME	trihydroxyoctadecenoic acid
UHPLC	ultra high-performance liquid chromatography
UHPLC-HRMS	ultra high-performance liquid chromatography coupled to high-resolution mass spectrometry
VIP	variable importance in projection
WCRF	World Cancer Research Fund
WHO	World Health Organization

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Chapter I - General introduction

1.1 Objectives and study outline

This PhD thesis envisioned a better understanding of differences in the formation of metabolites during the digestion of red and processed meat versus white meat. Over the last decades, several epidemiological studies have reported that a high consumption of red and especially processed meat increases the risk to develop various chronic diseases such as colorectal cancer, cardiovascular diseases and type 2 diabetes. In contrast, no such associations have ever been reported for the consumption of white meat. The main difference between red and white meat is the heme iron content, which has a catalysing effect on the formation of cyto- and genotoxic oxidation products. Although the heme-hypothesis is one of most advanced hypotheses in the red meat-colorectal cancer association, the exact underlying mechanisms remain unclear. Furthermore, there is probably not one single factor responsible and reciprocal interactions between the whole dietary pattern, microbiome and (epi)genetic factors are likely involved.

Meat is mostly consumed within a meal, which contains many components that could interact with each other. Indeed, previous studies have demonstrated that the cumulative and interactive effects of multiple components in the diet may attenuate or exert stronger effects than the sum of the single components, and more consistent results are obtained when focusing on dietary patterns instead of single nutrients or foods in nutritional epidemiology. Typically, diets associated with a reduced risk of various chronic diseases, frequently called prudent diets, have several common elements such as high intake of fruits, vegetables, whole grains and nuts, and lower intake of red and processed meats, sugar-sweetened foods and drinks and refined grains. In contrast, a dietary pattern high in fats and sweets, sugar-containing beverages, refined cereals, confectionaries and red and processed meat has been associated with higher risks to develop chronic diseases.

In this PhD thesis, both a targeted (hypothesis-driven) and an untargeted (non-hypothesis-driven) approach were applied to increase the understanding of postprandial processes initiated by red and processed meat consumption. The targeted approach primarily focused on the role of meat consumption in the formation of cyto- and genotoxic oxidation products during digestion and postprandial oxidative stress, whereas alterations in the gut metabolome and microbiome as a result of meat consumption were studied using an untargeted approach to generate new insights or to confirm previous suggested mechanisms.

Three main research questions were investigated by the targeted approach:

- Are the levels of oxidation products and postprandial oxidative stress higher after consumption of red and processed meat compared to white meat? (Chapter II)

- Is the formation of oxidation products and postprandial oxidative stress influenced by the dietary context in which meat is consumed? Can a dietary context rich in fruits and vegetables mitigate oxidation processes during meat digestion? (Chapter II)

- What is the variation in oxidation products formed during digestion of different types of processed meat and how are these levels situated across unprocessed red meat? (Chapter V)

CHAPTER I of this thesis comprises a brief perspective on the evolution of dietary patterns and nutrition research as well as a short overview of the importance of meat consumption and the link with chronic diseases. Furthermore, the basic principles of metabolomics were described and the use of *in vitro* models and pigs as experimental animals in nutrition research were introduced.

An extensive pig feeding trial as a digestion model for humans contributed to the main part of this PhD thesis. A 2×2 factorial design with chicken meat versus a mixture of red and processed meat on the one hand, in combination with a diet rich in fruits and vegetables or a diet with a high sugar and fat content on the other hand, was used to investigate the effects of meat consumption in a complex dietary context. After the four-week experimental period, the pigs were euthanized and blood, organ tissue and gastrointestinal digest samples were collected. This interesting range of samples was used to study the oxidative status of the pigs (**CHAPTER II**), to elucidate differences in the digestion metabolome (**CHAPTER III**) and to investigate differences in the microbiome and in colonic volatile organic compounds (**CHAPTER IV**).

The term red and processed meat covers a wide range of meats, originating from different animal species and subjected to different processing procedures that might induce differential health effects. Epidemiological studies consistently report a higher relative risk for processed meat compared to red meat, but no distinction is made between different types of processed meat. Therefore, and to gain more insight in the results obtained in Chapter II, a follow-up experiment with different types of processed meat was conducted in **CHAPTER V**. An *in vitro* digestion model

was applied to evaluate the concentration of lipid and protein oxidation products after gastrointestinal digestion of commercial luncheon and deep-fat fried meat products.

Finally, in **CHAPTER VI**, the main findings of the previous chapters were integrated and discussed. Limitations of the applied approaches and directions for future research were outlined.

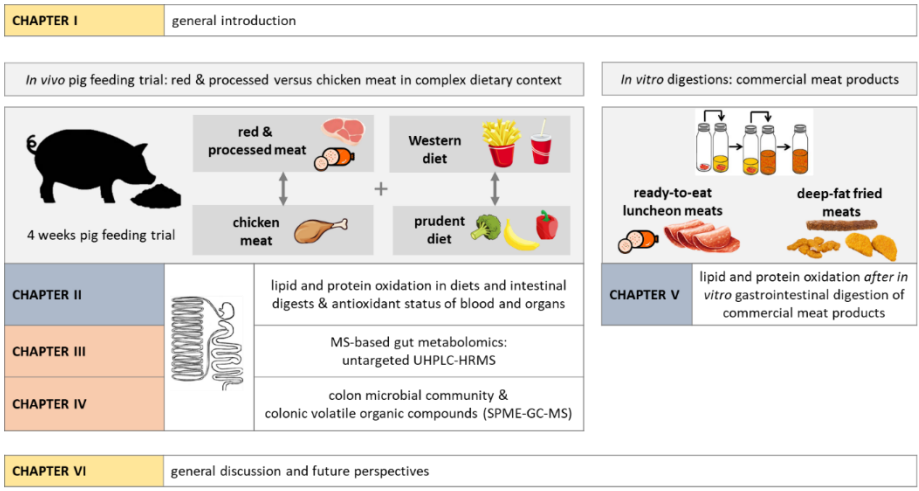


Figure 1.9 Schematic overview of this PhD thesis.

1.2 Historical perspective of dietary patterns and nutrition research

1.2.1 Collision between our ancient genome and the current Western diet

The diet and lifestyle that shaped the human digestive physiology for more than 2 million years has drastically changed starting with the agricultural revolution 11 000 years ago and followed by the industrial revolution and modern age. The timeframe starting from the agricultural revolution until now only accounts for 0.5% of the history of the genus *Homo* (Carrera-Bastos et al., 2011). The agricultural revolution introduced significant dietary changes such as the consumption of cereal grains, dairy products, meat from domesticated animals and cultivated plant foods. The industrial revolution and modern age were marked with faster, more radical and still ongoing changes such as the consumption of highly processed foods and a drastic shift in lifestyle including physical inactivity. The increased life expectancy during the previous two centuries, was due to factors such as improved medical care, sanitation and political stability rather than to a healthier diet and/or lifestyle (Eaton et al., 2002). On the contrary, the conflict between our ancient physiology and current lifestyle and diet is probably an important cause of many so-called diseases of

civilization including certain types of cancer, cardiovascular diseases, diabetes, obesity, and autoimmune diseases. Cordain et al. (2005) identified the following seven crucial dietary changes that could underlie many of the chronic civilization diseases: glycemic load, fatty acid composition, macronutrient consumption, micronutrient density, acid-base balance, sodium-potassium ratio, and fiber content.

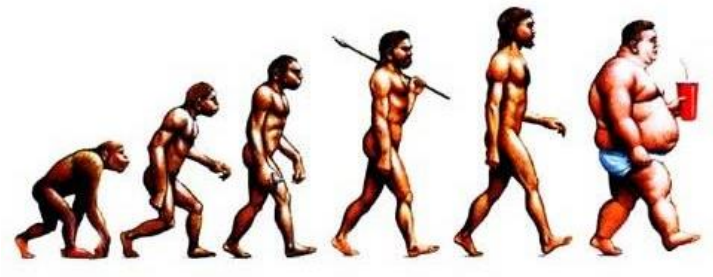


Figure 1.1 Cartoon of evolution theory of Darwin illustrating the obesity epidemic (anonymous source)

1.2.2 Shift in nutrition research and evolution of dietary guidelines

Traditionally, nutrition research favoured a reductionistic approach that focusses on specific mechanisms and health effects of single nutrients. This approach has among others led to the identification of various vitamins and minerals. Understanding the function and mode of action of specific nutrients is interesting in the prevention of deficiency diseases. Dietary guidelines in the first half of the 20th century mainly focussed on food groups to ensure adequacy of both micronutrients (vitamins and minerals) and macronutrients (proteins, carbohydrates, and fats) in addition to advice on food safety and food storage (Jahns et al., 2018). Increased knowledge on specific nutrients resulted in increased attention to the relation between nutrient intake such as calcium, vitamin C and iron intake and disease prevention. However, concerns about high calorie intake, fatty acid composition, sugar or salt and the lack of fiber in the diet shifted nutrition research and guidelines from a historical focus on nutrient adequacy towards the reduction of overweight and obesity and the prevention of chronic diseases. In 1980, the first Dietary Guidelines for Americans report was released in the United States and included concepts of food variety, body weight maintenance, nutrient adequacy, and moderation of certain nutrients and alcohol (USDA, 1980). In 1996, the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) of the United Nations published the report 'Preparation and Use of Food-Based Dietary Guidelines' to establish a scientific basis for the development of food-based dietary guidelines and to encourage the translation of the traditional national dietary guidelines that were mostly nutrient-based into food-based guidelines, intended to be understood and used by the

general public. The overall aim of the food-based dietary guidelines was to improve food consumption patterns and nutritional wellbeing of individuals and populations, while taking into account the nutritional status of the target population and the scientific evidence and epidemiology of diet-related health problems (FAO/WHO, 1996).

The traditional focus on nutrients in experimental, clinical and epidemiological nutrition research has helped to understand simple, independent food-health associations, to avoid deficiency conditions and to characterize food products in a uniform manner. However, the association between diet and chronic diseases with very complex etiologies might not be possible to explain in terms of nutrients or single food items (Tapsell et al., 2016). Food items and especially a dietary pattern contain many interacting compounds that may affect health in a multi-causal way. Studying the effects of isolated nutrients or food items may disregard overall effects caused by substitution, synergistic and/or antagonistic effects between nutrients within a complex diet. Indeed, results from nutrition studies using the traditional nutrient approach often seemed to be conflicting depending on whether they were performed *in vitro* or *in vivo*, or depending on the concentrations and surrounding food matrix used (Jacobs & Tapsell, 2007; Tucker, 2010). Also, the results of prospective cohort studies and randomized controlled trials focusing on specific nutrients have been paradoxical and have led to confusion among scientists, policy makers and the general population (Hennekens et al., 1996; Mente et al., 2009; Ness et al., 2001). These conflicting observations have forced researchers to consider a more holistic approach regarding nutrition research and chronic disease risk. The concept of food synergy was raised and defined as the concerted action of compounds within foods and diets and their interrelation with human physiology (Jacobs et al., 2009). Although the extent and nature of food synergy is difficult to uncover, more consistent results are obtained when focusing on dietary patterns instead of single nutrients or foods in nutritional epidemiology (Mente et al., 2009; Wirfält et al., 2013). Diet quality is generally comprised of nutrient adequacy, food variety or food diversity and moderation of foods, food groups, energy and nutrients (Alkerwi, 2014). Therefore, dietary pattern analysis aims to describe the overall diet, including nutrients, foods, food groups, combinations and variety, and frequency and quantity in which they are consumed (Hu, 2002). Although defining specific dietary patterns encounters several methodological issues and dietary complexities, systematic reviews reached similar conclusions (Hu et al., 1999; Tapsell et al., 2016; Wirfält et al., 2013). Typically, diets that were associated with a reduced risk of the majority of examined chronic diseases have several common elements such as high intake of fruits, vegetables, whole grains and nuts, and lower intake of red and processed meats, sugar-sweetened foods and drinks and refined grains (Schulze et al., 2018). Although specific food items and names of diets may vary (prudent

pattern, Dietary Approaches to Stop Hypertension diet, Mediterranean diet, High-Quality Diet, Nordic diet), the overall composition of those diets has great similarities. In this PhD thesis, the term prudent diet is used. In contrast, a dietary pattern, frequently characterized by high consumption of sweets, sugar-containing beverages, 'junk foods', refined cereals, confectionaries and red and processed meat has been frequently associated with higher risks to develop chronic diseases such as cardiovascular and metabolic diseases and colorectal cancer (Tapsell et al., 2016; Wirfält et al., 2013). Although this high energy and nutrient poor dietary pattern is frequently, and also in this thesis, named a Western diet, this dietary pattern is no longer restricted to Western countries but widespread across the globe. As clearly stated by Jacobs & Steffen (2003) and Tapsell et al. (2016), food synergy should be studied at different levels, namely at the level of dietary patterns, foods and nutrients, preferably using a 'top down' approach (Jacobs & Steffen, 2003; Tapsell et al., 2016). This 'top down' approach first investigates the relationship between dietary patterns and health outcomes, followed by the study of smaller units such as combinations of foods, food components, or nutrients to isolate the true causative agents and to improve the mechanical understanding of the observed health outcomes. The smaller units that have been proposed to contribute to a beneficial health effect of a prudent diet are the balanced amounts of micronutrients (vitamins and minerals) and other bioactive components such as antioxidants and phenolic compounds, whereas the lack or quantities of those components in addition to potential harmful components related to food processing are regularly proposed to be involved in the adverse health effects of a Western diet (Wirfält et al., 2013). However, food processing is not necessarily related to adverse health effects. On contrary, food processing can among others improve digestibility, shelf life and food safety. To conclude, the formulation of dietary guidelines should start with evidence of the relation between dietary patterns and health outcomes. A next step comprises the better understanding of those health outcomes, which requires multiple complementary approaches using both epidemiological, clinical and experimental studies at different levels of nutrients, foods and combinations of foods (Jacobs & Steffen, 2003).

1.3 Meat consumption and impact on health – a knife that cuts on both sides?

The consumption of meat has become a very controversial and sensational subject due to nutritional, environmental and ethical concerns. Although the environmental impact and ethics regarding meat consumption should not be overlooked, this thesis will particularly focus on the role of meat in health and disease. In recent years, high intake levels of meat have been linked to the increase in chronic diseases burden

typically associated with a Western diet and lifestyle. However, unlike the other characteristic elements of a Western diet such as sweets and sugar-containing beverages, consumption of meat is different, as meat is a food source of high nutritional value. Next to its well-known high-quality protein and rich vitamin and mineral content (B vitamins, iron, zinc, selenium etc.), meat also contains various bioactive components such as taurine, creatine, carnosine, carnitine, and glutathione (GSH) (Leroy & Cofnas, 2019; Williams, 2007). Many beneficial functions, including the optimal development of cognitive functions, have been ascribed to these compounds, however, the exact mechanisms are not always well known. Furthermore, meat consumption played an important role in human evolution and still harbors an important sociocultural function (Leroy & Praet, 2015). Presumably, the consumption of energy-dense meat has been crucial for the evolution of the brain size in the hominin lineage. The consumption of energy-dense meat required less energy compared to the digestion of bulky plant fibers, and as the brain and gut compete for energy, this extra amount of energy could have contributed to the development of a bigger brain (Aiello & Wheeler, 1995).

Nevertheless, high meat consumption is also one of the aspects of a Western lifestyle. Meat consumption became a symbol of wealth and the combination of economic growth, development in meat production technology and widespread urbanization has led to major dietary changes during the last two centuries including a significant rise in meat consumption per capita per year. This rise in meat consumption expressed as carcass weight availability in France and Great Britain has been illustrated by Smil (2002) as illustrated in Figure 1.2.

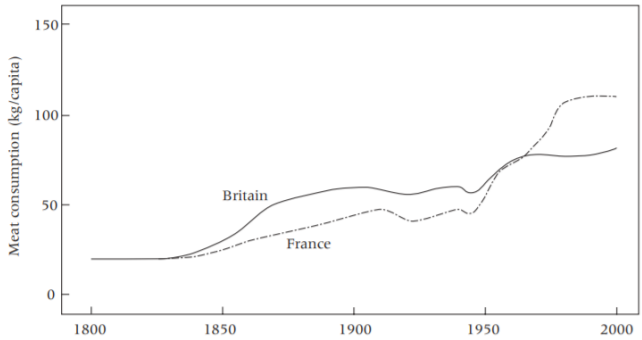


Figure 1.2 Meat consumption (as carcass weight availability) in France and Britain, 1800-2000 (Adapted from Smil 2002)

In addition, Figure 1.3 illustrates the global map of average meat consumption (excluding seafood and fish) as carcass weight availability per capita in 2013 as outlined by the FAO (Ritchie & Roser, 2020). The average global meat consumption in 2014 was around 43 kg of meat per person (expressed in carcass weight availability), with a 10-fold variation between high-consuming and low-consuming

populations. Meat consumption is highest in high-income countries (Australia, European countries and United States) with an average meat consumption of 80 – 116 kg per person in 2013. However, the direction and rate of change are also highly variable. Consumption in high-income countries have been stagnating, whereas countries subjected to major economic transitions have known fast increases in meat consumption over the last 50 years (Ritchie & Roser, 2020).

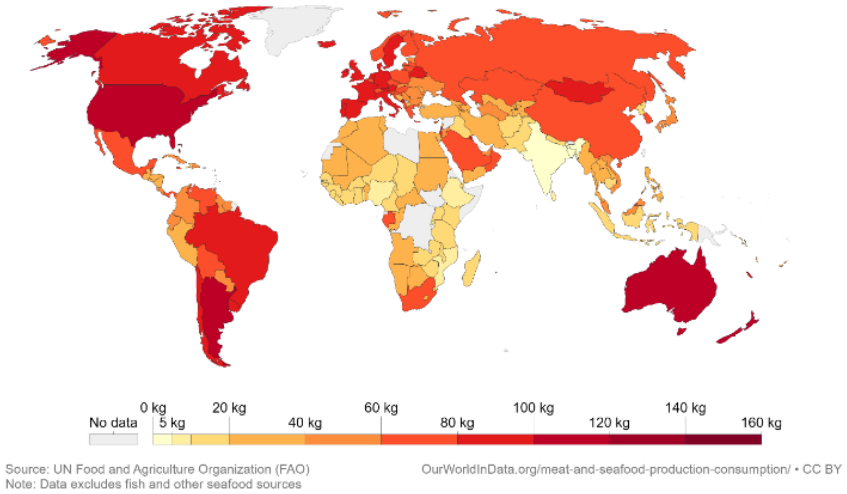


Figure 1.3 Global meat supply per person (carcass weight availability) in 2013 (Ritchie & Roser, 2020)

In parallel with this meat consumption pattern, also the burden of chronic non-communicable diseases is shifting. Whereas the epidemiological transition from infectious to chronic diseases began in Western countries approximately 100 years ago, nowadays, the mortality numbers and rates of chronic diseases is rising at a much faster rate in low- and middle-income countries than in developed countries (Amuna & Zotor, 2008; Omran, 2005; Stuckler, 2008). This clearly emphasizes the important role of nutrition in health policy.

Based on the outcome of a considerable number of observational epidemiological studies as recently reviewed by De Smet et al. (2019), high levels of meat consumption have been linked to the burden of chronic diseases. In most epidemiological studies and meta-analyses, a distinction is made between red and white meat, which relies on the myoglobin content in meat, and between red meat (unprocessed mammalian muscle meat) and processed meat. Processed meat is mostly defined as mammalian muscle meat that has been modified to improve taste and/or shelf life by salting, curing, fermentation and/or smoking. However, these definitions can be confusing (Demeyer et al., 2016) and within these broad categories of red meat and processed meat, an enormous variety of meat types and meat products, with potentially different health consequences, exists. Meta-

analyses combine data from multiple epidemiological studies and the calculated associations are expressed as relative risks (RR). A positive association was reported for meat consumption and (i) colorectal cancer (17%-27% increase in RR per 100 g red meat and 18%-29% increase in RR per 50 g processed meat) (Aune et al., 2013; Chan et al., 2011), (ii) coronary heart disease (no increased RR per 100 g red meat, but 42% increase in RR per 50 g processed meat) (Micha et al., 2012), and (iii) diabetes type 2 (19% increase in RR per 100 g red meat and 51% increase in RR per 50 g processed meat; (Micha et al., 2012; Pan et al., 2011), but also associations between other types of cancer (Farvid et al., 2015; Huang et al., 2013) and intestinal disorders (Cao et al., 2018) have been reported. These relative risks are consistently higher for processed meat than for red meat, and no negative associations have been reported for white meat consumption (Marangoni et al., 2015; Norat et al., 2002). The public health relevance of red meat consumption does not only depend on the relative risk ratios for chronic diseases, but is also determined by the prevalence of exposure to the risk factor and to the disease prevalence. For example, for colorectal cancer, the combination of the relative risk ratio related to red meat intake and the average red meat intake in a population can be used to calculate the fraction of colorectal cancers that is attributable to a high intake of meat. Varying outcomes have been reported for the population attributable fraction of colorectal cancer due to red meat intake, ranging from 5% in a recent French study and in New Zealand (Mota et al., 2019; Richardson et al., 2016) to 10-25% in regions where red meat intake is high (Norat et al., 2002) and 18% in an Australian study (Nagle et al., 2015), but in the latter, no distinction was made between red and processed meat. Epidemiological studies are often criticized because of other shortcomings such as the lack to establish causality, the risk of residual confounding and problems with accuracy of self-reported dietary intake (Leroy & Cofnas, 2019). More insight in the underlying mechanisms through mechanistic studies is of paramount importance in order to support a causal role of meat consumption in the etiology of chronic diseases, and to develop strategies to improve the composition and/or processing of meat.

In addition to these epidemiological studies, for colorectal cancer, a profound hazard analysis was conducted by the International Agency for Research on Cancer (IARC). The IARC working group, that evaluated not only epidemiological studies, but also evidence from animal and human intervention studies, classified processed meat consumption as ‘carcinogenic to humans’ (Group 1) and red meat consumption as ‘probably carcinogenic to humans’ (Group 2) (Bouvard et al., 2015). This outcome, that places processed meat consumption in the same group as tobacco smoke, is prone to misinterpretations since hazard analyses do not consider dose and exposure, in contrast to risk measurements.

1.4 Current hypotheses linking high meat consumption with chronic diseases – focus on oxidation

1.4.1 Suggested mechanisms for the association between high red and processed meat consumption and chronic diseases

Based on the composition of meat, several mechanisms have been proposed to explain the observed associations between meat consumption and chronic diseases. Several hypotheses, especially on the carcinogenic potential of meat intake, but also on the link with cardiovascular diseases and type 2 diabetes, have been proposed and were discussed in multiple reviews (Demeyer et al., 2016; Hammerling et al., 2016; Wolk, 2017). De Smet et al. (2019) classified the potential factors and processes that could be involved in the underlying mechanisms as (i) compounds intrinsic to meat (e.g. fatty acid profile, heme-Fe, N-glycolylneuraminic acid (Neu5Gc)), (ii) compounds that are formed during meat processing or preparation (e.g. heterocyclic amines, polycyclic aromatic hydrocarbons), (iii) compounds formed in the gastrointestinal tract or through subsequent metabolism during meat digestion (e.g. trimethylamine-N-oxide (TMAO)), or (iv) compounds that can be present in meat, but their formation can be catalyzed during digestion (e.g. N-nitroso compounds (NOCs), oxidation products). However, a complete understanding is still lacking, since many of these factors are not unique for red meat or cannot explain the higher epidemiological risk of processed meat compared to fresh red meat. Furthermore, there is probably not one single factor responsible for each of the associations, but rather a combination of different factors and a reciprocal impact of the whole dietary pattern, microbiome and (epi)genetic factors (Bultman, 2017; Montgomery & Srinivasan, 2019).

In addition to a non-hypothesis driven research approach in this PhD thesis by using untargeted metabolomics (section 1.5), also one of the most supported hypotheses was specifically investigated. In this targeted approach, we focused on the catalyzing effect of meat consumption on the formation of oxidation products as a potential factor contributing to the association between meat and chronic diseases. The focus on oxidation products originated from the fact that oxidative stress is a common factor in most aforementioned chronic diseases (Chen & Keaney, 2012; Rains & Jain, 2011) and that several digestion studies demonstrated an increase in oxidation products upon the consumption of red meat (Kanner & Lapidot, 2001; Sirota et al., 2013; Van Hecke et al., 2017a). An imbalance between reactive oxidation products and the body's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (antioxidant defense) is defined as oxidative stress. The various mechanisms through which oxidative stress as a result of an increase in oxidation products originating from red or processed meat consumption might play a role in

the aetiology and/or progression of chronic diseases was reviewed by Van Hecke et al. (2017a) and Estévez & Luna (2017).

1.4.2 Mechanism of oxidation in meat

Oxidation reactions are one of the main factors affecting shelf life and quality deterioration in meat, due to adverse changes in color, flavor, texture and nutritional value. Meat is very susceptible to oxidation since it contains fat, proteins and free and bound iron, all compounds that can take part in, or catalyze oxidation processes. One of the predominant hypotheses suggests that iron, present in the heme moiety of myoglobin, can promote oxidative stress. Transition metals, such as iron, are able to initiate and catalyze the free radical oxidative chain reaction via the so-called Fenton reaction. In this reaction, hydrogen peroxide (H_2O_2) catalyzes the oxidation of Fe^{2+} to Fe^{3+} (1), which is reduced back to Fe^{2+} (2). During this reaction the reactive oxygen species (ROS), hydroxyl radical ($HO^{\cdot}$) and hydroperoxyl radical ($HOO^{\cdot}$) are formed.

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$
(2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$

Those ROS are highly unstable and initiate further oxidative chain reactions by abstracting a hydrogen atom of polyunsaturated fatty acids (PUFA) or proteins, resulting in the formation of various potentially harmful lipid and protein oxidation products, which can eventually damage macromolecules. Among the lipid oxidation products, malondialdehyde (MDA) is considered a general marker for lipid oxidation, as it is derived from both n-3 and n-6 PUFA, whereas 4-hydroxy-2-nonenal (4-HNE) and hexanal are derived from the oxidation of n-6 PUFA (Guéraud et al., 2010). Free radical attack on proteins can take place at the protein backbone or the amino acid side chains of mainly cysteine, tyrosine, phenylalanine, tryptophan, histidine, proline, arginine, lysine and methionine, due to their susceptibility to hydrogen abstraction or hydroxyl addition to the aromatic rings (Lund et al., 2011). Protein carbonyl derivatives are considered the most abundant protein oxidation products. They can be formed via different pathways such as (i) direct oxidation of amino acid side chains (Requena et al., 2001), (ii) covalent binding of non-protein carbonyls, such as reactive lipid oxidation products (Stadtman & Levine, 2003), (iii) Maillard-mediated pathway with reducing sugars (Akagawa et al., 2002), (iv) fragmentation of the protein backbone through α-amidation reactions or oxidation of glutamyl side chains (Berlett & Stadtman, 1997; Hawkins & Davies, 2001). The most described protein carbonyl compounds (PCC) are α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) that originate from the oxidation of lysine, and of arginine and proline, respectively. Subsequently, AAS can be further oxidized to α-

amino adipic acid (AAA), which is suggested as a more stable end-product and a reliable protein oxidation marker (Estévez, 2011).

In muscular tissues of living animals, an endogenous multilevel antioxidant system is present to regulate oxidation processes. This antioxidant system consists of enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase (GSH-Px)), lipophilic non-enzymatic antioxidants (α-tocopherol, carotenoids and ubiquinone) and cytosolic non-enzymatic antioxidants (histidine containing dipeptides carnosine and anserine, GSH, ascorbic acid, iron binding proteins, and probably also polyamines and nucleotides) (Decker & Mei, 1996). After slaughter, the activity of many of those factors is lost, which results in a high susceptibility to oxidation in fresh meat and the need to add antioxidants to prolong the shelf life of meat products. Oxidation phenomena in meat products are even more complex. It is very likely that muscle structure disruption, thermal treatment, and functional ingredients such as salt, phosphate, and other additives, and processing conditions such as ripening and fermentation can considerably affect susceptibility to oxidation processes and the efficacy of antioxidants (Jiang & Xiong, 2016).

1.4.3 Oxidation during meat digestion

In addition to the oxidation reactions in meat during storage, processing and/or preparation, it has been shown that oxidation continues during gastrointestinal digestion and that oxidation products can be (partly) absorbed in the blood stream (Kanner & Lapidot, 2001; Van Hecke et al. 2017a). The consecutive exposure of meat to saliva, gastric juice, emulsifying pancreatic and bile juice, and microbiome can affect oxidation processes in various ways. For example, the study of Gorelik et al. (2007) demonstrated that saliva can exert either a prooxidant, antioxidant, or no effect on oxidation, depending on the composition of saliva of different donors. Evaluation of the isolated components of the salivary antioxidant defense system revealed that lactoperoxidase increased lipid peroxidation, whereas thiocyanate and nitrite reduced lipid peroxidation in a model simulating food digestion in stomach conditions. Gastric fluid, having a low pH and containing dissolved oxygen, was shown to be an excellent medium to promote lipid oxidation in grilled turkey muscle (Kanner & Lapidot, 2001). The occurrence of *in vivo* oxidation of dietary lipids during the consumption of a beef meal, either or not enriched with fruits and vegetables, or their polyphenol extracts, was also demonstrated in the stomach medium of cannulated minipigs (Gobert et al., 2014). This study additionally demonstrated the antioxidant effect of fruits and vegetables, and the importance of the chemical structure of the microconstituents and their respective bio-accessibility. The absorption of dietary related oxidation products was demonstrated in the postprandial plasma MDA concentration following red turkey meat consumption

either or not combined with red wine by Gorelik et al. (2008a, 2008b). In agreement with the study of Gobert et al. (2014), they also demonstrated the potential to limit oxidation processes by the simultaneous consumption of antioxidant rich food items.

In the subsequent digestion stages, oxidation can be affected by secreted lipases and bile salts in the small intestine as they influence the partitioning of aqueous, fat or emulsion phases (McClements & Li, 2010). The presence of heme-Fe in the large intestine could continue to stimulate further oxidation reactions in the colonic contents resulting in an increased cytotoxicity of fecal water (Sesink et al., 1999), but also shifts in the microbiome composition and related heme-Fe induced hyperproliferation of the colonic mucosa have been reported (IJssennagger et al., 2012a; 2015).

As oxidation products can be formed throughout the gastrointestinal tract on the one hand, but also be absorbed, degraded or metabolized into other compounds on the other hand, more kinetic studies are needed to enhance the understanding of oxidation mechanisms and fate of oxidation products during digestion.

1.5 Use of metabolomics to unravel complex relationships between dietary exposures and the metabolic phenotype

1.5.1 Metabolomics technologies

The central dogma of molecular biology refers to the concept that our hereditary information, in the form of DNA, is transcribed into RNA and translated into proteins that are involved in the modulation of our metabolome (Crick, 1970). Today's high-throughput innovative technologies, known as the 'omics' methodologies (genomics, transcriptomics, proteomics, and metabolomics), enable to assess thousands of genes, proteins and metabolites simultaneously. Metabolomics is one of the most recent 'omics' technologies (early 2000's) and refers to comprehensive analytical chemistry approaches aiming to provide a global description of all small molecular weight molecules (<1500 Da) present in a biofluid (urine, serum, plasma, feces, digestion fluids, saliva), tissue or cell at a given time (Courant et al., 2014). The metabolome is considered the closest link to the phenotype, since the metabolome reflects the information expressed and modulated by genetic, epigenetic and environmental factors as illustrated in Figure 1.4 (Krumsiek et al., 2016).

Metabolites are thus the final response of a biological system to genetic or environmental changes. The study of metabolites is applied in different areas such as medical, environmental, nutrition, and agricultural sciences, and in fundamental research. Metabolomics studies usually compare the metabolomes of a 'control' and a 'test' population, which can include the comparison between a healthy and diseased population, or a population either or not exposed to a drug or diet. The

differences between the metabolomes shed light on the alteration of the metabolome in response to the stimulus.

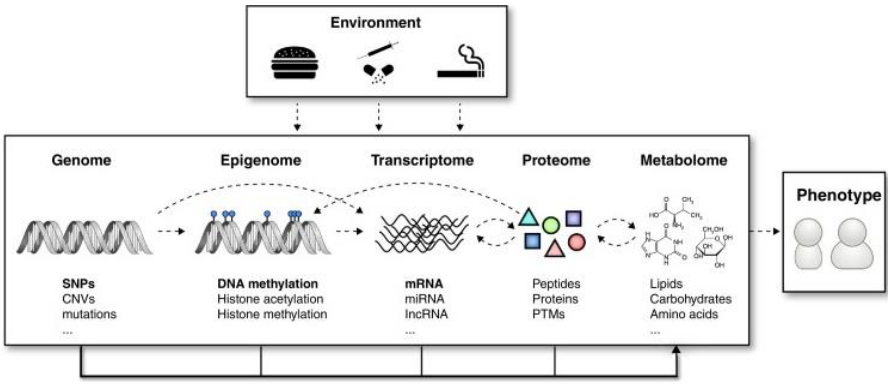


Figure 1.4 Simplified interplay between molecular omics, environment and the phenotype (adapted from Krumsiek et al. 2016)

A distinction can be made between untargeted and targeted approaches (Patti et al., 2012). Untargeted metabolomics aims to study a large set of metabolites without taking *a priori* knowledge of metabolites that could be of interest for the research question into account. This approach is also known as metabolic fingerprinting and is a hypothesis-generating, rather than a hypothesis-driven strategy. On the other hand, metabolomics technologies can also be used to focus on a limited number of specific compounds, or classes of metabolites, known as targeted metabolomics or metabolic profiling. The method of choice depends on the experimental design and the research question. The targeted approach has the advantage to increase sensitivity and absolute quantification of metabolites of interest, whereas the advantage for the untargeted approach lies in the potential for novel discoveries.

Untargeted metabolomics requires a series of key steps in between sample collection and biological interpretation, involving sample preparation, data acquisition, data analysis, metabolite identification and metabolic mapping (Figure 1.5). Unfortunately, it is not possible to assess the entire metabolome with one single analytical strategy due to various physico-chemical properties of the metabolites including differences in molecular weight, polarity and concentration. A variety of sample preparation methods and analytical platforms with different separation principles and detection methods are available. Each method has its own advantages and limitations, and the optimal method, or combination of methods, is chosen in function of the research question. A first important step is the selection of samples and sample preparation, as reviewed by Ulaszewska et al. (2019). Most commonly used biological samples are urine and blood, and the sample extraction method is frequently based on the polarity of the targeted metabolites where e.g. water,

water-methanol mixtures, or chloroform-methanol mixtures are used to extract highly polar, moderately polar, or lipid compounds respectively. It is important to optimize and validate sample extraction to provide a reproducible recovery and a wide coverage of target compounds.

The two main analytical platforms used in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Emwas, 2015; Scalbert et al., 2009). NMR is based on the energy absorption and re-emission of the atom nuclei (mostly ^{1}H) due to variations in an external magnetic field. NMR spectroscopy is non-destructive, highly reproducible, quantitative and offers structural information, but is not as sensitive as MS techniques. MS-based metabolomics is mostly preceded by chromatographic separation of the metabolites using liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE), with LC being the most frequently used. This separation step reduces the complexity of the samples, and the obtained retention time delivers additional information about the compounds, as it indicates the extent of interaction of the compounds with the chromatographic column. Following chromatographic separation, the eluted analytes are ionized and the abundances of the resulting mass-to-charge ratios (*m/z*) are detected in the MS system which is equipped with an ion source, mass analyzer and a detector. A wide range of ionization and mass selection methods are available and hybrid instruments combining ion traps and/or quadrupoles with HRMS enable to perform MS/MS fragmentation experiments.

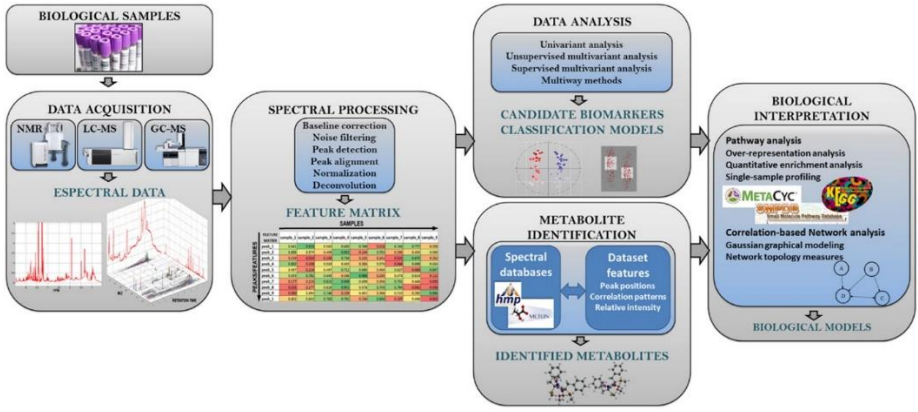


Figure 1.5 General workflow applied in untargeted metabolomics (adapted from Alonso et al. 2015)

The large and complex datasets acquired by the analytical platforms usually need preprocessing prior to statistical analysis (Liland, 2011; Ulaszewska et al., 2019; Worley & Powers, 2013). Data preprocessing can include chromatographic peak alignment, peak detection, noise removal, scaling and normalization. Metabolomics

data are mostly composed of a few observations and hundreds to thousands of variables. To understand the relations among and between the sample groups and the metabolites, and to extract discriminating metabolites between the sample groups, multivariate methods are required. Multivariate methods such as principal component analysis (PCA) and (orthogonal) projection to latent structures discriminant analysis ((O)PLS-DA) are frequently used in metabolomics studies (Worley & Powers, 2013). PCA is an unsupervised method which creates principal components by making linear combinations of the variables while maximizing samples variation. This method is used to explore the data, by identifying natural grouping of samples, and to detect potential outliers or instrumental drift. On the other hand, the supervised (O)PLS-DA method is used in predictive modelling and allows sample discrimination by reduction of dimensions, while maximizing correlation between variables. In (O)PLS-DA models, the Y-variable (sample group) is predicted from the X-matrix (abundances of metabolites). The OPLS method differs from a PLS method by its ability to separate the systemic variation in X into two elements, namely into the predictive variation (= correlated variation between X and Y) and orthogonal variation (= uncorrelated variation between X and Y). (O)PLS-DA models are suitable to determine differences between two treatment groups and to extract the metabolites with the largest discriminatory power. Because of the high number of variables compared to the number of observations, (O)PLS-DA models are sensitive to overfitting. To avoid overfitting, (O)PLS-DA model-validity is evaluated by cross-validation or permutation testing and inspection of the R^2X, R^2Y and Q^2Y model characteristics. R^2X and R^2Y are both goodness-of-fit parameters defining the variation that is explained by the model, while Q^2Y refers to the goodness of prediction calculated by cross-validation (Westerhuis et al., 2008).

The next, but probably most challenging step in untargeted MS metabolomics is the identification step, in order to transform the information from *m/z* ratios and retention times into interpretable biological knowledge. Identification of metabolites can be based on accurate mass, isotope pattern, MS/MS fragmentation data and retention times. The accurate mass and isotope pattern generate information about the molecular formula, however, one molecular formula can match multiple chemical structures. MS/MS fragmentation data provides additional information about the chemical structure. The obtained accurate masses and/or MS/MS data can be compared with biological databases such as the Human Metabolome Database (HMDB), PubChem or Kyoto Encyclopedia of Genes and Genomes (KEGG), or with *in silico* fragmentation algorithms offering candidate structures (Metfrag, Sirius). Of course, the highest level of confidence for identification is obtained by matching the data with analytical standards. Depending on the confidence of identification, the following four levels of identification were introduced by the Metabolomics Standards Initiative (Sumner et al., 2007):

1. Identified compounds. A minimum of two independent and orthogonal data relative to an authentic compound analyzed under identical experimental conditions are proposed as necessary to validate non-novel metabolite identifications (e.g. retention time/index and mass spectrum, accurate mass and tandem MS, accurate mass and isotope pattern).

2. Putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries).

3. Putatively characterized compound classes (e.g. based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class).

4. Unknown compounds—although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data.

The final step in metabolomics studies is the interpretation of the data. Databases and software tools such as MetExplore and Metscape are available to visualize metabolites in a metabolic network and thus help in biological interpretation (Basu et al., 2017; Cottret et al., 2018).

1.5.2 Advantages of metabolomics in nutrition research

Metabolomics is a recent but expanding experimental approach in nutrition science as it is an excellent tool to investigate the relation between dietary patterns and health outcomes from a top down approach. Foods contain thousands of small molecules that can be metabolized during digestion and in turn affect endogenous metabolic pathways. Metabolite profiling of foods prior to consumption can be referred to as food-omics (Puiggròs et al., 2011), whereas nutrimetabolomics focusses on the postprandial effects of food intake in order to improve the understanding of the relationship between nutritional status and disease risk predisposition (Ryan et al., 2013; Ulaszewska et al., 2019).

The traditional biofluids of choice in nutrimetabolomics are blood and urine. The blood metabolome mainly reflects the bioavailability of nutrients since blood transports chemicals to and from tissues, whereas urine typically contains metabolic breakdown products and thus reflects clearance effects. However, also other biofluids such as feces, saliva, sweat, exhaled breath, liver tissue or gastrointestinal fluids can reflect different responses to dietary intakes. Particularly the interest in the use of fecal samples in nutritional metabolomics is growing since the fecal metabolome provides a snapshot of the reciprocal interaction between diet, host and gut microbiome (Karu et al., 2018). The dietary pattern plays a major role in

shaping the composition and activity of the gut microbiome, which could in turn influence host homeostasis (Visconti et al., 2019). The study of the relation between the gut microbiome and human health is a hot and fast evolving topic, since multiple links between the gut microbiome and the host have been established including a role in immune defense, brain-gut axis, digestion and metabolism, inflammation and cell proliferation (Doré et al., 2013). Whereas many functions of the gut microbiome are beneficial and show co-evolution with the host, a dysbiosis of gut microbial homeostasis has been correlated with the development of several nutrition-related disorders, allergies and even neurological disorders and mental health (Carding et al., 2015; Guinane & Cotter, 2013). Most research on the gut microbiota has focused on profiling the bacterial composition using 16 S sequencing, which provides information on taxonomy and possible microbial entities, but not on their actual activity as sequencing does not differentiate between alive and dead bacteria and their metabolic potential (Zierer et al., 2018). Therefore, fecal metabolomics strongly complements sequence-based approaches, as fecal metabolites not only reflect the net result of nutrient ingestion, digestion and absorption by the gastrointestinal tract, but also the metabolites that are produced by the gut bacteria. Next to the tremendous biological importance of the fecal metabolome, the use of feces is also advantageous as it is non-invasive and encloses a high number of metabolites (De Paepe et al., 2018).

The two major aims of nutrimetabolomics are the identification of biomarkers of dietary intake and an enhanced understanding of the effect of dietary compounds on host metabolism after consumption (Llorach et al., 2012; Primrose et al., 2011; Ryan et al., 2013). One of the challenges of epidemiological studies is the objective and accurate measurement of food intake. Dietary exposure is traditionally assessed with self-reporting food frequency questionnaires, which has been criticized due to errors such as underreporting, recall errors and problems with assessment of portion sizes (Kipnis et al., 2002). Therefore, there is increasing interest in finding biomarkers of dietary intake to provide a more reliable measurement. Several putative biomarkers for a range of foods such as salmon, red meat, coffee, nuts, wine, broccoli, citrus fruits, raspberry, onions and legumes have been discovered (O'Gorman et al., 2013; Scalbert et al., 2014), however, their validity should still be tested in dose-response intervention studies and be examined for their sensitivity and specificity (Brennan & Hu, 2019). In accordance with the shift from nutrients towards food items and dietary patterns in nutrition research, a next level of metabolomics application is the use of metabolite profiles to classify individuals into dietary patterns and to identify so-called nutritypes. The most frequently reported candidate biomarkers of meat intake and metabolomics related dietary pattern research are discussed in the following two paragraphs.

1.5.3 Biomarkers of meat intake

The results from the recent and extensive literature review on biomarkers of meat and seafood intake reported various isotope ratios, anserine, carnosine, 1- and 3-methylhistidine, creatine, carnitine and acylcarnitines, TMAO and taurine as main markers of animal protein intake (Table 1.1) (Cuparencu et al., 2019a). The systematic review included potential biomarkers that were obtained by both targeted as well as untargeted methodologies from interventional and observational human studies. Many of these meat-associated metabolites cannot be used as food intake biomarkers because of the lack of specificity, inter-individual variability, or endogenous formation.

The isotope ratios of nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) could be indicative for the consumption of animal or plant protein intake, since the isotope ratios in animal tissues are affected by the animals' diet but with a tissue-dependent fractionation (Kuhnle et al., 2013). Tissue nitrogen derives for the greater part from dietary protein and since animals preferentially excrete the lighter isotope ^{14}N above ^{15}N, the $^{15}N/^{14}N$ ratio increases in the food chain, which is known as nitrogen isotopic enrichment. Carbon in plants derives from atmospheric CO_2 which is fixed in organic molecules by photosynthesis. Variations in the photosynthesis process of plants determines the $^{13}C/^{12}C$ ratio in plants. C3 plants such as wheat, rice, soy and potato have a stronger preference for CO_2 containing the lighter ^{12}C isotope of carbon, whereas C4 plants such as corn and sugarcane contain a higher $^{13}C/^{12}C$ ratio. Also, marine plants have a higher $^{13}C/^{12}C$ ratio compared to C3 plants, as many phytoplankton use bicarbonate which is higher in ^{13}C compared to atmospheric CO_2 for photosynthesis. Since animals incorporate carbon from the diet in their tissues, diet is the main determinant in the $^{13}C/^{12}C$ ratios in animals (O'Brien, 2015).

Table 1.1 Most frequently reported biomarkers related to meat intake obtained from interventional and observational human studies (adopted from Cuparencu et al. (2019a)). GC = gas chromatography; TEA = thermal energy analyser; HILIC = hydrophilic interaction liquid chromatography; HPLC = high performance liquid chromatography; IEC = ion-exchange chromatography; LC = liquid chromatography; MeIQx = 2-amino-3,8-dimethylimidazol4,5-f]quinoxaline; MS = mass spectrometry; NMR = nuclear magnetic resonance spectroscopy; PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine TMA = trimethylamine; TMAO = trimethylamine-N-oxide

Biomarkers	Sample	Approach	References
General biomarkers of all meat intake			
1-Methylhistidine	Urine	LC-MS; NMR; IEC; amino acid analyzer	(Altorf-Van Der Kuil et al., 2013; Block et al., 1965; Datta & Harris, 1951; Stella et al., 2006; Xu et al., 2010)
3-Methylhistidine	Urine; plasma	LC-MS; NMR; IEC; LC-MS/MS	(Altorf-Van Der Kuil et al., 2013; Block et al., 1965; Fraser et al., 2016; Mitry et al., 2019; Myint et al., 2000; Schmedes et al., 2016; Stella et al., 2006; Xu et al., 2010)

Carnitine	Urine; serum	NMR; colorimetry	(Delanghe et al., 1989; Schmedes et al., 2016; Stella et al., 2006)
Carnosine	Urine	LC-MS; IEC	(Altorf-Van Der Kuil et al., 2013; Block et al., 1965)
Anserine	Urine; plasma	IEC; paper chromatography; LC-MS	(Block et al., 1965; Datta & Harris, 1951; Mitry et al., 2019)
Guanidinoacetate	Urine	NMR	(Bertram et al., 2007; Schmedes et al., 2016)
Creatine	Urine; blood (serum and plasma)	NMR; LC-MS/MS; colorimetry	(Bertram et al., 2007; Delanghe et al., 1989; Lau et al., 2018; Pallister et al., 2015; 2016; Stella et al., 2006)
Creatinine	Plasma; serum; urine	LC-MS; colorimetry	(Delanghe et al., 1989; Schmidt et al., 2015)
Acylcarnitine	Urine; plasma	NMR; LC-MS	(Schmedes et al., 2016; Schmidt et al., 2015; Stella et al., 2006)
TMAO	Urine; plasma	NMR;	(Krüger et al., 2017; Stella et al., 2006; Xu et al., 2010)
Taurine	Urine	NMR; IEC	(Evered et al., 1969; Stella et al., 2006; Xu et al., 2010)
Histidine	Urine	NMR	(Bertram et al., 2007)
Trans-4-hydroxyproline	Blood (serum and plasma)	LC-MS/MS	(Pallister et al., 2015; 2016; Playdon et al., 2016)
Biomarkers of mammalian (red and offal) meat intake			
TMA and TMAO	Urine; plasma	LC-MS/MS	(Cho et al., 2017; Wang et al., 2018)
1-Methylhistidine	Urine	IEC, amino acid analyser	(Cross et al., 2011; Sjölin et al., 1987)
3-Methylhistidine	Urine; plasma	IEC: amino acid analyser; LC-MS	(Cross et al., 2011; Mitry et al., 2019; Sjölin et al., 1987)
Creatine	Urine; blood (serum and plasma)	NMR; LC-MS/MS	(Pallister et al., 2016; Rådjursöga et al., 2018)
(Trans-)4-hydroxyproline	Blood (serum and plasma)	GC-MS; LC-MS/MS	(Pallister et al., 2016; Ross et al., 2015)
Carnosine	Urine; plasma	LC-MS/MS; HPLC; LC-MS	(Mitry et al., 2019; Yeum et al., 2010)
Acylcarnitine	Urine; plasma	LC-MS or GC-MS, NMR	(O'Sullivan et al., 2010; Playdon et al., 2016)
Carnitine	Urine	LC-MS or GC-MS	(Playdon et al., 2016)
Biomarkers of poultry meat intake			
3-Methylhistidine	Urine; plasma	HILIC LC-MS; LC-MS; HPLC; LC-MS/MS; IEC	(Cheung et al., 2017; Mitry et al., 2019; Myint et al., 2000; Wang et al., 2018; Yin et al., 2017)
Anserine	Urine; plasma	HILIC LC-MS; LC-MS/MS; HPLC; LC-MS	(Cheung et al., 2017; Mitry et al., 2019; Yeum et al., 2010)
Carnosine	Urine; plasma	LC-MS/MS; HPLC; LC-MS	(Mitry et al., 2019; Yeum et al., 2010)
Guanidinoacetate	Urine	NMR	(Yin et al., 2017)
Creatine	Blood (serum and plasma)	LC-MS/MS	(Pallister et al., 2016)
Biomarkers of heated or processed meat intake			
PhIP and metabolites	Urine	GC-MS; HPLC-MS; LC-MS/MS	(Strickland et al., 2002; Friesen et al., 2001; Stillwell et al., 1997; Reistad et al., 1997; Kulp et al., 2004)
MeIQx and metabolites	Urine	GC-MS; HPLC-MS	(Stillwell et al., 1997; Reistad et al., 1997)
Nitrosoproline	Urine	GC-TEA	(Stich et al., 1984)

Histidine related compounds such as carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-3-methyl-L-histidine) are contained in significant amounts in red and white meat, with carnosine being the most abundant dipeptide and anserine being more common in poultry compared to red meat. The quantification of these dipeptides and their hydrolysis products such as β-alanine and 3-methylhistidine in urine and/or plasma can reflect the intake of animal protein (Cheung et al., 2017; Khodorova et al., 2019; Mitry et al., 2019; Stella et al., 2006; Xu et al., 2010). The hydrolysis products of anserine, 3-methylhistidine (N^{π}-methylhistidine), have frequently been suggested as a biomarker for meat and particularly chicken intake, whereas another histidine derivative, 1-methylhistidine (N^{τ}-methylhistidine), reflects general meat intake, as it is formed during degradation of actin and myosin. However, the excretion of 1-methylhistidine may depend on factors other than meat consumption, since the latter is also synthesized by humans and influenced by endogenous muscle catabolism and muscle mass (Khodorova et al., 2019; Mitry et al., 2019). Due to the different numbering of the atoms in the imidazole ring in biochemistry and organic chemistry, some confusion exists in literature, but here we use the IUPAC nomenclature, where the N^{π} atom (π for nearest to the side chain) in the imidazole ring is numbered as 3.

Carnitine was named after the Latin word for flesh (carnus) when it was discovered in 1905 in meat extracts by Gulewitsch and Krimberg (1905). Carnitine is a quaternary ammonium compound that plays an essential role in energy metabolism by the transfer of fatty acids across the inner mitochondrial membrane for subsequent β-oxidation via the carnitine shuttle (Figure 1.6).

Long-chain fatty acid-CoAs in the cytosol are first conjugated to carnitine to form acylcarnitines, by carnitine-palmitoyl transferase I (CPT1), which are then transported across the mitochondrial membrane via carnitine-acylcarnitine translocase (CACT). In the mitochondrial matrix, the acylcarnitines are reconverted to the respective long chain fatty acid-CoAs via the action of carnitine-palmitoyl transferase 2 (CPT2), making the long chain fatty acid-CoAs available for fatty acid β-oxidation. Short- and medium-chain fatty acids are thought to enter mitochondria directly. Finally, carnitine acetyltransferase (CrAT) converts acetyl-CoAs back into acetylcarnitine counterparts, allowing transport from the mitochondrial matrix to the cytoplasm (Melone et al., 2018).

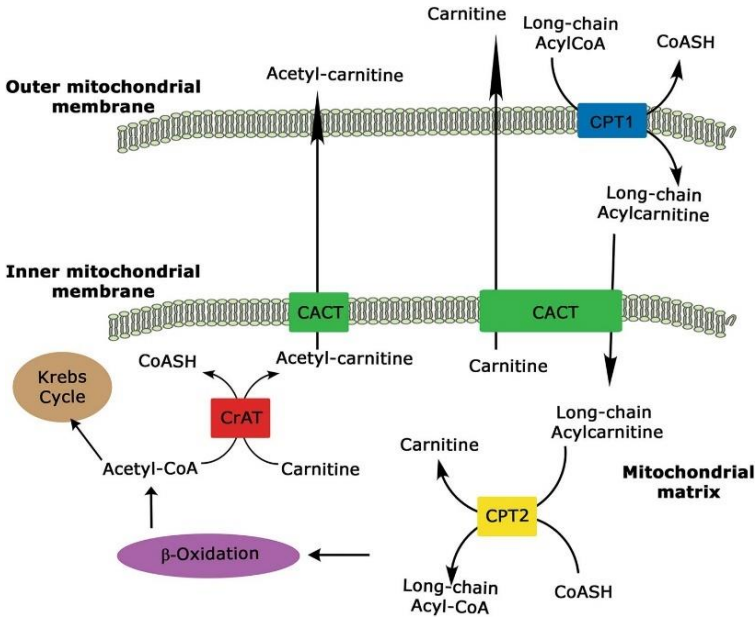


Figure 1.6 Carnitine shuttle (adapted from Melone et al. 2018). CPT = carnitine palmitoyl-transferase; CACT = carnitine/acylcarnitine translocase; CrAT =carnitine O-acetyltransferase; Acyl-CoA = coenzyme A with acylgroup; CoASH = coenzyme A without acylgroup

Most of the carnitine body pool, present as free carnitine, short-chain acylcarnitines or long-chain acylcarnitines, is localized in skeletal and heart muscle, as these tissues derive most of their energy from fat. Carnitine homeostasis in the body is maintained by absorption from the diet, endogenous biosynthesis, and efficient renal reabsorption (Rebouche, 2004). Red meat is one of the most important dietary sources of carnitine and higher concentrations of carnitine and acetylcarnitine were found in urine (Playdon et al., 2016; Stella et al., 2006) and serum (Delanghe et al., 1989) of subjects consuming high-meat diets in comparison to vegetarians. Also other acylcarnitines may reflect the intake of animal food sources (Khodorova et al., 2019; O'Sullivan et al., 2010; Playdon et al., 2016; Schmedes et al., 2016; Schmidt et al., 2015; Stella et al., 2006), however, acylcarnitine levels in plasma and urine can also be influenced by other factors such as age, gender, nutritional state, exercising and health status (Lehmann et al., 2010; Xu et al., 2016). Unabsorbed carnitine, as well as other dietary compounds containing the trimethylamine (TMA) moiety such as phosphatidylcholine and choline, can be precursors for the microbial formation of TMA and/or 3-dehydroxycarnitine (synonym for γ-butyrobetaine) (Figure 1.7). 3-Dehydroxycarnitine is primarily excreted in feces but can also be metabolized by particular species of the gut microbiota to TMA (Koeth et al., 2014). TMA is absorbed, converted to TMAO in the liver and excreted in urine (Rebouche & Chenard, 1991; Tang et al., 2019). In this regard, *in vitro* colonic digestion of beef versus chicken identified 3-dehydroxycarnitine as one of the most discriminating metabolites

characteristic for the beef digests (Rombouts et al., 2017). In addition, the influence of meat consumption on TMAO was supported by multiple studies (Cho et al., 2017; Jakobsen et al., 2017; Krüger et al., 2017; Stella et al., 2006; Xu et al., 2010), including a four-week intervention study that found higher levels of TMAO in plasma and urine in subjects consuming red meat compared to subjects consuming non-meat proteins or poultry (Wang et al., 2018). However, this metabolite is not unique to red meat, as TMAO is also naturally occurring in certain fish, and fish consumption could lead to substantially higher urinary TMAO levels compared to chicken, red meat, or processed meat intake (Cheung et al., 2017). Furthermore, TMAO formation and renal excretion is also dependent on the composition of the gut microbiota and kidney function (Cho et al., 2017; Johnson et al., 2018).

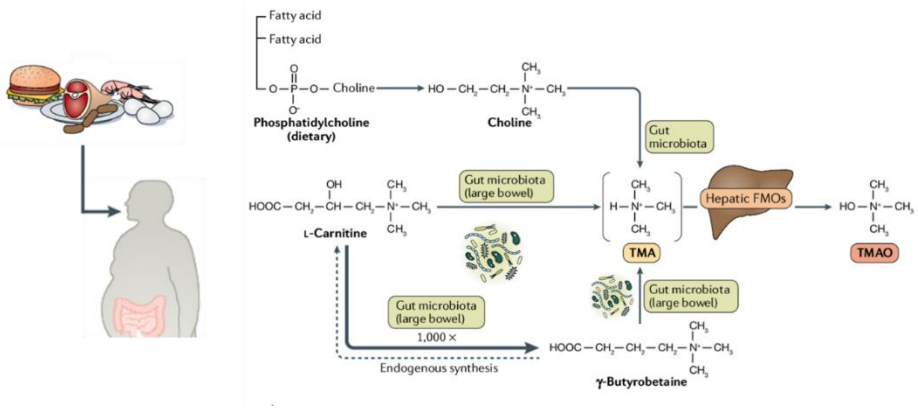


Figure 1.7 Relation between dietary trimethylamine (TMA) precursors and the formation of trimethylamine-N-oxide (TMAO) through gut microbiota transformation and liver flavin monooxygenase (FMO) enzymes. (Adopted from Tang et al., 2019)

Hydroxyproline in blood may be a general marker of consumption of animal foods rich in connective tissue, since hydroxyproline is a major component of the protein collagen and elastin, and only present in few other proteins (Pallister et al., 2015; 2016). Two other compounds that are frequently associated with meat intake are creatine and taurine. Creatine can be formed endogenously from arginine, glycine and methionine, however, the main dietary source is meat, including red meat, poultry and fish. Since elevated levels were detected in urine and blood after meat intake in multiple studies (Bertram et al., 2007; Delanghe et al., 1989; Lau et al., 2018; Pallister et al., 2015; 2016; Stella et al., 2006), creatine was suggested as a putative marker for habitual intake of meat (Cuparencu et al., 2019a). Taurine is the most abundant free amino acid in animal tissues and urinary taurine was higher in omnivorous subjects compared to vegetarians. However, this compound can also be synthetized endogenously and discrepancies between studies indicate that this marker is not robust enough to be used as food intake biomarker (Cuparencu et al., 2019a).

1.5.4 Metabolomics and dietary patterns

Instead of focusing on one specific nutritional determinant and one disease outcome, dietary patterns usually do not relate to one, but to multiple chronic diseases. Current technological advances, generating and analyzing multidimensional data, have the potential to study this complex interconnection between diet and health in a more holistic manner by focusing on dietary patterns (Collins et al., 2019). Two approaches can be used to derive dietary patterns from observational studies: *a priori* (based on current knowledge) and *a posteriori* (data driven). *A priori* defined patterns use scoring systems to assess the degree to which a subject complies with a predefined dietary pattern. Several theoretical scoring systems have been developed such as the Healthy Diet Indicator (HDI) (Huijbregts et al., 1997) or the modified Mediterranean Diet Score (mMDS) (Trichopoulou et al., 1995) to assess the adherence to the specific dietary recommendations defined by the WHO and a Mediterranean type like diet, respectively (Waijers et al., 2007). On the other hand, *a posteriori* or empirically derived dietary patterns are based on statistical analysis of actual collected food consumption data by using dimensional reduction or cluster analysis. Dimensional reduction by, for example, PCA tends to extract dietary patterns based on the correlation between food groups, whereas cluster analysis aims to aggregate individuals into relatively homogenous subgroups that share a similar dietary pattern. Both approaches led to the identification of two main dietary patterns in the EPIC-NL cohort, namely the so-called 'Western' and 'prudent' dietary pattern, reflecting a more unhealthy and healthy diet respectively (Stricker et al., 2013). The Western dietary pattern was characterized by high intakes of French fries, fast food, low-fiber products and soft drinks, whereas the prudent pattern was represented by high intakes of fish, vegetables and high-fiber products.

Several reviews summarized the current literature on metabolic signatures of dietary patterns or dietary interventions using metabolomics (Table 1.2) (Collins et al., 2019; Guasch-Ferre et al., 2018; Steffen et al., 2014). The dietary patterns included vegetarian and lactovegetarian diets, omnivorous diets, Western dietary patterns, prudent dietary patterns, Nordic diet and Mediterranean diet. Metabolomics can be used to discover biomarkers of *a priori* or *a posteriori* defined dietary patterns, but the identified biomarkers or the models obtained from the metabolomics studies can in turn be used to determine dietary patterns and to classify participants according to dietary patterns.

Since meat is an important determinant in the classification of dietary patterns, many of the studies and identified biomarker candidates have already been mentioned in the previous paragraph, discussing candidate biomarkers of meat intake.

Table 1.2 Dietary patterns related to metabolomic biomarker candidates (adopted from Collins et al., 2019; Guasch-Ferre et al., 2018 and Steffen et al., 2014). DP = dietary pattern; FIA = flow injection analysis; HPLC = high performance liquid chromatography; LC = liquid chromatography; MS = mass spectrometry; NMR = nuclear magnetic resonance spectroscopy; qTOF = quadrupole time-of-flight; TMA = trimethylamine; TMAO = trimethylamine-N-oxide

Dietary intervention/pattern	Results/biomarkers	Approach	Sample	References
DP1: vegetarian DP2: low meat DP3: high meat	DP1: p-hydroxyphenylacetate DP3: taurine, carnitine, acetylcarnitine, 1-methylhistidine, 3-methylhistidine, TMAO	NMR	Urine	(Stella et al., 2006)
DP1: lactovegetarian men DP2: lactovegetarian women DP3: omnivorous men DP4: omnivorous women	DP1: citrate DP3: TMAO, dimethylamine, phenylalanine, methylhistidine	NMR	Urine	(Xu et al., 2010)
DP1: high in vegetables, whole-grain, fish DP2: high in fruits, low-fat dairy, poultry DP3: high in meat products, refined grain, butter, low in fruits and vegetables	DP1: glycine, phenylacetylglutamine, acetoacetate DP3: TMAO, acetylcarnitine, N,N-dimethylglycine	NMR	Plasma and urine	(O'Sullivan et al., 2010)
DP1: high in fish and poultry, fruit, low in meat and snacks DP4: traditional Bavarian: high in meat, fish, snacks and low in potatoes, pasta, whole-grain DP6: high in whole-grain, eggs, milk, low in fish, fruit	DP1: less saturation of fatty acid side chains of glycerophosphatidylcholines DP4 and DP6: associated with several phospholipids	MS/MS	Plasma	(Altmaier et al., 2011)
DP1: high phytochemical diet DP2: diet free of fruits and vegetables	DP1: sulforaphane, hippuric acid, genistein, daidzein, equol, glycitein	LC-MS/MS	Urine	(May et al., 2013)
DP1: prudent (high in vegetables, fruits, whole-grain products) DP2: Western (high in refined grain products, desserts, sweets, processed meats)	DP2: short-chain acylcarnitines, leucine, phenylalanine	MS	Plasma	(Bouchard-Mercier, Rudkowska, Lemieux, Couture, & Vohl, 2013)
DP1: high in potatoes, dairy, cornflakes DP2: high in red meat, fish, low in whole grain DP3: high butter and low margarine intake	DP1: methionine, branched chain amino acids DP2: hexose, phosphatidylcholine DP3: acylcarnitines, acyl-alkyl-phosphatidylcholines and hydroxy-sphingomyelins	(FIA)-MS/MS	Serum	(Floegel et al., 2013)
DP1: high in fruits, vegetables DP2: high in low-fat dairy, low in sweets	DP1: sphingomyeline C26:1 and phosphatidylcholines DP2: phosphatidylcholines Acylcarnitine C9 was associated with hypocaloric dieting	(FIA)-MS/MS	Serum	(Menni et al., 2013)
DP1: New Nordic diet DP2: Average Danish diet	DP1: TMAO, hydroquinone-glucuronide and hippuric acid	UPLC-qTOF-MS	Urine	(Andersen et al., 2014)
DP1: healthy diet, whole grains, fatty fish DP2: whole-grain products DP3: control refined wheat bread	DP1: increased polyunsaturation of plasma fatty acids, increase in n-3 fatty acids, whereas n-6 and n-7 fatty acids decreased	NMR	Serum	(Lankinen et al., 2014)
DP1: meat eaters DP2: fish eaters DP3: vegetarians DP4: vegans	DP1: highest concentrations of 6 acylcarnitines, 61 glycerophospholipids, and 12 sphingolipids. DP2 and DP3: highest concentrations of amino acids and biogenic amines	LC-MS/MS	Plasma	(Schmidt et al., 2015)
DP1 and DP2: Mediterranean diet supplemented with olive oil or with nuts	DP1 and DP2: carbohydrates, creatine, creatinine, amino acids (proline, glycine, branched chain	NMR	Urine	(Vázquez-Fresno et al., 2014)

DP3: control low-fat diet	amino acids), lipids (oleic and suberic acids) DP3: hippurate, TMAO, histidine, methylhistidines, carnosine, anserine, xanthosine			
4 diets differing in compliance to the WHO healthy eating guidelines DP1: most concordant with guidelines DP4: least concordant with guidelines	DP1: hippurate, N-acetyl-S-methyl-cysteine-sulfoxide, dimethylamine, TMAO, methylhistidines DP4: acetylcarnitine, carnitine, creatine, glucose	NMR	Urine	(Garcia-Perez et al., 2017)
DP1: Healthy Eating Indes DP2: Alternate Mediterranean Diet Score DP3: Healthy Index Dietary Indicator DP4: Baltic Sea Diet	Differences in amino acids, cofactors or vitamins, lipids, exogenous xenobiotics and carbohydrates	UPLC-LC-MS/MS	Serum	(Playdon et al., 2016)
DP1: low-energy DP2: plant-based versus animal-based DP3: traditional Danish versus high sugar diet DP4: Danish versus modern DP5: Skim milk versus full fat milk products	DP1: more phosphatidylcholine DP2: animal-based: more lysine, arginine, glutamine, threonine, aspartate, citrate, polyol compounds DP3: Danish diet: more lysine, arginine, glutamine, glycine, phosphatidylcholine; high sugar diet: more triglycerides DP4: Danish diet: more alanine, lysine, glycine, phosphatidylcholine DP5: full fat milk products: more tyrosine and histidine	NMR	Plasma	(Peré-Trepat et al., 2010)
DP1: New Nordic diet DP2: Average Danish diet	DP1: pipecolic acid, betaine, TMAO, prolyl hydroxyproline, polyunsaturated phosphatidylcholines DP2: indolelactic acid, hydroxy-3-methylbutyrate, butyrylcarnitine	UPLC-qTOF-MS	Plasma	(Acar et al., 2019)
DP1: low fat DP2: low glycaemic index DP3: very low carbohydrate	Identified 152 metabolites whose concentrations differed for ≥1 diet compared with the others.	LC-MS/MS	Plasma	(Esko et al., 2017)
DP1: healthy cluster DP2: unhealthy cluster	Two-step cluster analysis was applied to the urinary data to identify clusters. Validation of the subsequent model revealed that 94% of subjects were correctly classified.	NMR	Urine	(Gibbons et al., 2017)

1.6 Different levels of nutrition research

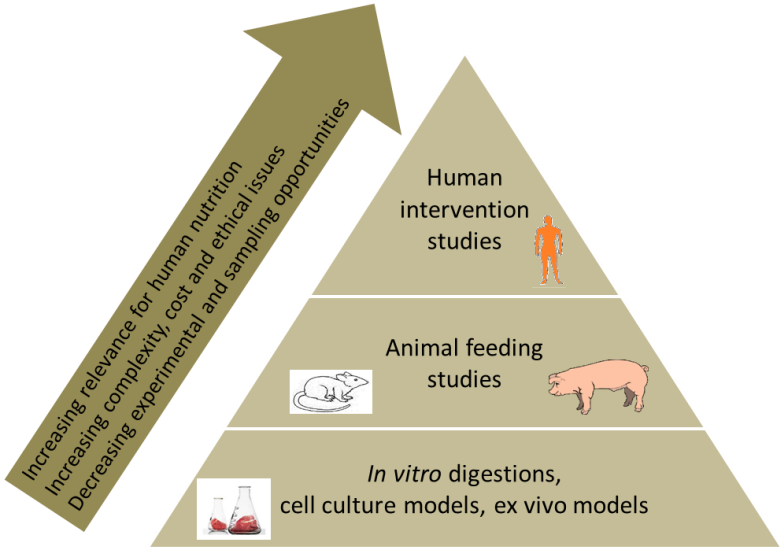
Nutrition research can be defined as the study of the interaction between diet (as whole diets, dietary components, dietary patterns) and the human body at the individual (cell, tissue and organism) or population level, in order to investigate the impact on normal biological function, health status or development of diseases. A classification of research methodologies can be made dependent on different criteria such as the strategy of data collection (retrospective or prospective), the degree of control (observational or experimental), the design (case-control, cohort

or cross-sectional) and the model (*in vitro, ex vivo* or *in vivo*, and animals or human subjects) (Martinez & Martinez-Gonzales, 2006).

Usually, observational epidemiological studies provide information on disease prevalence and diagnosis and generate hypotheses about disease etiologies. A main advantage is that findings from observational epidemiological studies are directly relevant to humans, in contrast to the results from *in vitro* or animal experiments. However, limitations of observational studies comprise the potential of residual confounding and the lack to establish causality (Patro-Gołąb & Szajewska, 2013). Experimental studies, on the other hand, provide stronger evidence for the effect of an exposure on an outcome, as they are conducted to test hypotheses, prove causality and to identify the underlying mechanisms by using *in vitro* or *in vivo* models. Experimental studies tend to use a reductionist approach by studying the effect of a specific exposure in a specific setting. The less controlled conditions in observational studies makes them more prone to confounding, however, they could also reveal potential combined effects that could have been missed by a reductionistic approach. Therefore, it is important to combine multiple approaches and to consider the strengths and limitations of the individual studies.

Processes involved in nutrient utilization, including digestibility, absorption, metabolism, excretion and some nutrient functions, are frequently investigated by using *in vitro* approaches that mimic a certain *in vivo* function. Common *in vitro* approaches used in nutrition research are *in vitro* digestion models, and everted intestine preparations or cell culture models. Whole animal systems have been used to assess nutrient utilization and fate, and nutrient functions. In many situations, animals are used as a substitute for humans. Using animals offers the advantages of feeding strictly controlled diets and to reduce intrinsic variability due to genetics and environmental conditions. Moreover, using animals permits sampling of biofluids, tissues and organs and minimizes costs and ethical constraints compared to human studies. Nevertheless, the selection of a suitable animal model and data extrapolation from animals to humans should be carried out with care, as the animal's metabolism and physiology is different from humans (Martinez & Martinez-Gonzales, 2006). Rats and mice are the most common laboratory animals, because of their availability, size, low costs, fast reproduction and ease of handling. However, except for nonhuman primates, the pig is one of the best animal models for nutrition research. The pig is an omnivorous animal with comparable nutritional requirements to humans and with a similar digestive anatomy and physiology (Miller & Ullrey, 1987; Roura et al., 2016). Several studies demonstrated the potential of pigs as a model for human metabolic studies in food research, as very similar postprandial responses in blood metabolome (Nielsen et al., 2014) and similar changes in microbial composition between the two species were found upon dietary

intervention (Heinritz et al., 2016). Moreover, because of the important role of the gut microbiome in digestion and health, human microbiota-associated pig models have been established to investigate how the gut microbiome is influenced by the diet and, in turn, impacts host health and disease (Wang & Donovan, 2015).



Experimental studies	Testing hypotheses and identifying mechanisms
Observational epidemiology	Obtain disease information, measure prevalence, develop hypotheses about disease etiologies

Figure 1.8 General overview of the different levels of nutrition research

Chapter II

Impact of red versus white meat consumption in a prudent or Western dietary pattern on the oxidative status in a pig model

Adapted from:

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Own contribution: input in study design, responsible for the formulation and preparation of the diets and the animal experiment, carrying out most of the laboratory analyses, interpretation of results and writing the manuscript with input of all co-authors.

Chapter II - Impact of red versus white meat consumption in a prudent or Western dietary pattern on the oxidative status in a pig model

Abstract

Human diets contain a complex mixture of antioxidants and pro-oxidants that contribute to the body's oxidative status. In this study, 32 pigs were fed chicken versus red & processed meat in the context of a prudent or Western dietary pattern for four weeks, to investigate their oxidative status. Lipid oxidation products (malondialdehyde, 4-hydroxy-2-nonenal and hexanal) were higher in the chicken versus red & processed meat diets (1.7- to 8.3-fold) and subsequent *in vitro* (1.3- to 1.9-fold) and *in vivo* (1.4 to 3-fold) digests ($P < 0.001$), which was presumably related to the higher polyunsaturated fatty acid content in chicken meat and/or the added antioxidants in processed meat. However, diet only had a marginal or no effect on the systemic oxidative status as determined by plasma oxygen radical absorbance capacity, malondialdehyde, glutathione and glutathione peroxidase activity in blood and organs, except for α-tocopherol, which was higher after the consumption of the chicken-Western diet. In conclusion, in contrast to the hypothesis, the consumption of chicken compared to red & processed meat resulted in higher concentrations of lipid oxidation products in the pig intestinal contents, however, this was not reflected in the body's oxidative status.

Keywords

red meat, oxidative stress, pigs, malondialdehyde, 4-hydroxy-2-nonenal

2.1 Introduction

Several large-scale epidemiological studies have associated a high consumption of red and especially processed meat with a higher risk of developing various chronic diseases (Bouvard et al., 2015; Micha et al., 2012). As opposed to red and processed meat, no such associations have been reported for the consumption of poultry meat (Norat et al., 2005). In addition, higher consumption of foods such as whole grains and fruits and vegetables have been linked with lower risks for chronic diseases (Boeing et al., 2012; Seal & Brownlee, 2015). However, foods are usually not consumed separately and higher intakes of certain foods are often associated with lower intakes of other foods. Furthermore, the cumulative and interactive effects of multiple components in the diet may attenuate or exert stronger effects than the sum of the single components (Jacobs & Steffen, 2003). The 2015 Dietary Guidelines Advisory Committee identified a healthy diet as a diet high in fruits and vegetables, whole grains, low- or nonfat dairy, seafood, legumes and nuts and low in red and processed meat, sugar-sweetened foods and drinks and refined grains (USDA, 2015). The dietary guidelines in Western Europe and Unites States carry this same central message, however, despite the recommendations, a Western diet characterized by high amounts of red and processed meat, refined grains, sweets, sugar drinks and fried food is still very popular in these countries.

Iron plays a central role in the human body since it exerts various physiological functions such as oxygen transport, DNA synthesis and electron transport. Although dietary iron intake is important in the prevention of anemia, the intake of high heme iron levels by high red meat consumption is hypothesized to stimulate the formation of oxidation products. An imbalance between ROS and antioxidant defense is defined as oxidative stress and is a common factor in many pathological conditions (Willcox et al., 2004). Iron is able to initiate and catalyze the Fenton reaction in the presence of hydrogen peroxide, through which ROS such as hydroxyl radicals are formed. These ROS are highly unstable and can initiate further oxidative chain reactions affecting PUFA and proteins, resulting in the formation of oxidized proteins and lipid oxidation products, of which some are potentially harmful (Esterbauer et al., 1991). Oxidation of n-6 PUFA leads to the specific formation of 4-HNE and hexanal, whereas MDA originates from both the oxidation of n-3 or n-6 PUFA. Previous studies demonstrated the formation of lipid and/or protein oxidation products during the digestion of red meat (Van Hecke et al., 2014a, 2016). However, not only meat, but also other dietary compounds are important sources of pro- and antioxidants, able to affect the extent of oxidation during the digestion of meals. Several *in vitro* and *in vivo* digestion experiments with red meat already demonstrated antioxidant effects of multiple herbs, spices, fruits, vegetables and beverages (Kanner et al., 2017; Pierre et al., 2013; Sirota et al., 2013; Van Hecke et

al., 2017b). Therefore, the rationale of the present study is that the formation of oxidation products during meat digestion is influenced by the dietary context in which meat is consumed. For example, Bastide et al. (2016) already concluded that the epidemiological link between red meat consumption and colorectal cancer depended on the ratio of heme iron to antioxidants in the diet. The review of Turner & Lloyd (2017) also addressed the need to compare meat consumption included in a healthy versus unhealthy dietary pattern.

Pigs offer a valuable model for human nutritional studies. Pigs and humans share many anatomical and physiological similarities related to the gastrointestinal tract resulting in similar nutritional requirements and digestive and metabolic processes (Roura et al., 2016). Furthermore, the ability of feeding a strictly controlled diet for several weeks and invasive sampling is a great advantage of using a pig model over a human intervention trial. Previously, (mini)pigs have been used to investigate the effect of meat-based diets albeit in combination with fruits and vegetables on oxidation (Gobert et al., 2014; Rezar et al., 2006). These studies concluded that the supplementation of fruits and vegetables could mitigate oxidation compared to the diets lacking fruits and vegetables. However, to the best of our knowledge, the relevance of meat consumption as part of a typical Western-style diet or a realistic healthy diet and its contribution to oxidative stress has not been investigated yet.

Therefore, the aim of the present study was to determine the impact of meat on the oxidative status in the context of either a prudent diet, conform current dietary guidelines, or a Western diet, representing the average dietary intake of the Belgian population. In this study, it was hypothesized that the consumption of red meat compared to chicken meat would lead to more oxidation products during digestion, and that this effect would depend on the dietary context of meat consumption. To that end, a four-week pig feeding study investigating meat type (red & processed meat, both associated with chronic diseases, versus chicken meat as control meat) and background diet (prudent versus Western background diet) was performed using a 2×2 factorial design comprising of the following four diets: chicken-prudent, red&processed-prudent, chicken-Western and red&processed-Western. The oxidative status was measured in blood and organ tissues, and formation of lipid and protein oxidation products during *in vitro* and *in vivo* digestion of the diets was assessed. The *in vitro* digestion was performed to compare the *in vitro* results with the *in vivo* study.

2.2 Materials and methods

2.2.1 Chemicals

All enzymes used in the *in vitro* digestion of the diets, namely α-amylase from porcine pancreas (~50 U/mg), mucin from porcine stomach type II, pepsin from porcine gastric mucosa (> 250 U/mg solid), lipase from porcine pancreas type II (10-400 U/mg protein), pancreatin from porcine pancreas (8 × USP specifications) and porcine bile extract as well as reagents used in the analytical procedures such as Trolox, 1-fluoro-2,4-dinitrobenzene, NADPH, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP), 2,4-dinitrophenylhydrazine (DNPH), 4-HNE, hexanal and 1,3-cyclohexanedione were purchased from Sigma Aldrich (Diegem, Belgium). The radical initiator 2,2'azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Across Organics (Geel, Belgium).

2.2.2 Diet formulation

Table 2.1 demonstrates the formulation of the four diets that were formulated to provide an equal meat intake per day. *Ad libitum* fed pigs tend to adapt their daily feed intake according to the energy content of the diet, resulting in a relatively constant daily energy intake. Since the Western diets are more energy dense compared to the prudent diets (approximately 1750 kcal/kg versus 1250 kcal/kg respectively, as shown in Table 2.2), it was expected that the pigs would consume higher absolute amounts of the prudent diets. The meat fraction (expressed as g meat/kg diet) is lower in the prudent diets compared to the meat fraction in the Western diets in order to provide an equal meat intake in all treatments. As piglets consume an equal amount of energy, expressing the proportion of food items in energy percentages, represents the relative proportions of the food items in the diets that are actually consumed. More than 90 food items (such as fruits, vegetables, bread, potatoes, rice and milk) and different methods of preparation (raw, cooking, grilling, frying) were used to mimic the diversity and nutrient levels of complex human diets. A detailed list of ingredients is provided in Supplemental Table S2.1. The amount of meat was based on the results from the Belgian Food Consumption Survey 2004, that reported a daily intake of 291 g of 'meat, fish, eggs and meat alternatives' at the 97.5 percentile of the Belgian population older than 15 years (De Vriese et al., 2005). Combined with the other food items, this fraction of meat corresponded to 21.5% of the total energy of the diets. The mixture of red & processed meat contained 62% fresh meat (mainly pork and beef) and 38% red processed meat (mainly cooked ham, filet de sax, salami and smoked bacon), which is a proportion based on the Belgian Food Consumption Survey 2004.

Table 2.1 Formulation of the four diets expressed in g/kg (fresh weight basis) and energy percentage (E%)

	prudent		Western		prudent		Western	
	chicken	R&P	chicken	R&P	chicken	R&P	chicken	R&P
	g/kg				E%			
red (62%) & red processed meat (38%)		145		197		21.5		21.5
chicken meat	145		197		21.5		21.5	
cereal products and potatoes	304	304	199	199	45.6	45.6	23.4	23.4
fruits and vegetables	299	299	173	173	10.4	10.4	4.50	4.50
dairy products and eggs	243	243	143	143	15.1	15.1	12.7	12.7
butter and cooking fats	8.62	8.62	14.3	14.3	7.06	7.06	8.54	8.54
fats and sweets	0.51	0.51	275	275	0.33	0.33	29.4	29.4

R&P = red & processed meat

Chicken thighs and breasts made up the chicken meat. To obtain isocaloric meat fractions in the diets and to provide a similar proportion of fat in both meat groups, chicken skin was added to chicken meat resulting in a final fat percentage of 9.6% in both meat types. The meat was collected from local supermarkets, slaughterhouses and meat processing plants. The raw meat from both meat types was prepared in a similar way by either baking (70%) or vacuum cooking (30%) and were stored (3-4 weeks) at -20 °C before addition to the background diets. The formulation of the Western background diet was based on the average food intake of the Belgian population and characterized by high amounts of refined grains, desserts and sweets (De Vriese et al., 2005). On the other hand, the prudent background diet was formulated based on the dietary guidelines of the Flemish active food guide pyramid (applied in the period 2005-2017) and was characterized by high amounts of fruits, vegetables, whole grains and dairy products (Vlaams Instituut voor Gezondheidspromotie, 2005). The Nubel databank, containing the nutrient composition of more than 3678 food items, was used to design the diets and to estimate their nutritional value (www.internubel.be). Table 2.2 provides the estimated macronutrient composition of the formulated diets according to Nubel and the analyzed composition of the experimental diets. As piglets were used as a digestion model for humans, and since the formulation of the diets was based on human diets, the composition of the diets was also expressed in equivalents for human consumption in Table S2.1.

All food items were mixed to avoid selection of particular food items by the piglets and to obtain homogenous diets. Because of the substantial volume (2000 kg in total), the food items of the background diets were collected from local supermarkets and were prepared over several weeks and stored at -20 °C. After collection and preparation, the food items were allocated to subgroups such as fruits, vegetables, fats & sweets, whole grains and refined grains. Within each subgroup, the frozen food items were grounded through a 10 mm plate equipped

Omega T-12 grinder, carefully mixed using a concrete mixer and immediately stored at -20 °C. Finally, the four diets were made in 20 batches of 100 kg per batch by mixing the subgroups according to the tailored proportions in the diets. The meat fractions were added last. Just before adding the meat fractions, separate samples of the background diets and the meat fractions were taken to analyze dietary characteristics of the meat and background diets as such, in addition to the analysis of the final diets. The resulting homogenous feed mixtures were divided in meal portions of 500 g to 1 kg and stored at -20 °C (with a maximum storage time of 3 months) until feeding. The temperature of the diets remained below 2 °C during the whole preparation process. Aliquots of the background diets, meat and final diets were stored at -20°C until the end of the feeding trial. Right after the trial, the feed aliquots were stored at -80°C until analysis to take eventual oxidation of the diets during storage at -20°C into account. All diet analyses were performed in duplicate on two randomly selected feed aliquots.

Table 2.2 Formulated (estimated using Nubel) and analyzed nutritional composition of the four diets

	formulated				analyzed			
	prudent		Western		prudent		Western	
	chicken	R&P	chicken	R&P	chicken	R&P	chicken	R&P
energy, kcal/kg	1089	1089	1482	1482	1263	1265	1743	1726
proportion of energy from proteins, %	22.6	23.8	18.1	19.3	20.7	20.4	17.7	16.4
proportion of energy from fat, %	29.2	28.8	40.2	39.6	22.5	24.9	32.5	35.6
crude protein, g/100 g	5.99	6.40	6.57	7.15	6.44	6.35	7.61	6.97
crude fat, g/100 g	3.45	3.46	6.53	6.54	3.13	3.47	6.24	6.76
dry matter, g/100 g	25.5	24.7	33.3	32.2	26.0	26.1	30.9	31.2

R&P = red & processed meat

Energy of the formulated diets corresponds with metabolizable energy and was estimated by the use of Atwater factors, whereas the analyzed energy corresponds with gross energy as determined by the amount of heat that was liberated in a bomb calorimeter.

2.2.3 Dietary characteristics

The pH was recorded and dry matter, crude protein and crude fat content were analyzed according to ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods, respectively. Gross energy was determined with an adiabatic bomb calorimeter (PARR 1261). Fatty acids were measured according to Raes et al. (2001). Briefly, lipids were extracted using chloroform/methanol (2/1; v/v) followed by methylation and GC to separate the fatty acid methyl esters. From the fatty acid composition, the peroxidizability index was calculated as an indication for its susceptibility to lipid peroxidation (Hu et al., 1989). Total heme and nitroso-heme were determined colorimetrically in the meat following its extraction in respectively acetone/H_2O/HCl 12M (40/4/1) and acetone/H_2O (40/5) according to Hornsey (1956).

2.2.4 In vitro digestion

The four diets were subjected to an *in vitro* digestion protocol as previously described by Van Hecke et al. (2014b) to better characterise the diets in terms of susceptibility to oxidation during digestion. In short, diet samples (4.5 g) were incubated consecutively with several enzymatic digestion buffers: 6 mL saliva (5 min), 12 mL gastric juice (2 h), followed by 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile juice (2 h). After the last incubation step, samples were homogenized with an Ultra Turrax (13500 rpm) and aliquots of 1 mL were stored at -80 °C. Incubations were performed in quadruplicate at 37 °C and for each diet, the four *in vitro* digest samples were used as replicates in further analysis.

2.2.5 Study design and animals

The experimental protocol was approved by the Ghent University Ethical Committee at the Faculty of Veterinary Medicine (EC 2016/26). Thirty-two castrated male piglets (Topigs×Piétrain) of three weeks old and originating from eight litters were purchased from a local commercial farm and allocated to eight pens. Each dietary treatment was repeated in two pens (n = 8 pigs per diet) and each diet was given to one of the piglets of each litter. On arrival, all piglets were fed a commercial pre-starter diet *ad libitum* for one week. Thereafter, the dietary treatments were gradually introduced during one week, after which the piglets were subjected to the dietary treatments for four weeks. The piglets were weighed twice a week and the average body weight at the start of the experimental feeding period did not differ significantly between the treatment groups (7.00 ± 0.88 kg). During the experimental feeding period, piglets were fed fresh meals at room temperature three times a day (8, 12, 18 h) during 30 min *ad libitum*. An individual feeding system was used to record their individual feed intake per meal. After every feeding period, leftovers were weighted. Since the meals consisted of homogenous mixtures to prevent selection of particular food items, the nutritional composition of the actual feed intake was assured to be very similar to the analysed composition of the feed. Water was always available *ad libitum*. Piglets were sampled at the end of the four-week feeding period, though due to practical reasons, euthanasia and sampling was spread over four consecutive days. Each day, two piglets from each dietary treatment were slaughtered, starting with the heaviest piglets. Two hours after receiving a last meal, piglets were euthanized by intramuscular injection of xylazine (20 mg/mL, 0.1 mL/kg bodyweight) and intraperitoneal injection of pentobarbital (60 mg/mL, 0.8 mL/kg bodyweight) followed by exsanguination. Postprandial blood samples were taken from the jugular vein just before exsanguination and collected in heparin tubes. Plasma and red blood cells were separated by centrifugation (1000 G, 15 min) and stored at -80 °C. Subsequently, all small and large intestinal contents were collected

separately, the pH was measured and the contents were gently mixed and stored at -80 °C. Organs (heart, liver (lobus hepatis dexter medialis), kidney) and tissue segments of ileum, proximal and distal colon were removed, weighted and gently rinsed with 0.9% NaCl solution. A 10 cm^2 segment of the ileum, proximal and distal colon tissue was scraped to collect mucosa. Diagonal sections of the tissue segments of the gastrointestinal tract were fixed in 10% formaldehyde buffer for histological examination. The organs and intestinal mucosae were chopped and homogenized before storage at -80 °C. All analyses on these biological samples were performed in duplicate. Before the analysis of thiobarbituric acid reactive substances (TBARS) and GSH-Px activity in organs and mucosae, buffered aqueous extracts were made by adding a 1% Triton X-100 phosphate buffer (pH 7; 50 mM) in a 1/10 (w/v) proportion to the organs and mucosae, followed by homogenization (Ultra Turrax, 13500 rpm), centrifugation (13800 G, 15 min, 4 °C) and subsequent filtration over glass wool.

2.2.6 Antioxidant capacity

The hydrophilic oxygen radical absorbance capacity (ORAC) of the diets and plasma was measured fluorimetrically (excitation at 485 nm and emission at 520 nm) and results were reported as µmol Trolox Equivalents (TE) per g diet and per mL plasma (Prior et al., 2003). Alpha-tocopherol in the diets, heart, liver, kidney and plasma was extracted with ethanol and hexane and quantified by reversed-phase HPLC and UV detection (292 nm) (Claeys et al., 2016; Desai, 1984). Concentrations of α-tocopherol (µg α-tocopherol/g sample) were determined by comparing the peak areas with a standard curve of α-tocopherol (0 – 15 µg/mL). Levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined in lysed erythrocytes and liver tissue using HPLC separation and UV detection (365 nm) (Reed et al., 1980; Yoshida, 1996). Results were expressed as area under the curve/g or mL sample. The GSH-Px activity was measured in plasma and buffered aqueous extracts from organs and gastrointestinal tissues (Hernández et al., 2004). The oxidation of NADPH was measured colorimetrically (340 nm) and recorded over time. One unit of GSH-Px activity was defined as the amount of sample (g) required to oxidize of NADPH per min at 25 °C.

2.2.7 Oxidation products

Total MDA concentrations in plasma and in buffered aqueous extracts of heart, liver, kidney and intestinal mucosae were analysed as TBARS according to the method of Grotto et al. (2007) with slight modifications. TBARS were determined after several reaction steps involving alkaline hydrolysis, acid deproteinization, derivatization with TBA and extraction with 1-butanol. The absorbance of the carbonyl-TBA complexes was measured colorimetrically at 532 nm, quantified using a standard curve with

TMP (0 – 30 nmol/ml) and expressed as nmol MDA-equivalents/g sample. Because of the complexity of the diets, a more specific HPLC method was applied to determine free reactive MDA in the diets, *in vitro* digests and intestinal contents according to Mendes et al. (2009). The samples were extracted with trichloroacetic acid (7.5%) and derivatized with DNPH. In case of the *in vitro* digests, the resulting MDA-DNPH hydrazine was subsequently separated by HPLC analysis, whereas for the diets and the intestinal contents, the MDA-DNPH was first concentrated through extraction with pentane, followed by evaporation to dryness with N_2 and resolved in 1 mL acetonitrile/H_2O (1/1, v/v) prior to HPLC analysis and UV/VIS detection (310 nm). Standard solutions (0 – 20 nmol/ml) were made with TMP and results were expressed as nmol MDA/g sample.

Levels of 4-HNE and hexanal were analyzed in the diets, *in vitro* digests and intestinal contents after formation of their fluorescent derivatives with 1,3-cyclohexanedione through HPLC as described previously (Van Hecke et al., 2017b). Concentrations were determined by comparing the peak areas with those obtained from the standard curves of 4-HNE (0 – 2 nmol/mL) and hexanal (0 – 40 nmol/mL) and results were provided in nmol/g sample.

PCC concentrations, indicating the extent of protein oxidation, were measured colorimetrically in diets, *in vitro* digests and small intestinal contents following their covalent reaction with DNPH according to Ganhão et al. (2010). PCC was not measured in the colonic contents because of the high digestion rate of dietary protein and the interference with bacterial protein. The carbonyl concentration (nmol DNPH/mg protein) was calculated from the absorbance at 280 nm and 370 nm of the samples using the equation below (Levine et al., 1994):

$$\frac{C_{hydrazone}}{C_{protein}} = \frac{A_{370}}{\varepsilon_{hydrazone,370} \times (A_{280} - A_{370} \times 0.43)} \times 10^6$$

Where $\varepsilon_{hydrazone,370}$ is 22000 M^{-1} cm^{-1} and the carbonyl concentrations obtained from the blanks were subtracted from the contribution obtained from the corresponding treated sample.

2.2.8 Intestinal morphology

Cross-sections of ileum, proximal and distal colon samples immersed in formalin solution were dehydrated, embedded in paraffin wax and cut into slides of 3 µm thickness using a microtome. Hematoxylin-eosin staining was performed to observe the morphology of the gut tissues. Olympus Cell B software (Olympus) attaches to an Olympus CX41 microscope at 10x magnification was used to measure villus length and crypt depth of the ileum. A minimum of 4 well-oriented and intact villi and crypts

was measured in three ileum cross-sections of each pig. The crypt depth of the proximal and distal colon was measured in at least 18 crypts per animal.

2.2.9 Statistical analyses

Data are presented as means ± SD, unless otherwise indicated. Data normality and homoscedasticity were verified using Kolmogorov-Smirnov and modified-Levene's test in SAS enterprise guide 7. If assumptions were met, a mixed model ANOVA procedure was used with meat, background diet and meat×background diet as fixed effects and litter, pen and euthanization day as random effects (if applicable). Tukey-adjusted *post hoc* tests were performed for all pairwise comparisons. If one of the assumptions for the use of ANOVA was violated, the non-parametric independent-samples Kruskal-Wallis test with pairwise multiple comparisons was performed using SPSS Statistics 25. P values < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Diet characterization

The analyzed nutritional composition of the diets presented in Table 2.2 was similar to the formulated nutritional values estimated according to the Nubel nutrient database (www.internubel.be). It is important to consider both the proportions of the nutrients in the diets (in g/100 g) as well as the actual nutrient intake (represented by the proportion of energy), since the piglets tend to consume a similar amount of energy, despite the differences in energy density in the diets. The Western diets had a higher energy density, higher fat content, more SFA, less MUFA and a higher n-6/n-3 fatty acid ratio compared to the prudent diets. The protein content expressed in g/100 g was slightly higher in the Western diets compared to the prudent diets. However, the actual protein intake was higher in the piglets that consumed the prudent diets, since the piglets tend to consume more of the less energy dense prudent diets.

A similar crude fat content of 9.6 g/100 g in both meat types was obtained by adding 9% of chicken skin (45% crude fat) to chicken meat (6% crude fat). Compared to the red & processed meat, chicken meat contained lower proportions of SFA and higher proportions of n-6 PUFA, a higher n-6/n-3 PUFA ratio and higher peroxidizability index. Total heme was 3.5-fold higher in red & processed meat than in chicken meat. Approximately 10% of total heme in red & processed meat was nitrosylated, whereas chicken meat contained very low amounts of nitroso-heme. No clear differences in ORAC values were observed between the four diets. The α-tocopherol concentration of the Western background diet was 2-fold higher compared to the prudent background diet, whereas the meat types had similar α-tocopherol contents. A 30%

higher α-tocopherol level was found in the chicken-Western diet compared to the other diets, indicating a loss of α-tocopherol through the combination of the red & processed meat and the Western background diet.

Table 2.3 Characteristics of the four diets, meat and background diets separately

	diets				meat		background diet	
	prudent		Western					
	chicken	R&P	chicken	R&P	chicken	R&P	prudent	Western
gross energy, kcal/kg	1263	1265	1743	1726	-	-	-	-
pH	5.83	5.39	5.69	5.28	-	-	-	-
dry matter, g/100 g	26.0	26.1	30.9	31.2	37.4	36.2	23.5	30.6
crude protein, g/100 g	6.44	6.35	7.61	6.97	26.2	21.7	3.55	2.96
proportion of energy from proteins, %	20.7	20.4	17.7	16.4	-	-	-	-
crude fat, g/100 g	3.13	3.47	6.24	6.76	9.61	9.59	2.28	5.87
proportion of energy from fat, %	22.5	24.9	32.5	35.6	-	-	-	-
SFA, g/100 g FA	31.6	35.6	37.0	38.2	29.7	38.3	32.5	40.0
MUFA, g/100 g FA	44.0	42.2	38.6	38.8	42.6	41.8	45.9	37.8
PUFA, g/100 g FA	21.2	19.5	21.6	20.2	26.1	17.7	20.1	21.0
linoleic acid, g/100 g FA	17.4	15.4	19.0	17.3	22.4	13.9	15.1	18.0
total n-6 PUFA, g/100 g FA	18.2	16.2	19.6	17.9	23.8	15.3	15.8	18.5
α-linolenic acid, g/100 g FA	2.74	2.89	1.87	1.98	1.93	2.03	3.77	2.20
total n-3 PUFA, g/100 g FA	3.00	3.29	2.05	2.28	2.25	2.44	4.22	2.52
ratio n-6/n-3 FA	6.08	4.94	9.57	7.93	10.6	6.26	3.8	7.33
peroxidizability index	25.9	24.6	24.8	23.8	31.3	22.6	26.1	24.8
total heme, mg hematin/kg meat	-	-	-	-	42.8	149	-	-
nitroso-heme, mg hematin/kg meat	-	-	-	-	0.58	14.24	-	-
ORAC (µmol Trolox equivalent/g)	10.2	8.96	9.50	9.29	11.58	12.89	8.77	8.38
α-tocopherol (mg/kg)	7.92	8.03	11.6	8.02	6.38	6.50	5.59	11.3
oxidation products in diet before digestion								
4-hydroxy-2-nonenal (nmol/g)	0.63	0.32	0.55	0.28	1.34	0.32	0.41	0.30
hexanal (nmol/g)	11.1	1.5	12.3	1.3	46.4	4.36	1.24	0.99
malondialdehyde (nmol/g)	2.56	1.22	4.04	2.74	19.1	4.46	0.52	0.42
PCC (nmol DNPH fixed/mg protein)	5.00	5.38	5.19	6.45	-	-	-	-
oxidation products after *in vitro* digestion								
4-hydroxy-2-nonenal (nmol/mL)	0.65	0.32	0.60	0.34	-	-	-	-
hexanal (nmol/mL)	1.69	1.19	1.63	1.28	-	-	-	-
malondialdehyde (nmol/mL)	1.11	0.51	1.24	0.75	-	-	-	-
PCC (nmol DNPH fixed/mg protein)	7.65	8.77	7.51	8.99	-	-	-	-

- = not analyzed; R&P = red & processed meat; FA = fatty acids; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; Total n-6 PUFA = C18:2,n-6 + C18:3,n-6 + C20:2,n-6 + C20:3,n-6 + C20:4,n-6 + C22:4,n-6 + C22:5,n-6; Total n-3 PUFA = C18:3,n-3 + C20:3,n-3 + C20:4,n-3 + C20:5,n-3 + C22:5,n-3 + C22:6,n-3; PCC = protein carbonyl compounds; ORAC = Oxygen Radical Absorbance Capacity; DNPH = 2,4-dinitrophenylhydrazine. Peroxidizability index (Hu et al., 1989) was calculated as follows: (% dienoic x 1) + (% trienoic x 2) + (% tetraenoic x 3) + (% pentaenoic x 4) + (% hexaenoic x 5).

Chicken meat contained approximately 4-fold more 4-HNE, 11-fold more hexanal and 4-fold more MDA compared to red & processed meat. The concentrations of hexanal and MDA in the background diets were considerably lower compared to those in the meat, except for 4-HNE where the concentrations in the red & processed meat were comparable to those in the background diets. The concentrations of lipid oxidation products in the diets corresponded to the combined fractions of lipid oxidation products in the meat and background diets, resulting in higher levels of lipid oxidation products in the diets containing chicken meat. The amount of PCC was

comparable between the chicken-prudent, red&processed-prudent and chicken-Western diet, but slightly higher in the red&processed-Western diet.

2.3.2 In vitro digestion

The differences in lipid oxidation products in the diets were reflected in the *in vitro* digests, as shown in Table 2.3. The concentrations of PCC in the *in vitro* digests were slightly higher after the digestion of the red & processed meat diets compared to the chicken diets.

2.3.3 Animal performance

The average daily energy intake of the pigs did not differ significantly between the four treatment groups, as illustrated in Figure 2.1. Since the meat fraction represented an equal energy percentage in the four diets, the piglets consumed an equal amount of meat per day, as intended. Because of the lower energy density of the prudent background diet, the average daily feed intake of the prudent diets (1268 ± 204 g/day) was higher (P = 0.001) compared to the Western diets (905 ± 81 g/day). In the second week of the experimental feeding period, there was a drop in feed intake and concomitant stagnation of body weight gain, regardless of dietary treatment. During this drop in feed intake, pigs presented loose stools but most of them recovered within 3-5 days. Nonetheless, two pigs fed the chicken-Western diet still suffered from diarrhea at the day of euthanasia, therefore, their colonic contents could not be collected. Pigs receiving the prudent diets (312 ± 91 g/day) had a higher body weight gain compared to those fed the Western diets (239 ± 55 g/day, P = 0.026), whereas meat consumption did not significantly affect body weight. As muscle mass increase is the most important factor in weight gain in growing piglets (and not fat accumulation), the higher absolute protein intake is probably contributing to the higher body weight gain in the pigs subjected to the prudent diets. Multiplying the average daily feed intake (1268 g/day and 905 g/day for the prudent and Western diets respectively) by the protein content of the diets (6.4 and 7.3 g protein/100 g diet for the prudent and Western diets respectively) illustrates that piglets subjected to the prudent and Western diets had an average daily protein intake of 81 and 66 g/day, respectively. Also, the liver tissue weight per kg body weight was 10% higher for pigs fed the prudent diets (P = 0.010), whereas the weight of heart and kidney was not significantly affected. The pH of the intestinal digest samples did not differ significantly among the dietary treatments (data not shown).

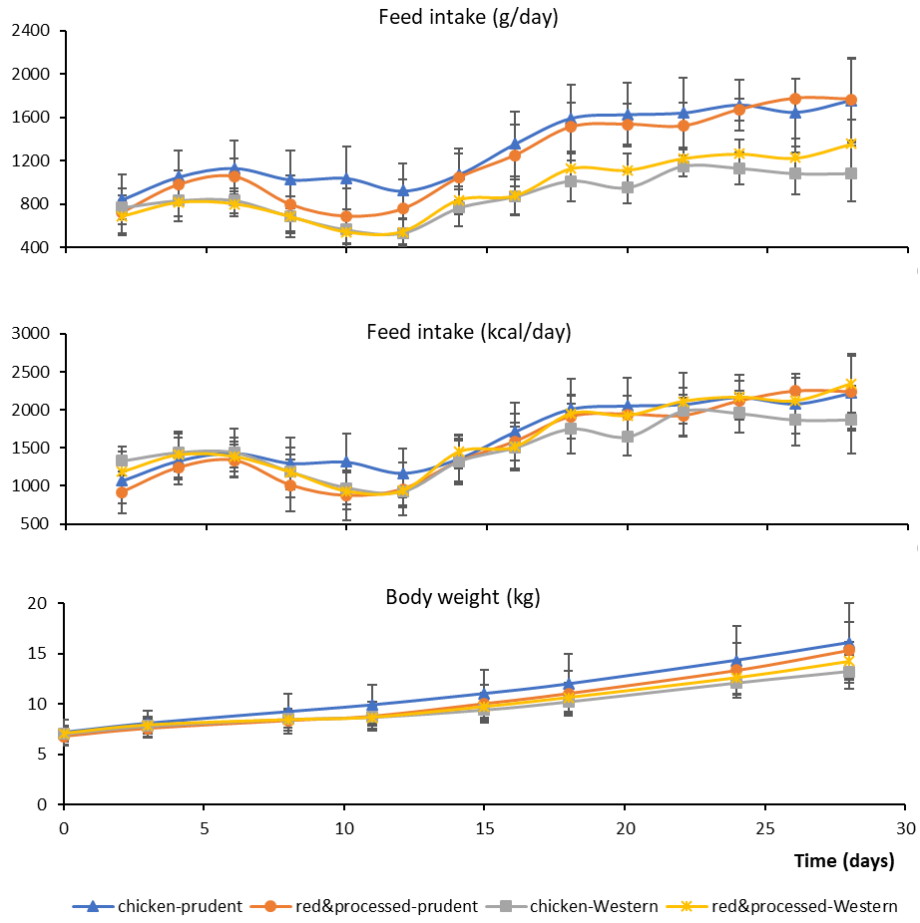

Figure 2.1 Feed intake in g/day, kcal/day and body weight during the experimental feeding period (n = 8 pigs per diet)

2.3.4 Oxidation products in intestinal contents

The concentrations of lipid oxidation products and PCC in small intestine and colonic contents are demonstrated in Figure 2.2. Lipid oxidation products were 1.4- to 3-fold higher in the intestinal contents of the pigs fed chicken meat compared to pigs fed red & processed meat diets ($P < 0.001$). The concentration of hexanal was higher in the small intestinal content when chicken meat was consumed in combination with the prudent versus Western background diet ($P = 0.038$). Significantly higher PCC concentrations were found in the small intestinal content of piglets consuming red & processed meat in the Western *vs.* the prudent background diet ($P = 0.010$).

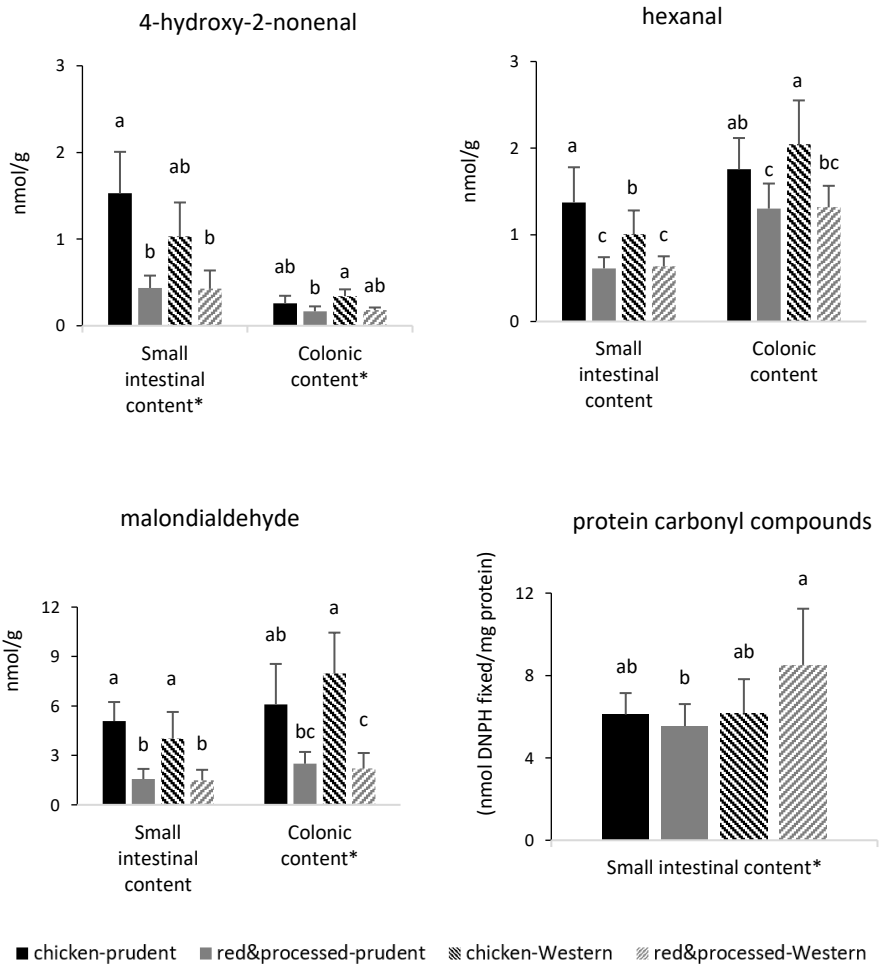

Figure 2.2 Lipid oxidation products (4-hydroxy-2-nonenal, hexanal and malondialdehyde) and protein carbonyl compounds in small intestinal (n = 8 pigs per diet) and colonic (n = 8 pigs per dietary treatment, except chicken-Western, where n = 6) contents. Bars with different superscripts differ significantly ($p < 0.05$) among dietary treatments within the same compartment of the intestinal tract. The error bars illustrate standard deviations. Samples with an asterisk (*) were analyzed using non-parametric statistical tests. DNPH= 2,4-dinitrophenylhydrazine.

2.3.5 α-Tocopherol

As illustrated in Figure 2.3, highest α-tocopherol concentrations were found in pigs fed the chicken-Western diet.

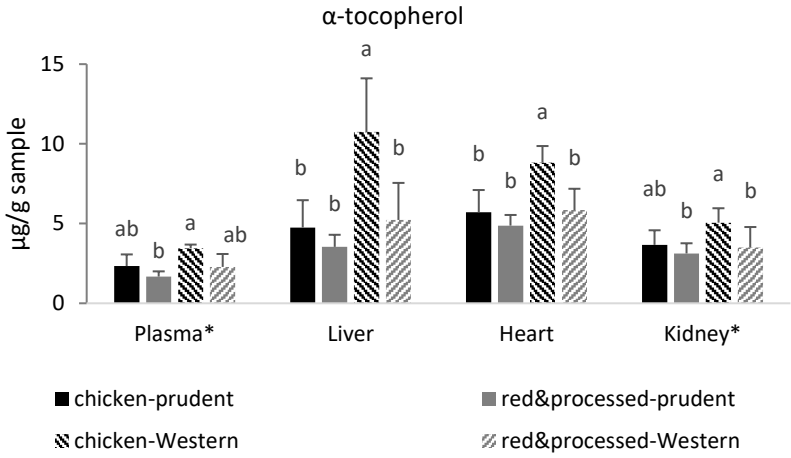

Figure 2.3 Alpha-tocopherol concentrations in plasma (n = 8 pigs per diet) and organs (liver, heart and kidney, n = 8 pigs per diet) of pigs following four weeks on the dietary treatments. Bars with different superscripts differ significantly ($p < 0.05$) among dietary treatments within the same type of sample. Error bars illustrate standard deviations. Samples with an asterisk (*) were analyzed using non-parametric statistical tests.

2.3.6 Oxidative status

The mean values and statistics of the analyzed parameters for the oxidative status are summarized in Table 2.4. No significant interaction effect of meat type and background diet was observed in the analyzed parameters. The ORAC values in plasma of pigs fed chicken meat tended to be slightly higher compared to those fed red & processed meat, irrespective of the background diet. The MDA concentrations were only significantly different in kidney, with slightly higher concentrations in kidneys following chicken meat consumption compared to red & processed meat ($P = 0.014$). Pigs fed the Western diets had higher GSH-Px activity in heart (+ 9%, $P = 0.029$) and showed a trend to be higher in kidney (+ 10%, $P = 0.055$) compared to the prudent diets. On the other hand, pigs on the red & processed meat diets tended to have higher GSH-Px activity in the liver (+ 15%, $P = 0.070$) compared to those fed the chicken diets. No significant differences were observed for GSH-Px activity in plasma or in the intestinal mucosae at three different locations. However, GSSG levels tended to be higher (+ 24%, $P = 0.057$) in erythrocytes of pigs fed red & processed meat diets compared to the chicken diets, whereas the type of meat had no effect

on GSH levels. In liver on the other hand, GSH levels tended to be influenced by the background diet, with slightly higher levels in pigs fed the prudent diets compared to the Western diets (P = 0.081), whereas no significant differences were observed for liver GSSG levels between the treatment groups.

Table 2.4 Parameters for the oxidative status in blood and organ tissues of pigs following four weeks on the dietary treatments (n = 8 pigs per diet)

		prudent		Western			P-values	
		chicken	R&P	chicken	R&P	SEM	P_{back}	P_{meat}
ORAC								
plasma*	µmol TE/mL	12.8	11.5	12.4	11.1	0.45	0.910	*0.060*
TBARS								
plasma*	nmol ME/mL	9.11	9.31	8.70	9.35	0.42	0.692	0.485
liver	nmol ME/g	184	221	174	175	7.67	0.115	0.270
heart	nmol ME/g	68.2	71.1	68.8	68.9	1.66	0.797	0.646
kidney*	nmol ME/g	59.5	57.9	63.5	58.3	0.82	0.274	**0.014**
mucosa of ileum*	nmol ME/g	63.2	56.8	44.4	46.7	3.45	0.110	1.000
mucosa of proximal colon*	nmol ME/g	30.7	31.8	29.3	24.9	1.27	0.155	0.429
mucosa of distal colon*	nmol ME/g	39.9	40.7	38.2	35.1	0.96	0.123	0.406
GSH-Px activity								
plasma*	µmol/min.mL	2.25	2.27	2.40	2.28	0.06	0.970	0.792
liver*	µmol/min.g	3.89	4.62	4.06	4.72	0.19	0.821	*0.070*
heart*	µmol/min.g	0.64	0.67	0.74	0.70	0.02	**0.029**	0.763
kidney*	µmol/min.g	4.01	3.76	4.48	4.15	0.12	*0.055*	0.187
mucosa of ileum	µmol/min.g	2.02	2.12	2.20	2.28	0.11	0.452	0.678
mucosa of proximal colon	µmol/min.g	3.13	3.68	4.24	3.82	0.24	0.176	0.892
mucosa of distal colon	µmol/min.g	2.40	2.62	2.41	2.29	0.11	0.671	0.791
reduced glutathione								
red blood cells	AUC×10^5/mL	2.66	2.82	2.75	2.58	0.10	0.768	0.979
liver	AUC×10^5/g	4.39	5.40	3.24	4.17	0.31	*0.081*	0.145
oxidized glutathione								
red blood cells	AUC×10^4/mL	3.51	4.57	3.28	4.39	0.29	0.699	*0.057*
liver*	AUC×10^4/g	2.11	2.98	1.92	1.69	0.27	0.291	0.792

R&P = red & processed meat, ORAC = Oxygen Radical Absorbance Capacity, TBARS = Thiobarbituric acid reactive substances, ME = malondialdehyde equivalents, TE = Trolox equivalent, GSH-Px = glutathione peroxidase, AUC = area under the curve, SEM = standard error of the mean, P_{back} = P-value of the fixed factor background diet, P_{meat} = P-value of the fixed factor meat. Data of parameters with an asterisk (*) were analyzed with non-parametric statistical tests. P-values ≤ 0.05 are highlighted in bold, P-values between 0.05 and 0.10 are highlighted in italic. P-values for the interaction term meat×background diet were not included since they were not significant.

2.3.7 Intestinal morphology

Villus length and crypt depth were not influenced by background diet, meat type, nor their interaction term (Table 2.5).

Table 2.5 Villus height and crypt depth (µm) in ileum and colon of the pigs subjected to four dietary treatments (n = 8 pigs per diet)

	prudent		Western			P-values		
	chicken	R&P	chicken	R&P	SEM	P_{back}	P_{meat}	$P_{back\times meat}$
villus length ileum	238	233	238	233	6	0.97	0.50	0.99
crypt depth ileum	439	426	433	425	13	0.79	0.48	0.85
crypt depth proximal colon	451	450	437	463	16	0.98	0.53	0.50
crypt depth distal colon	505	488	495	489	26	0.82	0.56	0.80

R&P = red & processed meat, P_{back} = P-value of the fixed factor background diet, P_{meat} = P-value of the fixed factor meat. $P_{back\times meat}$ = P-value for the interaction term meat×background diet.

2.4 Discussion

Although oxidative reactions are the basis of numerous biochemical pathways, oxidative stress has been linked to several pathological conditions and chronic diseases. Therefore, the role of dietary oxidation products as external factors of oxidative stress and the related health risks has become a subject of great interest. The aim of the present study was to investigate the effect of meat sources that have been linked with chronic diseases (red & processed meat) versus chicken meat as control in a complex dietary context (Western or prudent) on the oxidative status in pigs following a four-week experimental feeding period. Most studies investigating the impact of meat on oxidation during digestion used (semi-)purified diets and levels of meat or meat-derived compounds above common intake levels (Jacobs & Steffen, 2003; Turner & Lloyd, 2017). To the best of our knowledge, the present study is unique in its kind as it used a great diversity of whole foods and a lower meat fraction corresponding to the upper intake level of a Western diet.

We hypothesized that the consumption of red meat would lead to more oxidation products during digestion compared to chicken meat because of the catalyzing effect of iron in the Fenton reaction. In contrast, we found higher levels of lipid oxidation products in the chicken-based diets and subsequent digests. The results of the *in vitro* and *in vivo* digests correlated well, however, the oxidation products in the digests reflected the differences in the diets. In agreement with the hypothesis, the results of previously reported *in vitro* and *in vivo* experiments showed lower MDA levels throughout the digestion of chicken compared to beef and/or pork (Kanner et

al., 2017; Van Hecke et al., 2014a; 2016). In these studies, heme iron was mostly held responsible for the higher susceptibility of red meat to oxidation. However, Steppeler et al. (2016) indicated that the amount of unsaturated fatty acids and not the iron content was the most important factor in the formation of aldehydes during *in vitro* digestion of cooked meat and salmon loin, that both comprised of similar fat contents but different fatty acid profiles. The importance of fat content and fatty acid profile was also previously demonstrated upon digestion of meat or heme iron supplemented with different types of oils or by adding different proportions of lard to meat (Guéraud et al., 2015; Tirosh et al., 2015; Van Hecke et al., 2016). The chicken meat in the present study consisted of a mixture of breasts, thighs and added chicken skin, which led to a considerable proportion of n-6 PUFA. In contrast, in their *in vitro* digestions, Van Hecke et al. (2014a) normalized the fatty acid composition of chicken, pork and beef meat by adding lard, which might offer a possible explanation for the different results obtained. In the present study, the undigested cooked chicken meat was already more oxidized compared to the red & processed meat. Besides differences in heme iron and fatty acid profile, a range of other factors such as differences in the endogenous antioxidant capacity and in the ability of heme proteins from different animal species to promote lipid oxidation (Richards & Dettmann, 2003), processing methods and storage conditions could have played a role. The study of Rhee et al. (1994) demonstrated that among raw muscles from different animal species, beef was most susceptible to lipid oxidation during storage at -20°C, whereas cooking drastically changed the susceptibility of meat during storage resulting in significantly higher levels of TBARS in chicken thigh compared to beef, pork and chicken breast (Min et al., 2008). Although storage time in the present study was equal for all meat fractions, a different oxidative susceptibility during storage could be involved. In addition, also the added antioxidants such as nitrite salt in processed meat could have reduced the amount of lipid oxidation products in the red & processed meat (Van Hecke et al., 2014a).

The majority of lipid oxidation products in the diets originated from the meat and hence, the background diet did not affect lipid oxidation product concentrations in the diets and digestion as distinctly as expected. The prudent diet contained considerable amounts of compounds that could lower the oxidative load in the diets or during digestion of meat (Delosière et al., 2016; Pierre et al., 2013; Rezar et al., 2006). However, neither α-tocopherol concentrations nor ORAC values were higher in the prudent diets compared to the Western diets. Nevertheless, care should be taken in the interpretation of ORAC values since this method has been criticized regarding its biological relevance, its selective activity against particular radicals and the contribution of the protein fraction to the antioxidant capacity. In addition, feeding the piglets a mixture of many ingredients always including a certain fraction of protective compounds could have attenuated the adverse effects of a Western

dietary pattern. Indeed, in order to prevent postprandial oxidative stress, Prior (2007) concluded that antioxidant containing foods should be consumed in each meal, which was the case in this study. In this regard, next to the dietary composition, future studies should also consider additional aspects of a dietary pattern, e.g. portion size, temporal distribution of intake, variety etc.

PCC were higher in the small intestinal contents of pigs fed the red&processed-Western diet compared to the red&processed-prudent diet. The susceptibility to protein oxidation is primarily affected by the protein content and amino acid composition, but importantly, also lipid oxidation processes are involved (Estévez & Luna, 2017). Protein and lipid oxidation are interrelated since both lipids and proteins could be targets of ROS and because secondary lipid oxidation products can interact with the amino acid residues of proteins. Also, PCC can be formed during the Maillard-mediated pathway, in which reducing sugars react with the δ-amino group of alkaline amino acids (Villaverde et al., 2013). Possibly, this reaction pathway could have contributed to the higher PCC measured in the digests of the red&processed-Western diet. The evaluation of more specific protein oxidation products and further studies on this Maillard-mediated pathway will aid to elucidate this hypothesis. Additionally, the nature and action of antioxidants can also contribute to the outcome of PCC and the inconsistency with the results for lipid oxidation products in this study. Similar to our results, the studies of Delosière et al. (2016) and Raes et al. (2015) investigating the antioxidant potential of fruits and vegetables and a number of well-known antioxidants (carnosic acid, quercetin and α-tocopherol) during digestion, also found varying results comparing antioxidant effects on lipid and protein oxidation.

The α-tocopherol concentrations (the major vitamin E component) in plasma and organs reflected the dietary α-tocopherol levels, being 30% higher in the chicken-Western diet compared to the other diets. The two-fold higher α-tocopherol concentrations in the Western background diet compared to the prudent background diet was probably due to the high (vitamin E rich) fat content of the Western diet. Indeed, in two large food consumption surveys in Belgium and Ireland, the food group 'fats and oils' was identified as the main source of dietary vitamin E intake, followed by vegetable intake (Moyersoen et al., 2017; Zhao et al., 2014). Although both meat types had similar α-tocopherol concentrations in this study, the combination of red & processed meat with the Western background diet resulted in lower α-tocopherol levels in the red&processed-Western diet compared to the chicken-Western diet. This loss could possibly be explained by the catalyzing effect of heme iron on oxidation but was unexpected since more lipid oxidation products were present in the chicken meat. However, no clear antioxidant effect of the higher α-tocopherol concentration in the chicken-Western diet could be observed in the

oxidative stress parameters measured in organs, in contrast to what was expected as α-tocopherol is known to influence cellular responses to oxidative stress. On the other hand, the α-tocopherol concentrations in the present diets corresponded to a daily intake of 11.6 – 16.6 mg/day for humans (calculated with a theoretical daily energy intake of 2500 kcal), which is close to the European Food Safety Authority adequate intake levels (13 mg/day for men, European Food Safety Authority, 2015) and the usual vitamin E intake in the Belgian population (14 mg/day in men (Moyersoen et al., 2017)). The α-tocopherol concentrations used in experimental studies that clearly demonstrate a protective effect of α-tocopherol on oxidative stress markers, involve much higher doses of vitamin E supplementation such as 67 mg/day in diabetic patients and 200 mg/kg body weight per day in rats (Garg et al., 2005; Jain et al., 2000).

Recent reviews demonstrate involvement of dietary PCC and lipid oxidation products in oxidation markers in blood, urine or organs (Estévez & Luna, 2017; Van Hecke et al., 2017a). Since the diets and intestinal contents containing chicken meat had considerably more lipid oxidation products in the present study, an increase in oxidative stress was expected in pigs on chicken-based diets. However, only renal MDA was higher in pigs fed chicken meat compared to those fed red & processed meat. The increased GSH-Px activity in heart tissue could indicate a higher need for antioxidant action due to the Western background diet. In accordance, a lower erythrocyte GSH-Px activity together with a higher blood total antioxidant capacity was previously indicative for a beneficial oxidative status in pigs fed strawberries compared to other fruits (Pajk et al., 2006). Contrary, Chedea et al. (2018) demonstrated a higher total antioxidant status and higher GSH-Px activity in duodenum and colon mucosae of pigs after the addition of polyphenols to the control diet. These apparent inconsistencies emphasize the existence of different antioxidant mechanisms in different organs and emphasize that a proper interpretation of the oxidative status requires the analysis of multiple parameters. The rather inconsistent effects of the different diets on the measured parameters for the oxidative status (ORAC, α-tocopherol, MDA, GSH-Px, levels of GSH and GSSG), hampers a straightforward conclusion about the body's antioxidant status. The intestinal morphology, measured by villus length and crypt depth in the ileum and colonic crypt depth in the proximal and distal colon sections, was not different among the dietary treatments. Previous studies reported hyperproliferation and increased colonic crypt depth as a result of heme-feeding in rats (de Vogel et al., 2008) and mice (IJssennagger et al., 2012b), however, the concentrations of heme in these studies were much higher compared to the levels in the present study.

Previously, dietary factors such as resistant starch and polyphenols, characteristic for a prudent diet, or sucrose, highly present in Western diets, have been demonstrated to attenuate or stimulate, respectively, MDA formation in colon mucosae or kidney tissue (Chedea et al., 2018; Rosas-Villegas et al., 2017; Toden et al., 2010). However, in the present study, a mitigating or stimulating effect on lipid oxidation could not be attributed to those dietary compounds. Despite the close contact with the digests containing different lipid oxidation concentrations, no effect was observed at the level of the intestinal mucosae in terms of MDA concentrations or GSH-Px activity. In contrast, feeding pigs oxidized soybean oil (resulting in 270 – 414 nmol MDA/g oxidized diet versus 6.6 nmol MDA/g unoxidized diet) increased the concentrations of MDA in the mucosal samples from the jejunum (Rosero et al., 2015), while more oxidized diets (approximately 75 nmol MDA/g diet versus 45 nmol MDA/g diet) also yielded higher MDA concentrations in colonic mucosae in rats (Van Hecke et al., 2016). However, the concentrations of lipid oxidation products in the present study (with MDA levels below 5 nmol MDA/g diet) were relatively low compared to the MDA levels in the studies mentioned above. Furthermore, the concentrations of 4-HNE in this study (up to 0.63 nmol/g diet and up to 1.53 ± 0.48 nmol/g intestinal content) were also at the lower end compared to the cytotoxic concentration ranges of 4-HNE as documented by Esterbauer et al. (1991). In addition, besides the concentration of oxidation products in the diets and intestinal lumen, other factors such as bioavailability, the antioxidant defense system and barrier functionality of the intestinal mucosa, transit time and changes in microbiota could be involved in the oxidative status of the intestinal mucosae.

In conclusion, the hypothesis that red & processed meat consumption stimulates oxidation during digestion to a greater extent compared to chicken meat could not be confirmed. In contrast, chicken meat was more susceptible to lipid oxidation as observed already in the diets as such, and in the *in vitro* and *in vivo* digests. Not only heme iron, but other factors such as the fatty acid composition, the antioxidants in processed meat or other differences in the endogenous antioxidant capacity of the meat will have influenced the oxidative susceptibility during preparation, storage and subsequent digestion. Marginal differences between the two background diets were observed regarding oxidation product formation during digestion. In contrast to the hypothesis, little interaction effects were observed between meat type and background diet. The systemic oxidative status was not or only minimally affected by the background diet and meat type. More *in vivo* studies on the interaction of separate foods and dietary patterns on oxidation phenomena are warranted.

2.5 Acknowledgements

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2.6 Supporting information

A detailed list of food items used to compose the four diets is provided as Supplementary Table S2.1.

Table S2.1 List of food items used to compose the four diets, expressed in g/kg (fresh weight basis, left) and in g/day consumed by humans (right). The latter term was chosen since the diet formulation was based on human dietary guidelines (prudent) and on the average diet of the Belgian population (Western).

		prudent		Western		prudent		Western	
		chicken	R&P	chicken	R&P	chicken	R&P	chicken	R&P
		g/kg				g/day consumed (calculated for humans)			
red (62%) & red processed meat (38%)	total red & processed red meat		145		197		291		291
	total fresh red meat		89.4		121		180		180
	total processed red meat		55.3		75.2		111		111
	lean beef (Belgian Blue)		39.9		54.3		80.5		80.5
	lean pork meat		29.3		39.8		59.0		59.0
	minced pork		13.9		18.9		28.0		28.0
	minced meat (pork and beef)		4.46		6.06		8.98		8.98
	sheep meat		0.29		0.40		0.59		0.59
	beef liver		1.51		2.06		3.05		3.05
	smoked ham		1.43		1.94		2.88		2.88
	white sausage		1.68		2.29		3.39		3.39
	black pudding		0.84		1.14		1.69		1.69
	smoked horsemeat		0.34		0.46		0.68		0.68
	smoked bacon		4.88		6.63		9.83		9.83
	meatloaf		0.17		0.23		0.34		0.34
	filet d'Anvers		2.27		3.09		4.58		4.58
	filet de sax		10.1		13.7		20.3		20.3
	cooked ham		17.4		23.6		35.0		35.0
	ham sausage		1.18		1.60		2.37		2.37
	paté		1.60		2.17		3.22		3.22
	salami		13.5		18.3		27.1		27.1
chicken meat	chicken	145		197		292		292	
cereal products and potatoes	total	304	304	199	199	612	612	294	294
	white bread	20.2	20.2	68.6	68.6	40.7	40.7	102	102
	whole-wheat bread	104	104	11.4	11.4	210	210	16.9	16.9
	sandwiches			17.1	17.1			25.4	25.4
	biscuit	1.47	1.47			2.97	2.97		
	potatoes	109	109	72.4	72.4	220	220	107	107
	white rice			16.9	16.9			25.0	25.0
	whole-wheat rice	38.7	38.7			78.0	78.0		
	pasta	29.8	29.8	12.1	12.1	60.0	60.0	18.0	18.0
fruits	total	126	126	81.1	81.1	254	254	120	120
	apple	18.4	18.4	11.9	11.9	37.2	37.2	17.6	17.6
	banana	11.3	11.3	7.25	7.25	22.7	22.7	10.7	10.7
	pear	10.4	10.4	6.68	6.68	20.9	20.9	9.90	9.90
	orange	29.7	29.7	19.1	19.1	59.8	59.8	28.3	28.3
	pineapple	2.77	2.77	1.78	1.78	5.58	5.58	2.64	2.64
	strawberry	9.71	9.71	6.24	6.24	19.6	19.6	9.25	9.25
	grape	21.1	21.1	13.6	13.6	42.6	42.6	20.1	20.1
	mango	1.43	1.43	0.92	0.92	2.88	2.88	1.36	1.36
	melon	6.28	6.28	4.04	4.04	12.7	12.7	5.99	5.99
	lemon	2.97	2.97	1.91	1.91	5.98	5.98	2.83	2.83
	lime	1.08	1.08	0.70	0.70	2.19	2.19	1.03	1.03
	plum	0.14	0.14	0.09	0.09	0.29	0.29	0.14	0.14
	grapefruit	4.00	4.00	2.57	2.57	8.06	8.06	3.81	3.81
	blueberry	0.91	0.91	0.59	0.59	1.84	1.84	0.87	0.87
	papaya	1.08	1.08	0.70	0.70	2.19	2.19	1.03	1.03
	orange juice	2.28	2.28	1.47	1.47	4.60	4.60	2.18	2.18
	red currant	0.17	0.17	0.11	0.11	0.35	0.35	0.16	0.16
	pomegranate	0.91	0.91	0.59	0.59	1.84	1.84	0.87	0.87
	raspberry	0.86	0.86	0.55	0.55	1.73	1.73	0.82	0.82
	kiwi	0.63	0.63	0.40	0.40	1.27	1.27	0.60	0.60

Table S2.1 Continued

vegetables	total	173	173	91.5	91.5	349	349	136	136
	carrot	11.6	11.6	6.14	6.14	23.4	23.4	9.11	9.11
	beetroot	0.97	0.97	0.51	0.51	1.95	1.95	0.76	0.76
	parsnip	0.22	0.22	0.12	0.12	0.44	0.44	0.17	0.17
	turnip	2.12	2.12	1.12	1.12	4.27	4.27	1.66	1.66
	celeriac	0.76	0.76	0.40	0.40	1.53	1.53	0.59	0.59
	fennel	3.27	3.27	1.73	1.73	6.59	6.59	2.56	2.56
	celery	13.4	13.4	7.07	7.07	27.0	27.0	10.5	10.5
	onion	8.15	8.15	4.30	4.30	16.4	16.4	6.38	6.38
	garlic	0.30	0.30	0.16	0.16	0.61	0.61	0.24	0.24
	leek	11.1	11.1	5.86	5.86	22.3	22.3	8.68	8.68
	asparagus	2.76	2.76	1.46	1.46	5.55	5.55	2.16	2.16
	salsify	1.88	1.88	0.99	0.99	3.78	3.78	1.47	1.47
	mushroom	21.2	21.2	11.2	11.2	42.7	42.7	16.6	16.6
	zucchini	4.60	4.60	2.43	2.43	9.28	9.28	3.61	3.61
	eggplant	4.06	4.06	2.14	2.14	8.18	8.18	3.18	3.18
	bell pepper	3.03	3.03	1.60	1.60	6.10	6.10	2.37	2.37
	pumpkin	1.15	1.15	0.61	0.61	2.32	2.32	0.90	0.90
	haricot	2.36	2.36	1.25	1.25	4.76	4.76	1.85	1.85
	tomato	19.4	19.4	10.2	10.2	39.1	39.1	15.2	15.2
	soybean shoot	4.24	4.24	2.24	2.24	8.54	8.54	3.32	3.32
	cucumber	0.61	0.61	0.32	0.32	1.22	1.22	0.47	0.47
	broccoli	2.36	2.36	1.25	1.25	4.76	4.76	1.85	1.85
	cauliflower	2.76	2.76	1.46	1.46	5.55	5.55	2.16	2.16
	green cabbage	3.12	3.12	1.65	1.65	6.29	6.29	2.44	2.44
	white cabbage	8.42	8.42	4.45	4.45	17.0	17.0	6.59	6.59
	Brussels-sprouts	13.6	13.6	7.20	7.20	27.5	27.5	10.7	10.7
	red cabbage	2.63	2.63	1.39	1.39	5.31	5.31	2.06	2.06
	lettuce	11.5	11.5	6.08	6.08	23.2	23.2	9.01	9.01
	chicory	4.84	4.84	2.56	2.56	9.76	9.76	3.80	3.80
	avocado	6.72	6.72	3.55	3.55	13.5	13.5	5.27	5.27
	watercress	0.09	0.09	0.05	0.05	0.18	0.18	0.07	0.07
dairy products	total	243	243	143	143	490	490	212	212
and eggs	cheese (Belgian Gouda)	15.1	15.1	20.6	20.6	30.5	30.5	30.5	30.5
	semi-skimmed milk	223	223	97.7	97.7	450	450	145	145
	cream (35% fat)			18.3	18.3			27.1	27.1
	eggs	4.7	4.7	6.4	6.4	9.5	9.5	9.5	9.5
butter and	total	8.62	8.62	14.3	14.3	17.4	17.4	21.2	21.2
cooking fats	baking and frying fat	0.8	0.8	2.3	2.3	1.7	1.7	3.4	3.4
	margarine	0.2	0.2	0.6	0.6	0.4	0.4	0.8	0.8
	plant-based frying oil	0.0	0.0	4.6	4.6	0.0	0.0	6.8	6.8
	sunflower oil	0.0	0.0	4.6	4.6	0.0	0.0	6.8	6.8
	peanut oil	0.0	0.0	2.3	2.3	0.0	0.0	3.4	3.4
	rapeseed oil	3.4	3.4	0.0	0.0	6.8	6.8	0.0	0.0
	olive oil	4.2	4.2	0.0	0.0	8.5	8.5	0.0	0.0
fats & sweets	total	0.51	0.51	275	275	1.04	1.04	408	408
(and nuts)	hazelnuts	0.5	0.5	0.3	0.3	1.0	1.0	0.5	0.5
	milk chocolate			6.9	6.9			10.2	10.2
	fries			32.0	32.0			47.5	47.5
	mayonnaise			7.8	7.8			11.5	11.5
	ketchup			20.6	20.6			30.5	30.5
	fruitcake			20.6	20.6			30.5	30.5
	profiteroles			11.4	11.4			16.9	16.9
	pancakes			3.2	3.2			4.7	4.7
	millefeuille			12.6	12.6			18.6	18.6
	coke			68.6	68.6			101.7	101.7
	lemonade			68.6	68.6			101.7	101.7
	sugar			16.6	16.6			24.7	24.7
	croquettes			5.7	5.7			8.5	8.5
total diet		1000	1000	1000	1000	2015	2015	1482	1482

R&P = red & processed meat

Chapter III

Untargeted metabolomics to reveal red versus white meat-associated gut metabolites in a prudent and Western dietary context

Adapted from:

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Own contribution: input in the study design, responsible for the formulation and preparation of the diets and the animal experiment, carrying out most of the laboratory analyses, interpretation of results and writing the manuscript with input of all co-authors.

Chapter III - Untargeted metabolomics to reveal red versus white meat-associated gut metabolites in a prudent and Western dietary context

Abstract

Scope: To improve understanding of the epidemiological link between red and processed meat consumption and chronic diseases, more insight in the formation of metabolites during meat digestion is warranted.
Methods and results: Untargeted MS-based metabolomics was applied to explore the impact of red and processed meat consumption (compared to chicken), combined with a prudent or Western dietary pattern. A pig feeding study (n = 8 pigs per diet), as a sentinel for humans, was conducted in a 2 × 2 factorial design for four weeks. The luminal content of the small intestine and colon of the pigs were collected to determine their metabolic fingerprints. Seventy-six unique metabolites (38 in small intestine, 32 in colon, and 6 in both intestinal compartments) responsible for the distinct gut metabolic profiles of pigs fed either chicken or red and processed meat were (tentatively) identified. Consumption of red and processed meat resulted in higher levels of short- and medium-chain acylcarnitines and 3-dehydroxycarnitine, irrespective of dietary context, whereas long-chain acylcarnitines and monoacylglycerols were specifically associated with the red and processed-Western diet.
Conclusion: The identification of red and processed meat-associated gut metabolites in this study contributes to the understanding of meat digestion in a complex but controlled dietary context and its potential health effects.

Keywords

acylcarnitines, chronic diseases, dietary pattern, metabolomics, red and processed meat

3.1 Introduction

Although possible mechanisms explaining the association between high consumption of red and especially processed meat and several chronic diseases have been hypothesized, a complete understanding is still lacking. Untargeted metabolomics of biofluids collected in interventions with meat is therefore a promising approach to gain more insight in the metabolic effects of meat digestion. Up until recently, metabolomics studies investigating putative biomarkers related to exposure and/or effect of meat intake mainly focused on urine and plasma. In these studies, creatinine, creatine, carnitine, acylcarnitines, carnosine, taurine, 1-methylhistidine, 3-methylhistidine and TMAO were frequently revealed as important metabolites linked to meat intake (Cheung et al., 2017; Cuparencu et al., 2019b; Jakobsen et al., 2017; Khodorova et al., 2019; O'Sullivan et al., 2010; Stella et al., 2006). In addition to urine and blood, the use of feces as a matrix for metabolomics is increasing as the fecal metabolome provides a powerful snapshot of the reciprocal interaction between diet, host and gut microbiome (De Paepe et al., 2018; Van Meulebroek et al., 2017). The high metabolite coverage (De Paepe et al., 2018) and the direct contact between intestinal tissue and its content are additional arguments to include intestinal fluids as a matrix in metabolomics research when investigating dietary related gastrointestinal disorders. In this regard, Rombouts et al. (2017) applied metabolomics on *in vitro* colonic digests of beef and chicken, and identified 3-dehydroxycarnitine, tryptophan-derived metabolites and dityrosine as red meat related metabolites that could potentially be involved in red meat-associated diseases.

In this chapter, an untargeted metabolomics approach (UHPLC-HR-Q-Orbitrap-MS) was applied on the small intestinal and colon digest samples of the pig feeding trial (described in Chapter II) to investigate the differences in the digestion metabolome as a result of the dietary treatments. The main aim was to identify differentiating metabolites as a result of red and processed meat versus chicken meat consumption, but also an impact of the dietary context (prudent versus Western diet) on the formation and/or consequence of potentially harmful red meat-associated metabolites was hypothesized.

3.2 Materials and methods

3.2.1 Study design

The diet formulation and study design of the pig feeding trial were described in Chapter II (2.1-2.4). For the metabolomics analysis, the luminal contents of the small intestine (duodenum, jejunum and ileum together) and colon were collected separately. The collected samples were gently homogenized, lyophilized and stored at -80 °C until analysis. It should be noted that most piglets, regardless of dietary treatment, suffered from diarrhea during the second week of the experimental feeding period, but recovered quickly. Yet, two piglets fed the chicken-Western diet still presented diarrhea during sampling, and therefore, those colon samples were not included in the analysis.

3.2.2 Extraction and polar metabolomic profiling with UHPLC-Orbitrap-HRMS

The extraction protocol and subsequent UHPLC-Orbitrap-HRMS analysis procedure was developed and validated by Vanden Bussche et al. (2015). First, 100 mg of the lyophilized and homogenized luminal content of the small intestine and colon were resolved in 2 mL ultrapure water and 12.5 µL internal standard (valine-d8, 100 ng/µl) was added. After mixing, 0.5 mL of an ice-cold methanol/ultrapure water mixture (80/20, v/v) was added. This mixture was vortexed, centrifuged (13300 g, 10 min) and the supernatant was filtered through a polyamide filter (0.45 µm). Finally, the filtrates of the luminal content of the small intestine and colon were diluted with ultrapure water using a 1/9 and 1/1 dilution (filtrate/water, v/v), respectively. The dilution factors were determined in a preliminary dilution experiment with 9 dilution factors ranging from undiluted to 1/500 on pooled samples from small intestine and colonic digests separately. Metabolite coverage using an untargeted approach as well as linearity and peak shape of 25 targeted metabolites from an in-house database were assessed to select the optimal dilution factor. Quality control (QC) samples were prepared by combining small aliquots of the samples of each piglet and were named external or internal QC samples, depending on the time of injection. The external QC samples were used for column conditioning by injecting the QC samples 6 times prior to analysis of the analytical samples, which were analyzed in random order. Following a series of 10 analytical samples, 2 internal QC samples and a blank (100% acetonitrile) were injected to assess instrument performance. Also, an external standard mixture of ca. 300 gastrointestinal metabolites was injected at the beginning and end of the analytical batch to monitor instrument performance (Vanden Bussche et al., 2015). Analyses of the luminal content of the small intestine and colon were performed in two batches.

3.2.3 Untargeted chemometric data analysis

HRMS data, obtained in polarity switching mode, were processed with Compound Discoverer™ 2.0 (Thermo Scientific) to perform noise filtering, baseline correction, spectra alignment, peak detection and quantification, and spectral deconvolution. A blank sample (100% acetonitrile) was used for background subtraction and noise removal. The following parameter settings were applied: retention time window: 0.5 - 16 min; *m/z* range: 53.4 – 800 Da; peak intensity threshold: 500,000 arbitrary units; maximum retention time shift: 0.5 min; *m/z* width: 5 ppm. The CV of the internal QC samples were calculated for each metabolite and only those metabolites with a CV below 30% were retained.

Subsequently, regression analysis and predictive modelling were performed, thereby using PCA and (O)PLS-DA by means of R packages *pcaMethods* and *ropls*. Log-transformation and Pareto scaling were first implemented to induce data normality and standardize the features' intensity range. PCA models were created to visualize trends and possible outliers, whereas PLS-DA and OPLS-DA models were used to construct a prediction model that could explain and predict one Y-variable (dietary treatment) from the X-matrix (abundances of gut metabolites). PLS-DA models were constructed to compare the four treatment groups, whereas OPLS-DA models were used to retain discriminating metabolites between two groups. The number of predictive compounds in the latter two models were extracted applying autofit. PLS-DA and OPLS-DA models were evaluated by inspection of the R^2X and R^2Y (goodness-of-fit) and Q^2Y (goodness-of-prediction, based on cross-validation with seven segments) model characteristics and by comparison of the root mean squared error of estimation (RMSEE) of the permuted (n = 100) and the real model.

Since OPLS-DA models are not suited for multiple comparisons or interaction effects, the R package *limma* was applied to reveal statistical interaction effects between the type of meat and background diet (Ritchie et al., 2015). The lmFit function in *limma* was used to fit linear models. Empirical Bayes moderated t-statistics and associated Benjamini Hochberg adjusted p-values (=q-values) and (non-adjusted) p-values were computed for the contrasts of interest, namely the main effects meat and background diet and the interaction effect meat×background diet. Metabolites differing between two groups (chicken versus red and processed meat, and prudent versus Western background diet) with variable importance in projection (VIP) scores >1 (obtained from the OPLS-DA models) and q-values <0.05 (obtained from *limma*) were retained. For the interaction effect meat×background, a less stringent selection criterium, based on the (non-adjusted) p-values in *limma*, was used ($p < 0.05$). A workflow illustrating the selection of the metabolites was provided Figure 3.1.

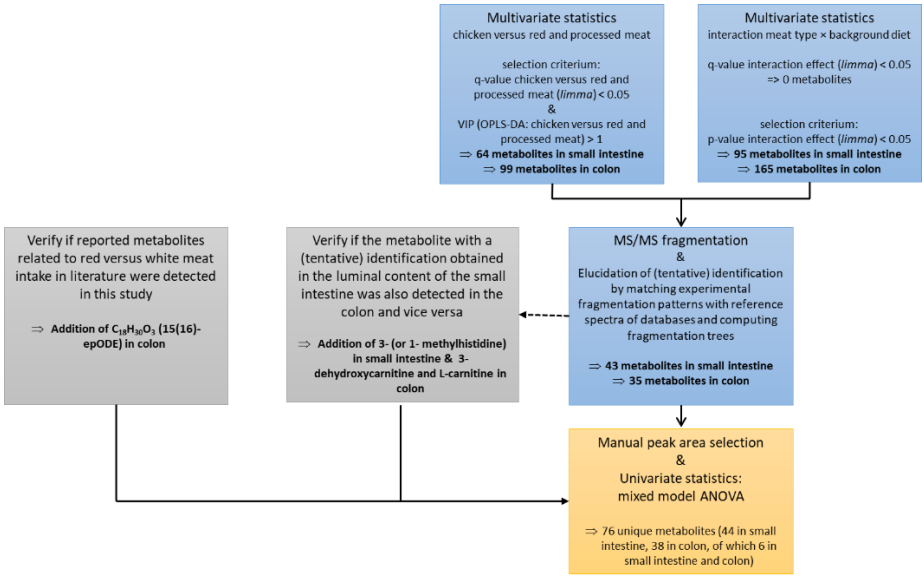

Figure 3.1 Workflow illustrating the selection of metabolites.

3.2.4 Metabolite identification

Identification was performed for those metabolites differentiating between the two meat sources and metabolites with meat×background diet interaction effects. Tentative identification was based on accurate mass (molecular ion and C-isotope profile) and MS/MS fragmentation patterns. Experimental MS/MS fragmentation spectra of the metabolites of interest were generated by Q-Orbitrap Exactive^{TM} MS (applying full-scan and parallel reaction monitoring scan events). Inclusion lists of parent ions with their associated accurate masses of $[M+H]^+$ or $[M-H]^-$ and expected retention times were constructed. Data-dependent fragmentation of the selected parent ions used the following MS/MS settings: resolution of 17,500 FWHM; AGC target of $2e^4$; maximum injection time of 40 ms; isolation window of 2.0 *m/z* and normalized collision energy at 20, 30 or 40 eV. More details on the instrumental settings of the UHPLC-Quadrupole-Orbitrap HRMS analysis are described in De Paepe et al. (2018). The experimental fragmentation patterns were implemented in open-source SIRIUS (CSI:FingerID)(Böcker & Dührkop, 2016) and Metfrag (http://msbi.ipb-halle.de/Metfrag) software to generate information about the identity of the metabolites by computing fragmentation trees and by matching the experimental fragmentation patterns with reference spectra of databases, notably the HMDB (http://www.hmdb.ca/). Subsequently, fragmentation patterns and

retention times of the metabolites were compared against commercial standards if available (Supporting Information Table S3.1). The recommendations for standard metabolite identification from the Chemical Analysis Working Group were followed (Sumner et al., 2007). Therefore, metabolites were described as identified metabolites by matching masses and retention times with authentic standards (Tier 1), putatively annotated metabolites by matching MS/MS spectra with library spectrum data (Tier 2), or putatively characterized metabolite class by spectral similarities to a similar compound class and knowledge from previous literature (Tier 3).

Next to the metabolites that were revealed by the untargeted chemometric analysis, some additional metabolites based on literature and the generated results were examined for their presence and significance, and were included in the list of (tentatively) characterized metabolites. All (tentatively) characterized metabolites were manually processed with XCalibur 2.1 and area ratios were obtained through normalisation of the peak intensities based on the associated internal QC samples. These area ratios were used to construct heat maps with R package *made4* and subjected to univariate analysis. Mixed model ANOVA with meat, background diet and meat×background diet as fixed effects, and litter, pen and euthanization day as random effects was applied with SAS Enterprise Guide 7. P-values <0.05 were considered statistically significant.

3.2.5 Pathway analysis

The *mummichog* algorithm (Li et al., 2013), which bypasses the bottleneck of metabolite identification, was used to evaluate altered biochemical pathways associated with meat intake. Based on *m/z*, p-values and statistical scores obtained in *limma* comparing chicken versus red and processed meat of all metabolites of the small intestine and colon digests, a likelihood list of affected pathways (p-value <0.05) was deduced. The following settings were applied: mass accuracy: 5 ppm; analytical mode: positive; p-value cutoff: 0.05; library: Homo sapiens [MFN].

3.2.6 Preliminary cytotoxicity experiments: Caco-2 cells incubated with colon supernatant

Colon supernatant was prepared by dissolving 100 mg of the individual freeze-dried colon samples in 5 mL phosphate buffered saline, followed by rotation for 1 h and centrifugation (2 h, 35000 G, 4 °C). Subsequently, the supernatant was first filtered over glass wool, followed by filter sterilization through a polyvinylidene fluoride membrane (0.22 µm). The human epithelial colorectal adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (ATCC, Manassas,

VA, USA) and was maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (Greiner), 1% non-essential amino acids and 1% penicillin/streptomycin (ThermoFisher). Cells were maintained in an incubator with 95% humidity and 10% CO_2 at 37°C. Medium was refreshed every two days and cells were sub-cultured using 0.25% trypsin/EDTA solution. Dependent on the passage number and incubation duration, Caco-2 cells undergo spontaneous differentiation, leading to a confluent monolayer of polarized cells with typical epithelial features (Jahn et al., 2011).

First, different dilutions of the colon supernatant samples were evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-22,3-diphenyl tetrazolium bromide, (Laparra et al., 2005)) and SRB (sulforhodamine B, (Vichai & Kirtikara, 2006)) assays as measures for cell viability by mitochondrial activity and protein content respectively to identify toxic and subtoxic concentration ranges. A pooled sample of the colon supernatant of two piglets, subjected to the prudent and Western diet, were diluted 1/2, 1/4, 1/6, 1/10, 1/20, 1/30, 1/40, 1/100, 1/200 and 1/2000 in serum-free DMEM. Caco-2 cells were seeded in 96 well plates in a density of 25000 cells per well and maintained with complete DMEM. After 24h, undifferentiated Caco-2 cells were exposed to the treatments, and after 72h of exposure, MTT and SRB tests were performed as previously described (Geirnaert et al., 2017). Three wells were used for each treatment and the dilution experiment was repeated on three different days.

After determination of a suitable concentration range, SRB tests and fluorometric measurements of GSH and GSSG were conducted according to Singh et al. (2017a) in undifferentiated Caco-2 cells. One day after seeding, cells were treated for 72h with colon supernatant samples of the individual piglets at three concentrations. In addition, GSH and GSSG were also determined in differentiated Caco-2 cells. For this purpose, cells were grown for five days until a confluent monolayer was formed. The differentiating cells were then treated for ten days with a subtoxic concentration of colon supernatant of the individual piglets. The supernatant was diluted with complete DMEM and was refreshed every three days. Results of the SRB tests were expressed as percentage of value of the untreated cells exposed the serum-free cell culture media, whereas concentrations of GSH and GSSG (µM) were calculated using standard curves of both components. In the latter experiments, N-acetyl-L-cysteine was used as negative control. Three wells were used per treatment, but the experiments were not repeated.

3.3 Results and discussion

3.3.1 Dietary induced changes in the gut metabolome

Data processing with Compound Discoverer™ resulted in the detection of 1851 and 1990 metabolites for the small intestine and colon digests, respectively. The PCA-X score plots (Figure S3.1, Supporting Information) confirmed an accurate instrumental performance by the close clustering of the internal QC samples and showed a clear separation of the piglets' samples according to background diet but not meat type. Score plots of PLS-DA models of small intestine and colon comparing the four dietary treatment groups are presented in Figure 3.2.

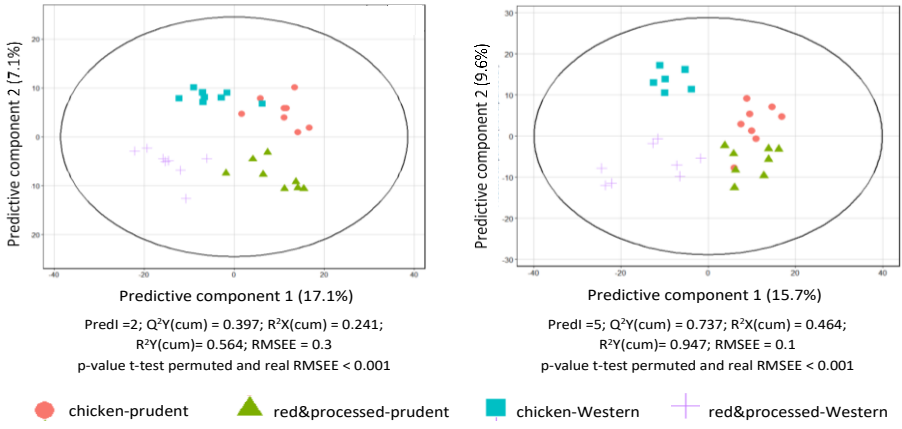

Figure 3.2. PLS-DA score plots demonstrating the diverging effects of the four diets on the small intestinal (left) and colon (right) metabolome. The number of predictive compounds (predI) was extracted applying autofit.

In both PLS-DA models, PC1 discriminates between the background diets (explaining 17.1% and 15.7% of the variance), whereas PC2 discriminates between meat type (explaining 7.1% and 9.6% of the variance). The PCA-X and PLS-DA models and the number of differentiating metabolites (Table 3.1) indicate a higher impact of the different background diets in comparison with the meat types on the intestinal metabolome of the pigs. About 16% of the measured metabolome was significantly different comparing the two background diets, whereas only 3.5% to 5% was influenced by the type of meat. This could be expected because of the more contrasting composition of the background diets compared to the two meat types. Based on the same selection criteria (q-value <0.05), no significant metabolites were found for the interaction effect meat×background diet. However, by using a less stringent criterium (non-adjusted p-value<0.05), 260 metabolites (95 in the small intestine and 165 in the colon metabolome) were retained for this interaction effect

(Figure 3.1). Nonetheless, many of these metabolites (n = 159) showed an overlap with the already retained meat- or background diet-associated metabolites.

Table 3.1 OPLS-DA model parameters and number of differentiating metabolites.

	Model parameters					Number of differentiating metabolites			
	R^2X	R^2Y	Q^2	RMSEE [a]	p-value [b]	chicken	red& processed	prudent	Western
Small intestine									
chicken *vs.* red&processed	0.379	0.993	0.749	0.044	< 0.001	26	38	-	-
prudent *vs.* Western	0.317	0.980	0.772	0.075	< 0.001	-	-	233	66
Colon									
chicken *vs.* red&processed	0.270	0.992	0.780	0.049	< 0.001	82	17	-	-
prudent *vs.* Western	0.412	0.996	0.859	0.035	< 0.001	-	-	193	128

[a] RMSEE, Root Mean Squared Error of Estimation.
[b] p-value corresponds with the t-test comparing the mean of the permuted RMSEEs with the RMSEE value of the real model.

3.3.2 Identification of differentiating metabolites

In digests of small intestine and colon respectively, 44 and 38 meat-associated metabolites mainly resulting from multivariate statistics, but also few originating from literature and results obtained in one of the two intestinal compartments were subjected to univariate statistics and (tentatively) identified (Tables 3.2 and 3.3). Six of those metabolites namely 3- (or 1-)methylhistidine, L-carnitine, C3-carnitine, C4-DC-carnitine, 3-dehydroxycarnitine and hydroxyprolyl-leucine, were present (and modulated by meat intake) in both matrices. Additional information on the metabolites such as retention time, scores from multivariate statistics, most abundant fragment ions, CSI:FingerID similarity and Metfrag scores is available in Supporting Information Tables S3.2 and S3.3. The identity of 17 metabolites could be confirmed with authentic reference standards (Tier 1). Retention times, most abundant fragment ions and suppliers of the standards are available in Supporting Information Table S1. Other metabolites (n = 59) were retained as putatively characterized metabolites based on good CSI:FingerID and Metfrag scores, previous annotation of certain metabolites in relation to meat in literature, and/or the putative identification of multiple compounds of the same classes. Heat maps of the (tentatively) characterized metabolites in small intestinal and colon digests of the piglets are presented in Supporting Information Figures S3.2 and S3.3.

3.3.2.1 Metabolites associated with red and processed meat intake

L-carnitine, short- and medium-chain acylcarnitines, lysophosphatidylcholines, carnosine, 3-dehydroxycarnitine, hydroxyprolyl-leucine, and some linoleic acid derivatives were responsible for the distinct metabolic profile related to red and processed meat consumption. Interestingly, higher abundances of long-chain acylcarnitines and monoacylglycerols were only found following consumption of red and processed meat in combination with the Western background diet. Potential involvement of the (putatively) identified red and processed meat-associated metabolites in chronic diseases is further discussed below.

Acylcarnitines are formed during mitochondrial fatty acid oxidation. Plasma acylcarnitine profiles are therefore indicative of metabolic state, and disturbances in the relative composition have been linked with mitochondrial dysfunction and tissue damage (Reuter & Evans, 2012), as well as with insulin resistance, even though the causative link is not clear yet (Aichler et al., 2017). For example, it was demonstrated that intraperitoneal injection of C6-carnitine and C8-carnitine in mice impaired glucose tolerance, insulin tolerance and insulin secretion (Batchuluun et al., 2018), and that acylcarnitine profiles were characteristic for different diabetic states in mice (Weiser et al., 2018), whereas other studies also reported positive effects such as increasing insulin sensitivity upon oral supplementation with acetyl-L-carnitine in insulin-resistant subjects (Reuter & Evans, 2012). Several studies identified altered acylcarnitine concentrations in plasma or urine following consumption of (red) meat (Khodorova et al., 2019; O'Sullivan et al., 2010; Stella et al., 2006), or a high fat diet (Acar et al., 2019; Bouchard-Mercier et al., 2013). Although most studies reported elevated levels of acylcarnitines, different responses can be observed for short-, medium and long-chain acylcarnitines. However, caution in the interpretation of acylcarnitine profiles in biofluids is warranted, as the study of Schooneman et al. (2014) demonstrated that plasma acylcarnitine levels do not reflect tissue levels. In addition, the exact physiological role of acylcarnitines ingested through the diet or formed during gastrointestinal digestion, should be further investigated.

Table 3.2 List of (tentatively) characterized metabolites influenced by meat intake in the small intestine (n = 8 pigs per dietary treatment).

			Average peak area ratio (SD)				p-values mixed model univariate statistics		
m/z	tentative metabolite identity	ID level	chicken-prudent	red&processed-prudent	chicken-Western	red&processed-Western	background diet	meat	meat × background diet
Chicken									
103.123	Cadaverine[b]	1	1.56(0.87)	1.07(0.84)	1.80(1.57)	0.43(0.59)	0.604	0.022	0.247
170.092	3- (or 1-)Methylhistidine[a]	3	1.83(0.78)	0.59(0.11)	1.39(0.46)	0.47(0.14)	0.103	<0.001	0.341
173.139	Acetylagmatine	3	2.53(1.52)	0.15(0.13)	2.29(2.06)	0.03(0.04)	0.725	<0.001	0.906
215.139	Valyl-Proline	3	1.71(0.39)	0.61(0.16)	1.72(0.58)	0.60(0.16)	0.999	<0.001	0.936
241.129	Anserine[b]	1	2.27(1.73)	0.27(0.08)	2.11(1.18)	0.26(0.10)	0.821	<0.001	0.840
265.085	Aspartyl-Methionine	3	1.69(0.40)	0.94(0.43)	1.47(0.50)	0.66(0.21)	0.096	<0.001	0.823
281.113	Aspartylphenylalanine	3	1.41(0.25)	0.92(0.32)	1.68(0.37)	0.93(0.10)	0.143	<0.001	0.169
304.150	DL-alpha-Asp-Gly-DL-leu	3	1.36(0.33)	0.76(0.19)	1.44(0.27)	0.82(0.11)	0.440	<0.001	0.973
362.155	H-Asp-Asp-Leu-OH	3	1.17(0.27)	0.73(0.30)	1.50(0.36)	1.10(0.33)	0.004	0.001	0.843
401.202	H-Gly-DL-Asp-DL-Pro-DL-Leu-OH	3	1.76(0.51)	0.29(0.12)	2.35(0.40)	0.36(0.15)	0.015	<0.001	0.047
520.224	H-Val-Glu-Thr-Asp-Gly-OH	3	1.07(0.32)	0.77(0.17)	1.52(0.49)	1.01(0.28)	0.063	0.035	0.561
484.308	Litocholyltaurine	3	1.31(0.81)	0.62(0.29)	1.64(1.48)	0.49(0.22)	0.959	0.042	0.674
Red&processed meat									
162.112	L-carnitine[b]	1	0.17(0.05)	2.15(0.36)	0.11(0.04)	1.93(0.67)	0.517	<0.001	0.704
204.123	C2-carnitine[b]	1	0.23(0.05)	2.01(0.34)	0.15(0.04)	1.79(0.55)	0.347	<0.001	0.653
218.138	C3-carnitine[b]	1	0.52(0.20)	1.77(0.57)	0.33(0.14)	2.08(0.59)	0.661	<0.001	0.097
232.154	C4-carnitine[b]	1	0.30(0.13)	1.89(0.53)	0.25(0.04)	2.17(0.78)	0.501	<0.001	0.332
246.169	C5-carnitine[b]	1	0.43(0.17)	1.88(1.11)	0.37(0.15)	1.87(0.95)	0.907	<0.001	0.945
248.149	C4-3-OH-carnitine	3	0.34(0.16)	2.14(0.42)	0.24(0.11)	1.96(0.54)	0.390	<0.001	0.784
260.185	C6-carnitine[b]	1	0.36(0.21)	1.69(0.59)	0.34(0.07)	1.96(0.78)	0.549	<0.001	0.492
262.128	C4-DC-carnitine	3	0.14(0.03)	2.36(0.59)	0.20(0.12)	2.14(0.60)	0.590	<0.001	0.337
146.117	3-Dehydroxycarnitine	3	0.49(0.15)	1.83(0.59)	0.27(0.09)	1.97(0.67)	0.805	<0.001	0.263
227.113	Carnosine[b]	1	0.80(0.66)	1.58(0.51)	0.66(0.37)	1.46(0.56)	0.503	0.001	0.964
245.149	Hydroxyprolyl-Leucine	3	0.00(0.00)	2.53(0.85)	0.04(0.10)	2.28(0.69)	0.562	<0.001	0.415
452.313	LysoPC(O-14:1)	3	0.60(0.43)	1.36(0.46)	0.57(0.18)	1.44(0.55)	0.848	<0.001	0.722
480.344	LysoPC(P-16:0)	3	0.58(0.46)	1.17(0.63)	0.78(0.20)	1.60(0.97)	0.175	0.006	0.617
508.375	LysoPC(P-18:0)	3	0.38(0.25)	1.59(0.74)	0.53(0.13)	1.82(1.05)	0.425	<0.001	0.875
510.391	LysoPC(O-18:0)	3	0.54(0.35)	1.20(0.59)	0.97(0.27)	1.36(0.64)	0.103	0.009	0.456
Red&processed meat (× Western background diet)									
314.232	C10:1-carnitine	3	0.28(0.09)	1.26(0.42)	0.65(0.27)	2.42(1.02)	<0.001	<0.001	0.033
316.247	C10-carnitine[b]	1	0.36(0.14)	1.50(0.69)	0.43(0.20)	2.50(1.43)	0.070	<0.001	0.107
342.263	C12:1-carnitine	3	0.53(0.39)	1.10(0.39)	0.62(0.40)	1.97(0.99)	0.096	0.003	0.170
344.279	C12-carnitine	3	0.42(0.25)	0.89(0.37)	0.75(0.34)	2.35(1.57)	0.008	0.003	0.075
368.279	C14:2-carnitine	3	0.32(0.22)	0.87(0.35)	0.84(0.62)	2.24(1.75)	0.093	0.086	0.433
370.294	C14:1-carnitine	3	0.34(0.24)	0.97(0.37)	0.69(0.41)	2.22(1.47)	0.081	0.023	0.309
372.310	C14-carnitine[b]	1	0.38(0.22)	0.96(0.39)	0.78(0.39)	2.46(1.39)	0.008	0.003	0.102
398.325	C16:1-carnitine[c]	3	0.43(0.27)	1.19(0.55)	0.76(0.41)	2.36(1.50)	0.115	0.018	0.359
400.341	C16-carnitine[b]	1	0.42(0.27)	0.88(0.29)	0.97(0.52)	2.08(1.30)	0.002	0.004	0.172
424.341	C18:2-carnitine	3	0.47(0.30)	0.93(0.38)	0.98(0.43)	2.26(1.21)	0.009	0.012	0.195
426.357	C18:1-carnitine[b]	1	0.54(0.41)	1.18(0.48)	0.81(0.36)	1.99(1.10)	0.027	0.001	0.239
428.372	C18-carnitine[b]	1	0.31(0.17)	1.10(0.35)	0.85(0.52)	2.25(1.17)	0.001	<0.001	0.179
329.268	Palmitoleoylglycerol	3	0.19(0.22)	0.22(0.24)	0.84(0.88)	3.42(2.63)	0.001	0.009	0.011
331.283	Palmitoylglycerol[c]	3	0.16(0.06)	0.41(0.26)	0.84(0.61)	2.66(1.43)	<0.001	0.001	0.006
353.268	Linolenoylglycerol	3	0.51(0.65)	0.50(0.56)	0.87(0.74)	2.77(2.00)	0.002	0.017	0.015
357.299	Oleoylglycerol[b]	1	0.37(0.27)	0.31(0.22)	0.78(0.54)	2.13(1.33)	<0.001	0.014	0.008
359.315	Stearoylglycerol[c]	3	0.29(0.11)	0.41(0.21)	0.94(0.51)	1.83(0.89)	<0.001	0.011	0.041

a) Metabolites did not result from untargeted analysis, but were added based on literature or obtained results in the other gastrointestinal compartment. b) Identification was confirmed with commercial standards. c) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list. *m/z* corresponds with $[M+H]^+$ mass of the parent ion. ID level = level of identification according to Sumner et al. (2007) with Tier 1 representing identified metabolites and Tier 3 representing putatively characterized metabolite classes. SD = standard deviation

Table 3.3 List of (tentatively) characterized metabolites influenced by meat intake in the colon (n = 8 pigs per dietary treatment, except chicken-western, where n = 6)

			Average peak area ratio (SD)				p-values mixed model univariate statistics		
m/z	tentative metabolite identity	ID level	chicken-prudent	red& processed -prudent	chicken-Western	red& processed -Western	back-ground diet	meat	meat × back-ground diet
Chicken									
127.087	2-(4-Methylimidazol-1-yl)ethanol	3	1.65(1.04)	0.14(0.05)	2.38(1.09)	0.23(0.23)	0.099	<0.001	0.188
157.061	4-Imidazolone-5-propionic acid	3	1.41(1.11)	0.43(0.14)	2.77(1.37)	0.54(0.34)	0.024	<0.001	0.050
160.108	Guanidinovaleric acid	3	1.08(1.12)	0.20(0.12)	3.01(4.13)	0.21(0.11)	0.188	0.021	0.194
162.076	Methylglutamate	3	1.81(0.48)	0.43(0.13)	1.87(0.73)	0.32(0.11)	0.848	<0.001	0.587
170.092	3- (or 1-)Methylhistidine	3	1.31(0.72)	0.30(0.31)	2.69(2.39)	0.25(0.17)	0.092	<0.001	0.071
186.087	N-(4,5-Dihydro-1-methyl-4-oxo-1H-imidazol-2-yl)alanine	3	1.20(0.38)	0.85(0.26)	1.61(0.46)	0.68(0.40)	0.434	<0.001	0.044
203.103	Prolylserine	3	1.85(0.79)	0.13(0.08)	2.35(0.77)	0.16(0.13)	0.200	<0.001	0.257
212.103	Acetyl-3-(or 1-)methylhistidine	3	2.04(1.11)	0.33(0.11)	1.58(1.24)	0.26(0.15)	0.478	0.001	0.601
257.124	2-(3-Carboxy-3-aminopropyl)-L-histidine	3	1.61(0.39)	0.07(0.04)	2.46(1.02)	0.08(0.06)	0.212	<0.001	0.229
314.207	H-Pro-Val-Val-OH	3	1.73(0.77)	0.71(0.34)	1.52(0.39)	0.43(0.25)	0.220	<0.001	0.879
403.234	deamino-hPhe-Ala-Ala-Pro-NH2	3	1.30(1.06)	0.11(0.07)	1.96(1.42)	0.16(0.12)	0.266	<0.001	0.346
175.097	Suberic acid	3	1.15(0.39)	0.98(0.45)	1.21(0.32)	0.54(0.26)	0.078	0.012	0.131
217.143	$C_{11}H_{20}O_4$ (Undecanedioic acid)[b]	1	1.53(0.50)	0.81(0.18)	1.12(0.47)	0.55(0.16)	0.014	<0.001	0.518
227.128	$C_{12}H_{18}O_4$ (Dioxo-dodecenoic acid)	3	1.20(0.35)	0.95(0.29)	1.20(0.19)	0.57(0.22)	0.020	<0.001	0.043
247.154	$C_{12}H_{22}O_5$ (Hydroxydodecanedioic acid)	3	1.25(0.37)	1.03(0.22)	1.34(0.15)	0.53(0.21)	0.008	<0.001	0.002
279.232	$C_{18}H_{30}O_2$ (Octadecatrienoic acid)	3	1.07(0.59)	0.50(0.21)	1.94(1.42)	0.62(0.17)	0.099	0.004	0.196
343.211	$C_{18}H_{30}O_6$ ((-)-11-Hydroxy-9,15,16-trioxooctadecanoic acid)	3	1.26(0.39)	0.92(0.35)	1.39(0.28)	0.47(0.26)	0.107	<0.001	0.020
433.245	Glucosyl (2E,6E,10X)-10,11-dihydroxy-2,6-farnesadienoate	3	1.16(0.36)	0.70(0.15)	1.57(0.22)	0.70(0.26)	0.047	<0.001	0.055
Chicken (× Western background diet)									
318.166	H-DL-Leu-Gly-DL-Glu-OH	3	0.97(0.43)	1.14(0.48)	1.68(0.42)	0.72(0.22)	0.352	0.019	0.002
213.160	Cyclo(L-Valyl-L-Leucyl)	3	0.30(0.33)	0.65(0.75)	3.31(3.31)	0.24(0.24)	0.036	0.029	0.008
247.144	Cyclo(L-Valyl-L-Phenylalanyl)	3	0.17(0.11)	0.37(0.35)	3.00(1.50)	1.14(0.70)	<0.001	0.010	0.002
261.160	Cyclo(L-Leucyl-L-Phenylalanyl)	3	0.50(0.27)	0.79(0.41)	2.41(1.12)	1.02(0.26)	<0.001	0.014	0.001
Chicken (× prudent background diet)									
295.228	$C_{18}H_{32}O_3$ (HODE)[c]	3	2.12(1.81)	0.42(0.09)	0.48(0.20)	0.75(0.54)	0.094	0.070	0.018
299.258	$C_{18}H_{34}O_3$ Hydroxyoctadecenoic acid	3	3.09(3.36)	0.28(0.17)	0.34(0.13)	0.69(0.65)	0.098	0.084	0.031
317.268	$C_{18}H_{36}O_4$ Dihydroxyoctadecanoic acid	3	3.13(3.25)	0.28(0.17)	0.32(0.13)	0.67(0.62)	0.078	0.070	0.026
Red&processed meat									
146.117	3-Dehydroxycarnitine[a]	3	0.70(0.18)	1.33(0.36)	0.95(0.31)	1.10(0.53)	0.890	0.017	0.094
162.112	L-carnitine[a]	1	0.98(0.44)	1.51(0.88)	0.78(0.19)	1.12(0.35)	0.112	0.028	0.657
218.139	C3-carnitine	1	0.48(0.24)	1.49(0.49)	0.69(0.18)	1.05(0.80)	0.255	<0.001	0.140
262.128	C4-DC-carnitine	3	0.82(0.40)	1.25(0.38)	0.82(0.24)	0.86(0.45)	0.131	0.079	0.195
311.223	$C_{18}H_{32}O_4$ (HPODE)[c]	3	1.00(1.17)	2.25(1.79)	0.31(0.17)	0.77(0.53)	0.040	0.093	0.419
329.234	$C_{18}H_{34}O_5$ (TriHOME)	3	0.96(0.64)	1.63(0.62)	0.28(0.17)	0.70(0.39)	<0.001	0.008	0.413
295.226	$C_{18}H_{30}O_3$ (15(16)-epODE)[a]	3	0.82(0.59)	1.28(0.50)	0.40(0.14)	0.70(0.30)	0.007	0.031	0.594
130.086	D-pipecolic acid[b]	1	0.53(0.29)	1.24(0.68)	0.54(0.20)	1.47(1.20)	0.788	0.054	0.760
148.097	2-amino-6-Hydroxyhexanoic acid	3	0.81(0.46)	1.28(0.30)	0.54(0.09)	1.15(0.36)	0.230	0.007	0.616
243.195	$C_{14}H_{26}O_3$ (Oxotetradecanoic acid)	3	0.35(0.13)	0.76(0.30)	0.57(0.30)	2.13(1.69)	0.024	0.010	0.105
181.122	Norecasantalic acid or Jasmolone	3	0.90(0.32)	1.32(0.22)	0.68(0.12)	0.89(0.15)	0.001	0.001	0.107
245.149	Hydroxyprolyl-Leucine	3	0.00(0.00)	1.99(0.81)	0.00(0.00)	1.95(0.89)	0.960	<0.001	0.957
246.108	Hydroxyprolyl-Asparagine	3	0.59(0.18)	1.34(0.44)	0.79(0.21)	1.20(0.64)	0.970	0.004	0.417

a) Metabolites did not result from untargeted analysis, but were added based on literature or obtained results in the other gastrointestinal compartment. b) Identification was confirmed with commercial standards. c) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list. *m/z* corresponds with $[M+H]^+$ mass of the parent ion, except for $C_{18}H_{32}O_3$ (HODE), $C_{18}H_{32}O_4$ (HPODE) and $C_{18}H_{34}O_5$ (TriHOME), where *m/z* corresponds with $[M-H]^-$. ID level = level of identification according to Sumner et al. (2007) with Tier 1 representing identified metabolites and Tier 3 representing putatively characterized metabolite classes. SD = standard deviation

Present acylcarnitine profiles reflect dietary intake of carnitine, originating from red meat, and dietary fatty acid composition. Pigs fed the red&processed-Western diet contained higher levels of medium- and especially long-chain acylcarnitines in their small intestines, presumably explained by their concurrent high presence of medium- and especially long-chain fatty acids and carnitine in their diets (Supporting Information Figure S3.4). Acylcarnitines in the intestinal lumen have been hypothesized to originate from intestinal tissue release, intraluminal esterification, or microbial production (Sachan & Ruark, 1985), or through the involvement of the enterohepatic cycle (Hamilton & Hahn, 1987). Whereas most acylcarnitines were detected in the luminal content of the small intestine and no longer in the colon, 3-dehydroxycarnitine was present in both gastrointestinal compartments, but more abundant in the colon. 3-Dehydroxycarnitine is a known fecal metabolite produced from carnitine by gut microbes (Koeth et al., 2014; Rombouts et al., 2017), whereas TMAO is the urinary counterpart of carnitine degradation originating from microbial conversion of carnitine to TMA, either or not through 3-dehydroxycarnitine formation, followed by the hepatic conversion into TMAO (Koeth et al., 2013).

Lysophosphatidylcholines, detected at higher abundances following red and processed meat intake in the present study, can also serve as precursors for TMAO formation (Tang et al., 2013). Although TMAO is also found at elevated levels following fish consumption (Cheung et al., 2017), which is generally associated with lower cardiometabolic risk, the formation of TMAO is one of the proposed mechanisms by which red meat is linked with cardiovascular diseases through the acceleration of atherosclerosis (Koeth et al., 2013; Tang et al., 2013). It remains unclear however if TMAO comprises a causal agent or merely serves as a biomarker for an underlying phenomenon, since circulating TMAO can be confounded by many factors, among which kidney function and colonic microbial composition (Cho et al., 2017). Besides a link with atherosclerosis, the levels of TMAO and lysophoshatidylcholines have also been linked to colorectal cancer development, as plasma TMAO was positively associated with rectal cancer risk in postmenopausal women in a case-control study (Bae et al., 2014), and lysophoshatidylcholines were found to be increased in colorectal tumor tissue (Pakiet et al., 2019). However, the favorable health effects of the conditionally essential nutrient carnitine should be emphasized as well. Indeed, carnitine supplements have been shown to exert beneficial health effects in patients with severe cardiovascular disorders (Flanagan et al., 2010) and to improve insulin resistance as carnitine might prevent the accumulation of intracellular lipids, enhance glucose metabolism and exert antioxidant effects (Bene et al., 2018).

Monoacylglycerols originate from hydrolysis of triacylglycerols. Because of the higher fat content and/or differences in lipolysis rate, more monoacylglycerols could

be expected in pigs consuming the Western diets. However, it is unclear why particularly pigs fed the red&processed-Western diet had higher levels of monoacylglycerols compared to pigs fed the chicken-Western diet. Slightly higher levels could be expected in red and processed meat compared to chicken meat since monoacylglycerols may be used as emulsifier in meat products (Younes et al., 2017) and since hydrolysis also occurs during storage and processing of meat (and especially in meat products) by microbial and endogenous lipases (Molly et al., 1996). Nevertheless, this does not fully explain the outcome as no substantial differences in monoacylglycerols were observed between pigs consuming the red&processed-prudent and chicken-prudent diets.

Carnosine is a well-known dipeptide present in meat and has been described as a urinary marker for meat intake with higher concentrations found in response to the consumption of pork followed by beef and chicken (Cheung et al., 2017; Cuparencu et al., 2019b). The higher levels of hydroxyprolyl peptides (hydroxyprolyl-leucine and hydroxyprolyl-asparagine) observed in this study can likely be attributed to the higher collagen content of meat products in the red and processed meat mixture (Cuparencu et al., 2019b).

3.3.2.2 Metabolites associated with chicken meat intake

Chicken meat consumption mainly resulted in higher abundances of di- and oligopeptides, many of them containing an imidazole moiety and a range of fatty acids. In combination with the Western and prudent background diet respectively, cyclodipeptides and linoleic acid derivatives were putatively characterized.

Anserine, a dipeptide with diverse biological activities (including antioxidant activity) is generally present in large amounts in chicken and was detected in the small intestine. Its hydrolysis product 3-methylhistidine is frequently suggested as a candidate urinary biomarker for chicken intake. On the other hand, 1-methylhistidine, with the same accurate mass and highly similar fragmentation pattern, reflects general meat intake but is also influenced by endogenous muscle catabolism and muscle mass (Cheung et al., 2017; Khodorova et al., 2019; Mitry et al., 2019). Based on its higher abundance, particularly following chicken meat consumption, the metabolite detected both in the small intestine and colon in this study was tentatively ascribed as 3-methylhistidine.

Cadaverine and agmatine are biogenic amines that originate from food directly and/or are produced in the gut by microbial decarboxylation of respectively lysine and arginine, with many physiological functions (Neis et al., 2015). Lithocholyltaurine is a conjugated bile salt formed in the liver from lithocholic acid and taurine, whereas

taurine is an abundant amino acid present in dark meat of chicken that may play a protective role in cardiovascular (Yamori et al., 2010) and gut health (Yu et al., 2016). The putatively characterized metabolite N-(4,5-dihydro-1-methyl-4-oxo-1H-imidazol-2-yl)alanine is the keto tautomer of N-(1-methyl-4-hydroxy-3-imidazolin-2,2-ylidene)alanine and was recently described as potential urinary marker for general meat intake (Cuparencu et al., 2019b). Notably, urinary excretion was higher for chicken and beef compared to pork, which is in agreement with the present results since pork represented about 70% of the red and processed meat. Whereas guanidinoacetic acid has been reported as a urinary marker for chicken intake (Yin et al., 2017), less is known about guanidinovaleric acid found in the present study. Higher abundances of cyclic dipeptides were mainly detected following consumption of the chicken-Western diet. Cyclic dipeptides can be produced by bacteria and yeasts or during thermal processing of foods, have been identified in chicken essence and beef, and exert a wide variety of biological activities including antiviral, antibacterial and antioxidant functions (Borthwick & Da Costa, 2017).

3.3.2.3 Oxygenated lipids in the digestion metabolome of the colon

Two other classes of metabolites that were detected upon the consumption of chicken or red and processed meat, either or not modulated by the background diets, were putatively characterized as dicarboxylic acids (undecanedioic acid and dodecanedioic acid) and oxidized linoleic acid metabolites. Linoleic acid is a direct precursor of the bioactive hydroxy-octadecadienoic (HODEs) and oxo-octadecadienoic acids (oxoODEs), which have been mechanistically linked with inflammation and pathological conditions (Choque et al., 2014). These linoleic acid derivatives can occur in food or be formed during digestion through oxidation of linoleic acid via the intermediate hydroperoxyl-octadecadienoic acid (HPODE). HPODE can be reduced to HODE but other reaction pathways also occur, resulting in the formation of among others 4-HNE, dioxododecenoic acid (DODE) or trihydroxyoctadecenoic acid (TriHOME) (Lee et al., 2005; Thomas et al., 2013). The exact identity of the oxidized linoleic acid metabolites is difficult to determine as many potential fatty acid derivatives with identical accurate mass, and very similar fragmentation profiles exist. In this study, the exact identity of the fatty acid derivatives could not be confirmed and is therefore not discussed in detail. However, more research is warranted with respect to the dietary contribution and biological potency of these oxidized fatty acids as these may possess important health implications.

3.3.3 Pathway analysis

The *mummichog* algorithm identified nine metabolic pathways that were significantly altered in the gut digestome comparing chicken versus red and processed meat consumption (Figure 3.3). The carnitine shuttle was the most significantly altered pathway followed by several amino acid pathways. The linoleate metabolism pathway was nearly significant. Most of the (tentatively) identified metabolites in the present study were related to the altered pathways proposed by this algorithm.

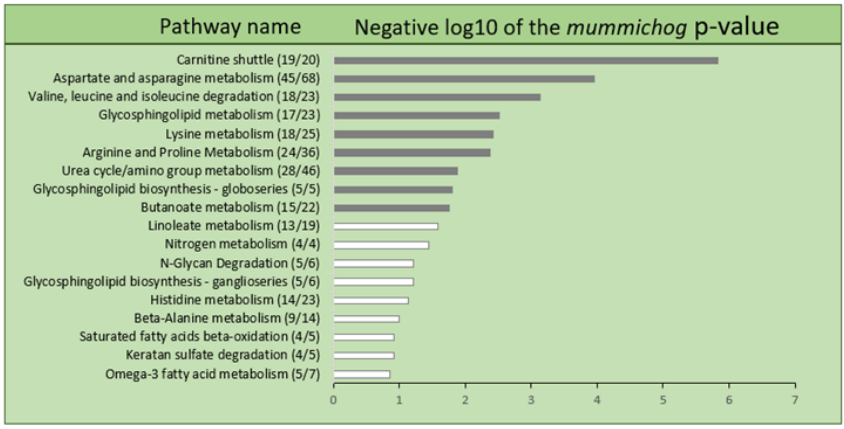

Figure 3.3 Nine metabolic pathways were significantly altered in the small intestinal and/or colon metabolome after the consumption of chicken versus red and processed meat (Fisher's exact test p-value ≤ 0.05, grey filled bars). The ratio in brackets represents the number of significant matches to the total number of matched metabolites in the pathway.

3.3.4 Preliminary cytotoxicity experiments: Caco-2 cells incubated with colon supernatant

Based on the results obtained from the preliminary MTT and SRB tests with pooled colon supernatant samples (data not shown), the dilution factors 1/4 and 1/40 were determined as toxic and subtoxic concentrations respectively, whereas 1/20 was chosen as an intermediate concentration. Although considerably inter-day variation was present, the dilution factor of 1/40 did not show toxic effects in the three repeated experiments. Nevertheless, in the following experiment, when assessing the effect of the colon supernatant of the individual piglets on the protein content of the Caco-2 cells, the dilution factor of 1/40 showed toxic effects (Figure 3.4). Therefore, a dilution factor of 1/80 was included in the determination of GSH and GSSG (Figure 3.5) in the undifferentiated Caco-2 cells. Because of the longer treatment of the differentiated Caco-2 cells, a dilution factor of 1/100 was used (Figure 3.6). No statistical differences were observed as a result of dietary

treatments. It is possible that the considerable inter-individual and inter-day variation outweigh potential treatment effects.

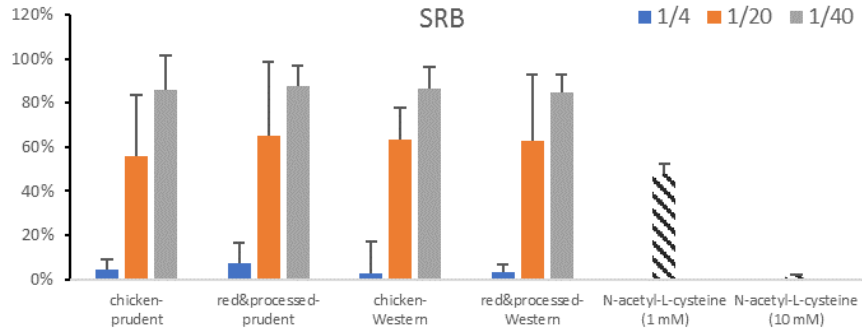

Figure 3.4 Protein content (SRB assay) of Caco-2 cells treated with different dilutions of colon supernatants of piglets subjected to four dietary treatments (chicken-prudent, n = 7; red&processed-prudent, n = 8; chicken-Western, n = 6; red&processed-Western, n = 7). The data is expressed as percentage compared to the untreated condition. Error bars indicate standard deviations. SRB = sulforhodamine B

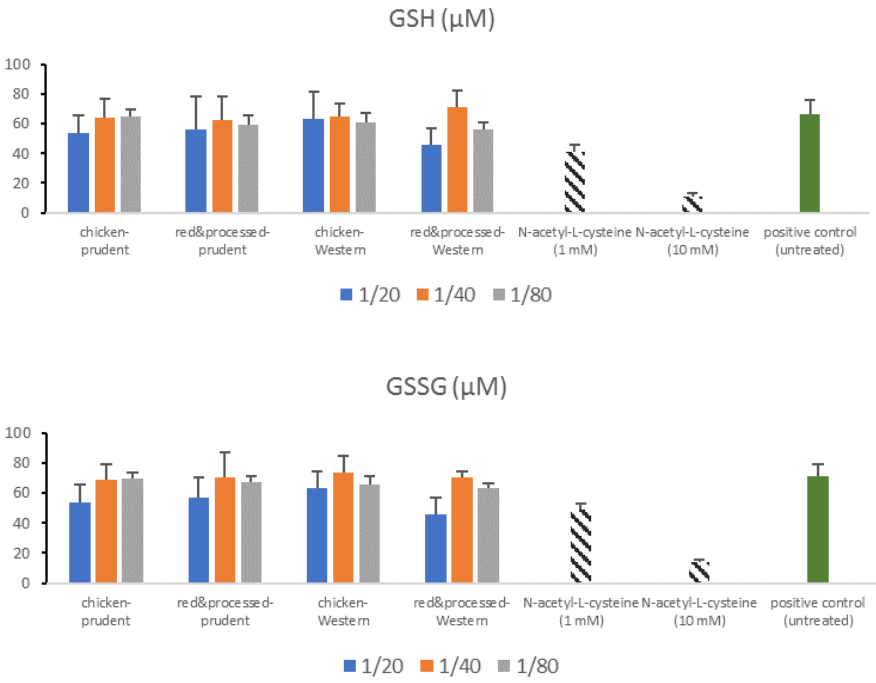

Figure 3.5 Reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations in undifferentiated Caco-2 cells treated with different dilutions of colon supernatants of piglets subjected to four dietary treatments (chicken-prudent, n = 7; red&processed-prudent, n = 8; chicken-Western, n = 6; red&processed-Western, n = 7). N-acetyl-L-cysteine was used as negative control. Error bars indicate standard deviations.

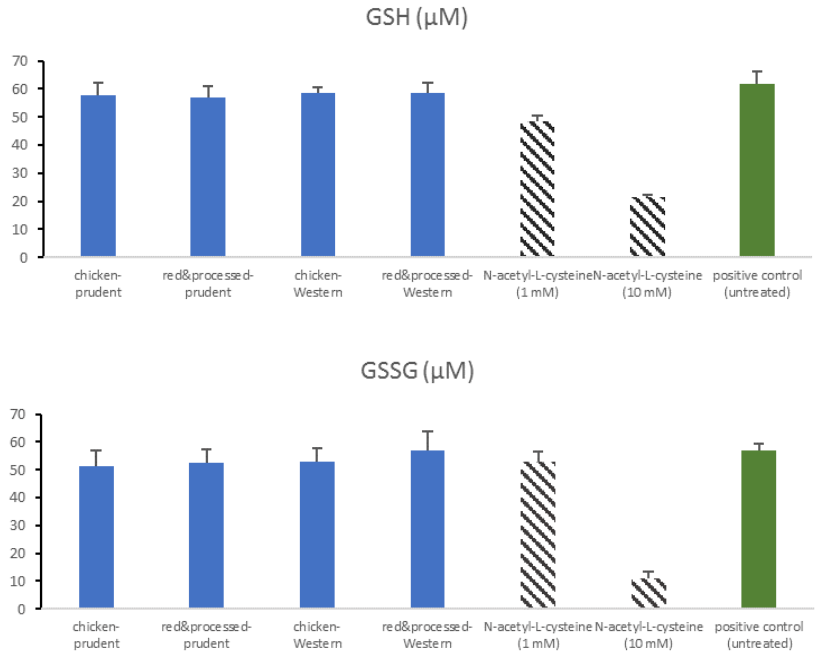

Figure 3.6 Reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations in differentiated Caco-2 cells treated with 1/100 dilution of colon supernatants of piglets subjected to four dietary treatments (chicken-prudent, n = 7; red&processed-prudent, n = 8; chicken-Western, n = 6; red&processed-Western, n = 7). N-acetyl-L-cysteine was used as negative control. Error bars indicate standard deviations.

3.3.5 Study limitations

Studying meat-associated metabolites within a complex but controlled diet brings along some limitations. All food items were mixed to obtain homogenous diets in order to avoid selection of particular food items by the piglets and to obtain digest samples of a whole diet instead of one particular meal. This implicates (i) a loss of food structure, hereby also influencing digestibility and bioavailability, (ii) potential increased chemical interaction between the food items already during preparation and storage of the experimental diets and (iii) the repeated concomitant intake of about 90 food items instead of variable and distinct meals.

The explorative study design allows to investigate the alterations in the metabolite profiles of the luminal content of the small intestine and colon following consumption of chicken versus red and processed meat across different diets. However, more research is needed to reveal if the differentiating metabolites mainly result from the food itself, or from the digestion and transformation of food-derived compounds, or from differences in physiological response. The differentiating

metabolites are not necessarily specific to chicken or red meat intake and do not necessarily serve as dietary intake biomarkers.
The focus on the gut metabolome in an *in vivo* model is unique in this research area and could be responsible for the detection of the high number of acylcarnitines as differentiating metabolites following different types of meat intake. It is possible that these differences observed in the luminal content of the small intestine diminish after further digestion and absorption and have therefore not been detected to a same extent in common metabolomics matrices such as urine and blood. A multi-matrix approach including blood, urine, intestinal and tissue samples in future nutrimetabolomic studies could generate more insights in metabolite distribution, transformation, absorption and clearance effects.

3.4 Concluding remarks

This study revealed a range of gut metabolites discriminating between chicken versus red and processed meat intake across different background diets and confirmed the presence of several metabolites that had been reported to occur in urine or blood following meat intake. The metabolites were mainly related to protein degradation and lipid metabolism, pointing towards the use of proteomics/peptidomics and lipidomics platforms as promising complementary approaches in this line of work. The differences in metabolic profiles may on the one hand be linked to compositional differences between red and processed meat and chicken meat, but may also be explained by induced changes in host metabolism or microbial composition/activity. Although the abundance of most meat-associated metabolites did not depend on the dietary context, long-chain acylcarnitines and monoacylglycerols only occurred at higher levels following the consumption of red and processed meat in a Western dietary pattern. As several of the digestion metabolites can be linked to red meat-associated diseases as reported in literature, these findings advocate in-depth research on the relevance of these metabolites in the relationship between meat consumption in its dietary context and human health.

3.5 Acknowledgements

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3.6 Supporting information

Table S3.1 Retention time and fragmentation pattern of the commercially available standards.
Figure S3.1 PCA-X score plots of the small intestinal and colon digestion metabolome.
Table S3.2 and S3.3 Statistical scores and additional information on the identification of the meat-associated metabolites in the luminal content of the small intestine (Table S3.2) and colon (Table S3.3).
Figure S3.2 and S3.3 Heatmap of normalized peak intensities of (tentatively) characterized meat-associated metabolites in the luminal content of the small intestine (Figure S2) and colon (Figure S3).
Figure S3.4 Fatty acid composition of the diets.

Commercial standards

The authentic standards of cadaverine, L-carnitine, acetyl-L-carnitine (C2-carnitine), propionyl-L-carnitine (C3-carnitine), butyryl-L-carnitine and isobutyryl-L-carnitine (C4-carnitine), valeryl-L-carnitine and isovaleryl-L-carnitine (C5-carnitine), hexanoyl-L-carnitine (C6-carnitine), decanoyl-L-carnitine (C10-carnitine), tetradecanoyl-L-carnitine (C14-carnitine), hexadecanoyl-L-carnitine (C16-carnitine), trans-2-hexadecenoyl-L-carnitine (C16:1-carnitine), octadecanoyl-L-carnitine (C18-carnitine), octadecenoyl-L-carnitine (C18:1-carnitine), undecanedioic acid, 1-oleoylglycerol (oleoylglycerol), 1-stearoylglycerol (stearoylglycerol), 9(S)-hydroxy-10E,12Z-octadecadienoic acid (HODE) and D-pipecolic acid were purchased from Sigma-Aldrich (Overijse, Belgium), whereas 1-palmitoylglycerol (palmitoylglycerol) and 13(S)-Hydroperoxy-9Z,11E-octadecadienoic acid (HPODE) were obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Anserine and carnosine were gifts from a collaborating laboratory.

Standards were obtained for 22 metabolites and the identity of 17 metabolites was confirmed. The identity of the remaining five (trans-2-hexadecenoyl-L-carnitine (C16:1-carnitine), 1-palmitoylglycerol (palmitoylglycerol), 1-stearoylglycerol (stearoylglycerol), 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid (HPODE) and 9(S)-hydroxy-10E,12Z-octadecadienoic acid (HODE)) was not confirmed due to differences in retention time (> 0.2 min) and/or different MS/MS spectra. However, they were retained in the list as putatively characterized metabolites based on good CSI:FingerID and Metfrag scores, previously annotation of certain metabolites in relation with meat in literature, and/or the putative identification of multiple compounds of the same classes. The retention time of the standard trans-2-hexadecenoyl-L-carnitine did not correspond with the retention time of the compound that was tentatively identified as C16:1-carnitine. However, the presence of the specific 85.029 Da fragment in its fragmentation profile increases the likelihood that this unidentified metabolite is an acylcarnitine (and an isomer of trans-2-hexadecenoyl-L-carnitine). For palmitoylglycerol and stearoylglycerol, the fragmentation patterns of the standards and the unidentified metabolites corresponded to a great extent, but not the retention times. For another group of compounds, the linoleic acid derivatives, (of which the standards 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid and 9(S)-hydroxy-10E,12Z-octadecadienoic acid were available) multiple potential identity candidates are present and difficult to identify, as multiple possible structures with an identical molecular mass, molecular formula and very similar fragmentation patterns for this class of metabolites exist.

Fragmentation patterns of butyryl-L-carnitine (retention time: 6.27 min) and isobutyryl-L-carnitine (retention time: 6.08 min), and of valeryl-L-carnitine (retention time: 8.20 min) and isovaleryl-L-carnitine (retention time: 8.02 min) were very

similar. However, based on the retention times of the standards and the metabolites in the samples, eluting at 6.28 min and 8.03 min, the metabolites C4-carnitine and C5-carnitine could be respectively identified as butyryl-L-carnitine and isovaleryl-L-carnitine.

Table S3.1 Retention time and fragmentation pattern of the commercially available standards

(Tentatively) characterized metabolites	Corresponding standards	RT sample (min)	RT standard (min)	MS/MS fragment ions standards (relative abundancy)
Standards compared with metabolites detected in small intestinal digests				
Cadaverine[a]	cadaverine	0.73	0.68	102.128 (100) 74.097 (16) 103.131 (13) 58.066 (6) 72.081 (2)
Anserine[a]	anserine	0.80	0.80	102.128 (100) 142.948 (40) 241.129 (25) 170.092 (15) 109.076 (13)
L-carnitine[a]	L-carnitine	0.85	0.84	162.112 (100) 103.039 (17) 60.082 (16) 102.092 (4) 85.029 (3)
C2-carnitine[a]	acetyl-L-carnitine	1.27	1.26	85.029 (100) 204.123 (39) 60.082 (17) 145.050 (14) 144.102 (3)
C3-carnitine[a]	propionyl-L-carnitine	3.06	3.03	85.029 (100) 218.138 (28) 159.065 (16) 60.082 (13) 86.032 (2)
C4-carnitine[a]	butyryl-L-carnitine	6.28	6.27	85.029 (100) 232.154 (24) 173.081 (16) 60.082 (12) 144.101 (2)
C5-carnitine[a]	isovaleryl-L-carnitine	8.01	8.02	85.029 (100) 246.169 (26) 187.096 (19) 60.082 (12) 85.065 (4)
C6-carnitine[a]	hexanoyl-L-carnitine	9.82	9.80	85.029 (100) 260.185 (21) 201.112 (14) 60.082 (11) 99.081 (4)
Carnosine[a]	carnosine	0.80	0.81	210.087 (100) 156.077 (97) 110.072 (83) 227.114 (64) 181.108 (33)
C10-carnitine[a]	decanoyl-L-carnitine	11.10	11.10	85.029 (100) 316.248 (19) 60.082 (12) 257.174 (8) 155.143 (3)
C14-carnitine[a]	tetradecanoyl-L-carnitine	12.01	11.99	85.029 (100) 372.310 (16) 60.082 (12) 313.237 (6) 144.102 (3)
C16:1-carnitine[b]	trans-2-hexadecenoyl-L-carnitine	12.17	12.43	85.029 (100) 398.326 (11) 339.252 (9) 237.221 (9) 255.232 (9)
C16-carnitine[a]	hexadecanoyl-L-carnitine	12.83	12.81	85.029 (100) 400.342 (20) 60.082 (13) 341.269 (5) 144.102 (3)
C18:1-carnitine[a]	octadecenoyl-L-carnitine	12.96	12.97	85.029 (100) 426.358 (17) 60.082 (14) 144.102 (3) 95.086 (2)
C18-carnitine[a]	octadecanoyl-L-carnitine	13.10	13.11	85.029 (100) 428.372 (16) 60.082 (13) 369.299 (3) 144.102 (2)
Palmitoylglycerol[b]	1-palmitoylglycerol	15.28	15.48	313.273 (100) 57.071 (44) 95.086 (38) 293.237 (31) 71.086 (31)
Oleoylglycerol[a]	1-oleoylglycerol	14.82	14.82	95.086 (100) 69.071 (95) 81.071 (83) 83.086 (72) 121.102 (63)
Stearoylglycerol[b]	1-stearoylglycerol	14.13	13.65	341.304 (100) 95.086 (36) 57.071 (34) 97.102 (30) 109.101 (27)
Standards compared with metabolites detected in colon digests				
Undecanedioic acid[a]	undecanedioic acid	11.05	11.04	135.117 (100) 153.127 (63) 69.071 (48) 97.065 (35) 217.143 (27)
$C_{18}H_{32}O_3$ (HODE)[b]	9(S)-hydroxy-10E,12Z-octadecadienoic acid	13.88	13.38	277.217 (100) 295.228 (99) 171.102 (64) 296.232 (9) 278.221 (8)
$C_{18}H_{32}O_4$ (HPODE)[b]	13(S)-Hydroperoxy-9Z,11E-octadecadienoic acid	12.57	13.41	113.096 (100) 293.212 (47) 195.138 (17) 57.033 (15) 59.012 (13)
D-pipecolic acid[a]	D-pipecolic acid	1.26	1.22	84.081 (100) 130.086 (34) 85.084 (3) 131.090 (1) 84.045 (1)

a) Identification was confirmed with commercials standards. b) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list. Note that retention time of the metabolites in the samples shown in this table may deviate from the retention time in Tables S3.2 and S3.3, due to the analysis of the samples with the UHPLC-Orbitrap-HRMS procedure (Vanden Bussche et al., 2015) and the additional fragmentation analysis with the slightly adapted UHPLC-Q-Orbitrap-HRMS procedure (De Paepe et al., 2018).

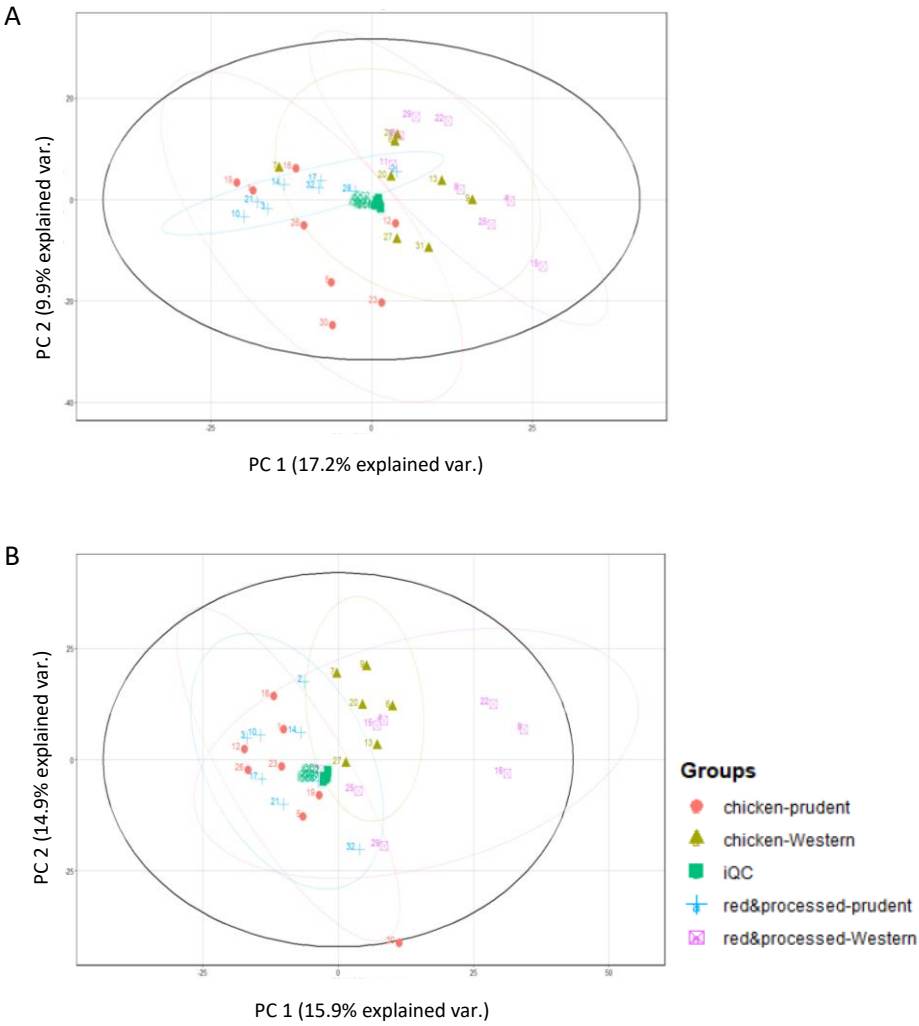

Figure S3.1 PCA-X score plots based on the small intestinal (A) and colon (B) metabolome of pigs fed four dietary treatments. Separation of the two background diets was observed, with the prudent diets situated at the left, and the Western diets situated at the right. The close clustering of the internal quality control samples (iQC) indicated good instrumental performance. Data were subjected to log-transformation and Pareto scaling and only those components with a coefficient of variance lower than 30% for the iQCs were included.

Table S3.2 Additional information on the (tentatively) identified metabolites that were influenced by meat intake in the luminal content of the small intestine (n = 8 pigs per dietary treatment).

				multivariate statistics		information for identification			
Tentative metabolite identity	*m/z*	RT (min)	average peak intensity	VIP[d]	q-value[e]	MS/MS fragment ions samples (relative abundancy)	Collision energy (eV)	CSI:FingerID similarity (%)	Matched fragments Metfrag (HMDB)
Chicken									
Cadaverine[b]	103.123	0.76	854496	2.9	0.016	102.128 (100) 103.131 (15) 74.097 (15) 58.066 (6) 72.081 (3)	30	ND	4/8
3- (or 1-)Methylhistidine[a]	170.092	0.85	753411	NA	NA	ND	NA	ND	ND
Acetylagmatine	173.139	1.69	198037	4.9	<0.001	173.139 (100) 114.091 (68) 156.113 (55) 113.107 (27) 115.087 (14)	30	62.72	NA
Valyl-Proline	215.139	1.77	575702	4.1	<0.001	72.081 (100) 215.139 (38) 216.142 (2) 73.085 (2)	20	77.11	3/11
Anserine[b]	241.129	0.86	1012975	5.5	<0.001	241.129 (100) 102.128 (77) 170.092 (62) 109.076 (54) 126.103 (28)	30	76.13	14/18
Aspartyl-Methionine	265.085	2.68	209304	2.8	<0.001	265.085 (100) 150.058 (91) 171.076 (77) 219.029 (74) 70.029 (37)	20	81.48	11/17
Aspartyl-Phenylalanine	281.113	5.83	657929	2.4	0.006	120.081 (100) 166.086 (53) 70.029 (41) 235.107 (34) 74.024) (11)	30	90.48	9/16
DL-alpha-Asp-Gly-DL-leu	304.150	5.55	805697	2.9	<0.001	132.102 (100) 86.097 (95) 240.134 (82) 280.867 (75) 173.056 (70)	20	76.23	NA
H-Asp-Asp-Leu-OH	362.155	5.39	699242	2.0	0.028	231.061 (100) 86.097 (41) 132.102 (39) 203.066 (33) 158.045 (32)	20	78.69	NA
H-Gly-DL-Asp-DL-Pro-DL-Leu-OH	401.202	6.64	269170	4.6	<0.001	229.154 (100) 70.066 (25) 252.098 (19) 401.202 (16) 224.102 (12)	20	70.76	NA
H-Val-Glu-Thr-Asp-Gly-OH	520.224	5.17	264789	1.9	0.024	385.134 (100) 403.145 (62) 229.118 (61) 502.214 (40) 274.103 (40)	20	52.95	NA
Litocholyltaurine	484.308	9.47	7647667	2.9	0.033	448.287 (100) 110.027 (15) 430.294 (14) 449.291 (11) 431.298 (10)	20	NA	7/20
Red&processed meat									
L-carnitine[b]	162.112	0.91	12370378	7.1	<0.001	162.112 (100) 103.039 (20) 60.082 (14) 163.116 (4) 85.029 (4)	30	83.88	5/12
C2-carnitine[b]	204.123	1.41	16799270	6.3	<0.001	85.029 (100) 204.123 (42) 60.082 (19) 145.050 (15) 144.102 (3)	30	88.49	4/12
C3-carnitine[b]	218.138	2.69	1097364	5.2	<0.001	85.029 (100) 218.138 (29) 159.065 (16) 60.082 (13) 102.055 (2)	30	85.22	5/11
C4-carnitine[b]	232.154	5.24	2570936	5.8	<0.001	85.029 (100) 232.154 (33) 173.081 (20) 60.082 (13) 70.0657 (4)	30	72.59	NA
C5-carnitine[b]	246.169	6.58	1296199	4.8	<0.001	85.029 (100) 246.169 (40) 187.096 (22) 60.082 (12) 85.065 (4)	30	84.17	4/12
C4-3-OH-carnitine	248.149	1.73	377410	5.5	<0.001	85.029 (100) 248.149 (66) 189.075 (21) 103.039 (17) 60.082 (17)	30	78.83	10/18
C6-carnitine[b]	260.185	8.22	172532	4.8	<0.001	85.029 (100) 260.185 (29) 201.112 (14) 60.082 (12) 99.081 (3)	30	84.09	5/10
C4-DC-carnitine	262.128	1.64	705597	6.9	<0.001	85.029 (100) 262.128 (74) 60.082 (26) 203.054 (14) 103.039 (8)	30	76.50	8/11
3-Dehydroxycarnitine	146.117	0.98	3460704	5.3	<0.001	84.081 (100) 87.045 (96) 130.050 (83) 84.045 (77) 146.117 (64)	20	52.01	6/16
Carnosine[b]	227.113	0.85	1523574	3.2	0.002	210.087 (100) 156.077 (97) 110.072 (90) 227.114 (60) 181.108 (37)	20	89.96	11/19
Hydroxyprolyl-Leucine	245.149	1.55	365075	4.0	<0.001	245.149 (100) 70.066 (15) 246.153 (5) 86.097 (5) 100.076 (4)	30	45.85	5/17

LysoPC(O-14:1)	452.313	12.35	351349	2.9	0.002	280.299 (100) 298.310 (32) 281.303 (10) 155.010 (7) 378.277 (6)	30	60.31	NA
LysoPC(P-16:0)	480.344	11.88	2042803	2.8	0.024	104.107 (100) 86.097 (36) 184.073 (32) 480.345 (28) 181.026 (20)	30	88.32	9/18
LysoPC (P-18:0)	508.375	12.90	247191	3.4	<0.001	104.107 (100) 184.073 (17) 86.096 (8) 124.999 (5) 508.376 (4)	30	89.28	8/10
LysoPC (O-18:0)	510.391	12.96	376767	2.5	0.022	104.107 (100) 184.073 (14) 91.055 (9) 86.097 (8) 167.979 (6)	30	83.12	12/17
Red&processed meat (× Western background diet)									
C10:1-carnitine	314.232	9.39	180965	2.8	<0.001	85.029 (100) 314.232 (22) 255.159 (11) 60.082 (10) 128.143 (5)	30	70.95	13/18
C10-carnitine[b]	316.247	9.55	528536	5.0	<0.001	85.029 (100) 316.248 (27) 60.082 (12) 257.174 (10) 155.143 (4)	30	86.46	4/10
C12:1-carnitine	342.263	9.77	473147	2.8	0.012	85.029 (100) 342.263 (18) 283.190 (12) 60.081 (8) 181.159 (6)	30	66.21	15/18
C12-carnitine	344.279	9.97	821863	3.4	0.001	85.029 (100) 344.279 (23) 60.082 (12) 285.206 (7) 183.174 (3)	30	91.06	5/9
C14:2-carnitine	368.279	9.94	369617	2.6	0.014	85.029 (100) 368.279 (23) 60.082 (15) 189.163 (7) 81.070 (5)	30	73.87	14/26
C14:1-carnitine	370.294	10.22	2236296	3.9	<0.001	85.029 (100) 370.295 (22) 60.082 (15) 311.222 (5) 191.179 (4)	30	78.77	7/9
C14-carnitine[b]	372.310	10.49	962582	3.3	0.001	85.029 (100) 355.263 (30) 372.311 (25) 60.082 (13) 161.132 (7)	30	68.17	4/17
C16:1-carnitine[c]	398.325	10.69	805908	3.7	0.001	85.029 (100) 398.326 (21) 60.081 (16) 81.070 (15) 357.278 (9)	30	76.59	NA
C16-carnitine[b]	400.341	11.08	1178251	2.7	0.020	85.029 (100) 400.342 (18) 60.082 (12) 341.267 (5) 144.102 (3)	30	86.38	4/11
C18:2-carnitine	424.341	10.85	533444	2.9	0.009	85.029 (100) 424.341 (23) 60.082 (16) 69.071 (8) 83.086 (5)	30	73.77	5/13
C18:1-carnitine[b]	426.357	11.22	2646494	3.3	0.004	85.029 (100) 426.357 (16) 60.082 (12) 144.102 (3) 427.361 (2)	30	80.44	10/11
C18-carnitine[b]	428.372	11.64	560047	3.6	0.001	85.029 (100) 428.372 (21) 60.082 (12) 369.299 (5) 144.102 (3)	30	84.54	8/12
Palmitoleoylglycerol	329.268	12.85	168654	1.6	0.200	237.221 (100) 311.258 (97) 219.211 (77) 121.101 (38) 135.116 (33)	20	89.78	14/19
Palmitoylglycerol[c]	331.283	13.59	2140733	2.9	0.006	313.273 (100) 57.071 (37) 95.086 (36) 71.086 (34) 109.101 (29)	20	91.11	12/20
Linolenoylglycerol	353.268	12.55	280085	2.3	0.023	184.073 (100) 103.076 (48) 81.019 (28) 194.115 (21) 165.113 (15)	20	49.01	6/20
Oleoylglycerol[b]	357.299	13.80	1203880	1.3	0.701	81.070 (100) 95.086 (96) 69.071 (95) 83.086 (66) 67.055 (65)	40	94.24	13/18
Stearoylglycerol[c]	359.315	14.67	211623	1.4	0.231	341.304 (100) 57.071 (35) 109.102 (34) 95.086 (32) 267.268 (31)	20	91.49	11/19

a) Metabolites were not selected based on untargeted statistical analysis, but were added based on literature or obtained results in the other gastrointestinal compartment. b) Identification was confirmed with commercial standards. c) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list d) VIP scores obtained from OPLS-DA models comparing chicken versus red and processed meat. e) Benjamini Hochberg adjusted p-values (q-value) to correct for false discovery rates obtained in *limma* investigating the main effect meat (chicken vs. red and processed meat). NA= not available, ND=not determined. *m/z* corresponds with the accurate $[M+H]^+$ mass of the parent ion.

Table S3.3 Additional information on the (tentatively) identified metabolites that were influenced by meat intake in the luminal content of the colon (n = 8 pigs per dietary treatment, except chicken-western, where n = 6).

				multivariate statistics		information for identification			
tentative metabolite identity	*m/z*	RT (min)	average peak intensity	VIP[d]	q-value[e]	MS/MS fragment ions samples (relative abundancy)	Collision energy (eV)	CSI:FingerID similarity (%)	Matched fragments Metfrag (HMDB)
Chicken									
2-(4-Methylimidazol-1-yl)ethanol	127.087	1.01	1595392	5.4	<0.001	127.087 (100) 109.076 (11) 96.069 (6) 128.090 (5) 126.066 (3)	30	47.34	NA
4-Imidazolone-5-propionic acid	157.061	2.45	301947	2.0	0.016	157.061 (100) 106.992 (66) 125.002 (24) 78.998 (18) 111.056 (17)	30	40.28	4/17
Guanidinovaleric acid	160.108	1.88	946166	4.5	<0.001	160.108 (100) 101.060 (27) 100.076 (15) 118.086 (5) 128.951 (5)	30	72.53	NA
Methylglutamate	162.076	1.89	1743357	5.3	<0.001	144.065 (100) 102.055 (24) 72.045 (13) 126.055 (13) 90.055 (12)	30	52.85	11/17
3- (or 1-)Methylhistidine	170.092	0.89	447080	2.0	0.014	170.092 (100) 152.071 (60) 109.076 (30) 126.103 (21) 96.069 (18)	20	91.9	7/17
N-(4,5-Dihydro-1-methyl-4-oxo-1H-imidazol-2-yl)alanine	186.087	1.04	2269198	2.1	0.028	186.087 (100) 140.082 (12) 163.940 (7) 117.980 (7) 128.951 (5)	30	50.65	5/17
Prolylserine	203.103	2.30	570015	3.9	<0.001	114.055 (100) 90.055 (16) 126.055 (15) 185.092 (14) 203.102 (10)	20	45.31	8/17
Acetyl-3-(or 1-)methylhistidine	212.103	1.03	505607	3.1	<0.001	212.103 (100) 109.076 (61) 91.039 (33) 85.029 (29) 145.049 (22)	30	62.76	
2-(3-Carboxy-3-aminopropyl)-L-histidine	257.124	1.48	380016	3.1	<0.001	168.077 (100) 257.124 (62) 258.094 (26) 114.066 (24) 126.055 (10)	20	35.4	6/18
H-Pro-Val-Val-OH	314.207	5.96	436362	3.8	<0.001	314.207 (100) 268.201 (35) 110.072 (24) 156.077 (22) 315.212 (8)	30	52.52	NA
deamino-hPhe-Ala-Ala-Pro-NH2	403.234	9.13	325146	4.6	<0.001	293.186 (100) 168.102 (39) 403.233 (31) 86.097 (20) 180.109 (18)	20	49.81	NA
Suberic acid	175.097	8.05	1119094	2.0	0.566	83.086 (100) 157.086 (43) 111.081 (39) 175.097 (22) 55.055 (18)	20	83.94	9/19
$C_{11}H_{20}O_4$ (Undecanedioic acid)[b]	217.143	9.53	134362	2.9	0.003	135.117 (100) 153.127 (62) 86.097 (40) 69.071 (38) 217.143 (27)	20	73.73	7/17
$C_{12}H_{18}O_4$ (Dioxo-dodecenoic acid)	227.128	9.14	331716	2.5	0.003	111.044 (100) 181.122 (71) 163.112 (62) 153.127 (48) 135.117 (31)	30	53.82	NA
$C_{12}H_{22}O_5$ (Hydroxydodecanedioic acid)	247.154	8.99	284416	3.1	<0.001	229.143 (100) 211.132 (25) 147.117 (19) 193.122 (16) 165.127 (15)	20	65.16	NA
$C_{18}H_{30}O_2$ (Octadecatrienoic acid)	279.232	10.16	292477	3.3	<0.001	117.070 (100) 149.023 (66) 279.231 (46) 279.158 (30) 95.086 (21)	20	62.22	8/14
$C_{18}H_{30}O_6$ ((-)-11-Hydroxy-9,15,16-trioxooctadecanoic acid)	343.211	9.69	258061	2.5	<0.001	343.199 (100) 133.101 (35) 125.096 (33) 97.065 (28) 109.101 (26)	30	50.63	9/18
Glucosyl (2E,6E,10X)-10,11-dihydroxy-2,6-farnesadienoate	433.245	9.37	293184	2.8	<0.001	293.211 (100) 414.230 (80) 146.027 (65) 432.241 (53) 415.233 (48)	20	46.76	5/18
Chicken (× Western background diet)									
H-DL-Leu-Gly-DL-Glu-OH	318.166	4.89	346464	1.1	0.315	319.136 (100) 143.118 (49) 148.060 (18) 86.097 (17) 130.050 (5)	20	72.54	NA
Cyclo(L-Valyl-L-Leucyl)	213.160	7.94	548245	1.4	0.544	213.160 (100) 72.081 (76) 86.097 (74) 140.143 (30) 168.138 (29)	30	61	NA

Cyclo(L-Valyl-L-Phenylalanyl)	247.144	8.69	134999	0.5	0.568	120.081 (100) 72.081 (45) 247.144 (29) 174.128 (18) 219.149 (18)	30	57.78	NA
Cyclo(L-Leucyl-L-Phenylalanyl)	261.160	9.24	845659	0.2	0.805	120.081 (100) 86.097 (43) 261.159 (25) 216.138 (16) 233.164 (15)	30	62.19	5/18
Chicken (× prudent background diet)									
$C_{18}H_{32}O_3$ (HODE)[c]	295.228	10.90	1493774	1.6	0.456	295.228 (100) 296.231 (11) 230.986 (3) 134.894 (3) 158.977 (2)	30	79.38	15/18
$C_{18}H_{34}O_3$ (Hydroxyoctadecenoic acid)	299.258	10.60	719699	2.2	0.234	263.237 (100) 245.226 (91) 97.101 (87) 83.086 (62) 111.117 (58)	20	79.64	13/20
$C_{18}H_{36}O_4$ (Dihydroxyoctadecanoic acid)	317.268	10.61	1001222	2.2	0.302	263.237 (100) 97.102 (76) 245.226 (72) 83.086 (52) 111.117 (47)	20	75.29	6/20
Red&processed meat									
3-Dehydroxycarnitine[a]	146.117	0.96	55768092	1.5	0.349	146.117 (100) 87.044 (52) 60.082 (34) 147.121 (7) 84.081 (4)	20	73.69	5/17
L-carnitine[a]	162.112	0.93	4751179	1.3	0.437	ND	NA	ND	ND
C3-carnitine	218.139	3.34	582040	2.4	0.036	85.029 (100) 218.138 (29) 159.065 (16) 60.082 (13) 102.055 (2)	30	85.22	5/11
C4-DC-carnitine	262.128	1.59	827900	1.3	0.024	85.029 (100) 262.128 (74) 60.082 (26) 203.054 (14) 103.039 (8)	30	76.50	8/11
$C_{18}H_{32}O_4$ (HPODE)[c]	311.223	10.96	714170	2.6	0.021	311.223 (100) 311.168 (79) 171.102 (52) 293.212 (48) 211.133 (32)	30	84.62	13/16
$C_{18}H_{34}O_5$ (TriHOME)	329.234	9.65	1843683	1.9	0.039	329.233 (100) 171.101 (26) 330.236 (14) 229.144 (13) 211.133 (13)	30		8/15
$C_{18}H_{30}O_3$ (15(16)-epODE)[a]	295.226	9.66	622158	1.2	0.523	ND	NA	ND	ND
D-pipecolic acid[b]	130.086	1.04	16366507	2.7	0.032	84.081 (100) 130.086 (37) 84.045 (34) 70.066 (4) 130.035 (4)	40	63.41	6/11
2-amino-6-Hydroxyhexanoic acid	148.097	0.98	1470157	2.5	0.006	84.045 (100) 130.050 (42) 102.055 (38) 148.061 (12) 147.121 (9)	20	65.66	NA
$C_{14}H_{26}O_3$ (Oxotetradecanoic acid)	243.195	11.87	284812	2.9	0.003	95.086 (100) 109.101 (78) 83.086 (36) 165.164 (35) 107.967 (31)	30	63.19	7/19
Norecasantalic acid or Jasmolone	181.122	10.13	381323	2.1	0.001	181.122 (100) 163.112 (11) 135.117 (11) 182.126 (6) 116.972 (4)	30	50.55	13/18
Hydroxyprolyl-Leucine	245.149	1.55	1229321	5.9	<0.001	245.149 (100) 246.153 (7) 60.082 (5) 100.076 (3) 70.066 (3)	30	41.47	8/18
Hydroxyprolyl-Asparagine	246.108	1.42	5567373	2.8	0.001	246.108 (100) 132.077 (22) 186.087 (19) 228.098 (7) 246.1319 (6)	30	34.31	7/16

a) Metabolites were not selected based on untargeted statistical analysis, but were added based on literature or obtained results in the other gastrointestinal compartment. b) Identification was confirmed with commercial standards. c) The identity of the detected metabolite does not correspond with the available specific standard, but is likely an isomer of the available standard, which is covered by the nomenclature used in the list d) VIP scores obtained from OPLS-DA models comparing chicken versus red and processed meat. e) Benjamini Hochberg adjusted p-values (q-value) to correct for false discovery rates obtained in *limma* investigating the main effect meat (chicken vs. red and processed meat). NA= not available, ND=not determined. *m/z* corresponds with the accurate $[M+H]^+$ mass of the parent ion, except for $C_{18}H_{32}O_3$ (HODE), $C_{18}H_{32}O_4$ (HPODE) and $C_{18}H_{34}O_5$ (TriHOME), where m/*z* corresponds with $[M-H]^-$

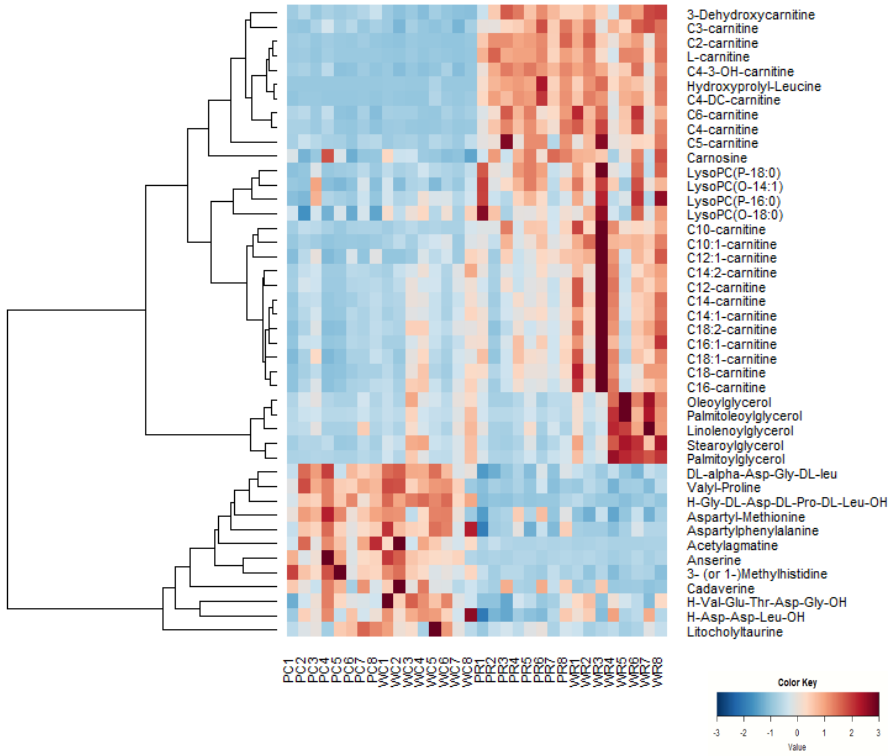

Figure S3.2 Heatmap of normalized peak intensities of (tentatively) characterized meat-associated metabolites in the luminal content of the small intestine. Columns represent samples (n = 8 pigs per dietary treatment) and rows represent metabolites. Data was by default scaled to limits of -3,3. PC = chicken-prudent, WC = chicken-Western, PR = red&processed-prudent, WR = red&processed-Western.

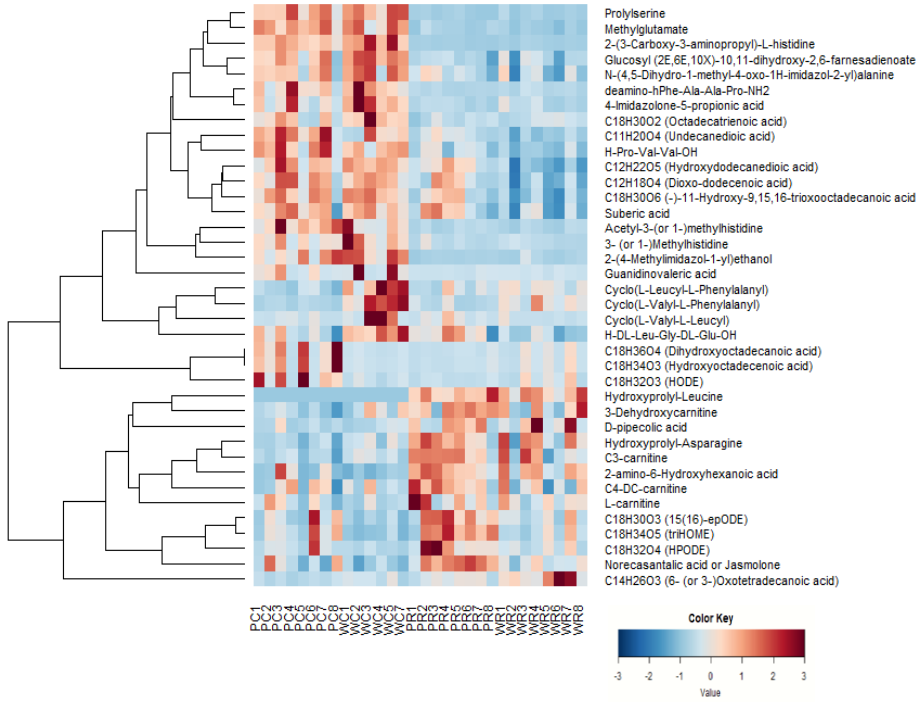

Figure S3.3. Heatmap of normalized peak intensities of (tentatively) characterized meat-associated metabolites in the luminal content of the colon. Columns represent samples (n = 8 pigs per dietary treatment, except chicken-western, where n = 6) and rows represent metabolites. Data was by default scaled to limits of -3,3. PC = chicken-prudent, WC = chicken-Western, PR = red&processed-prudent, WR = red&processed-Western.

Figure S3.4 Contribution of the most abundant fatty acids to the diet.

Chapter IV

Red and processed meat consumption within two different dietary patterns: Effect on the colon microbial community and volatile metabolites in pigs

Adapted from:

Vossen E., Goethals S., De Vrieze J., Boon N., Van Hecke T., De Smet S. (2020). Red and processed meat consumption within two different dietary patterns: Effect on the colon microbial community and volatile metabolites in pigs. *Food Research International*, doi: 10.1016/j.foodres.2019.108793

Own contribution: input in the experimental design, responsible for the formulation and preparation of the diets and the animal experiment, input in finalization of the manuscript. Analysis of the microbial community and volatile metabolites, data analysis and preparation of the paper was performed by the first author and the other co-authors. Despite my modest contribution to this paper, it was decided to include this experimental chapter in this PhD thesis to provide a complete picture of the available data of the pig feeding trial and to integrate these findings in the general discussion.

Chapter IV - Red and processed meat consumption within two different dietary patterns: Effect on the colon microbial community and volatile metabolites in pigs

Abstract

Pigs were fed either red and processed meat or chicken meat within either a prudent or a Western dietary pattern for four weeks (2 × 2 full factorial design). The colon microbial community and volatile organic compounds were assessed (either quantified or based on their presence). Results show that *Lactobacilli* were characteristic for the chicken-prudent dietary pattern treatment and *Paraprevotella* for the red&processed-prudent dietary pattern treatment. Enterobacteriaceae and *Desulfovibrio* were characteristic for the chicken-Western dietary pattern treatment and *Butyrivibrio* for the red&processed-Western dietary pattern treatment. *Campylobacter* was characteristic for chicken consumption and *Clostridium XIVa* for red and processed meat, irrespective of the dietary pattern. Ethyl pentanoate and 1-(methylthio)-propane were observed more frequently in pigs fed red and processed meat compared to chicken meat. The prevalence of 3-methylbutanal was >80% for pigs receiving a Western dietary pattern, whereas for pigs fed a prudent dietary pattern the prevalence was <35%. The concentration of butyrate was significantly higher when the prudent dietary pattern was given, compared to the Western dietary pattern, but no differences for other short chain fatty acids or protein fermentation products were observed.

Keywords

red meat consumption, Western diet, microbiota, volatile organic compounds, digestion

4.1 Introduction

Global meat production hovers around 337 million tons in 2019 and is forecasted to increase to a global total of 365 million tons in 2030 (FAO, 2019). It is estimated that the demand for animal-derived foods and meat in particular in the coming decades will continue to grow strongly in developing countries, whereas in high income countries meat consumption may stagnate or even decline in future (FAO, 2009; Vranken et al., 2014). The current high levels of red and processed meat consumption in many countries have been criticized for contributing to the burden of chronic diseases (Ekmekcioglu et al., 2018; WCRF, 2007). Associations with chronic diseases are not observed for chicken meat consumption and no definite mechanisms have been found so far (De Smet et al., 2019). Knowing the factors that determine the nutritional value of meat and the impact on human health and disease is important. Since meat is always eaten as part of a meal, the interactive effects of meat with other food components on the gut microbiome and the metabolome in general needs to be considered (De Smet & Vossen, 2016).

Dietary influences are mediated in part by the gut microbes, which consist of hundreds of different bacterial species as well as fungi and viruses. These microbes metabolize certain dietary components, including complex carbohydrates that are otherwise indigestible, and produce hundreds of novel molecules, some of which are absorbed into the circulation and have (patho)physiological effects (Danneskiold-Samsøe et al., 2018; Senghor, et al. 2018). Multiple studies have found evidence for lower incidence of mortality risk from chronic metabolic diseases with adherence to a healthy Mediterranean-type diet (Jin et al., 2019). Substantial changes in microbiome composition have been measured in response to changes in dietary intake, such as those seen with a shift from plant-based to animal-based diets (David et al., 2014; Graf et al., 2015). In recent years, there is increasing information regarding the effect of protein type on the gut microbial composition (Lang et al., 2018; Oliphant & Allen-Vercoe, 2019; Singh et al., 2017b; Zhu et al., 2015). However, inter-individual differences, such as genetics, obesity, medications and previous dietary habits increase the complexity and uniqueness of the microbiome. For instance, Lang et al. (2018) showed that the effects of individual differences outweighed the effect of experimental diets and that the protein source is less influential than saturated fat level.

Zhu et al. (2015) demonstrated differences between the cecal microbial community of rats fed diets based on poultry proteins versus a diet containing pork and beef proteins, with, among others, a higher abundance of *Lactobacillus* in rats fed poultry. Similarly, a higher abundance of *Lactobacillus* was observed in the colon of rats fed a diet containing chicken and starch, compared to a diet high in beef and sucrose

(Van Hecke et al., 2019a). It has been hypothesized that meat, high in sulfur-containing amino acids, promotes the growth of sulfur-reducing bacteria (Vipperla & O'Keefe, 2013). Indeed, abundantly more *Desulfovibrionaceae* were found in pigs fed a diet containing 30% cooked red meat (Belobrajdic et al., 2012) and in rats receiving 65% beef and sucrose (Van Hecke et al., 2019a) compared to their opposing diets. Intriguingly, in these studies the opposing diets were respectively red meat with wheat fiber components and red or chicken meat with corn starch instead of sucrose, showing the importance of other food compounds in the diet. In contrast, Le Leu et al. (2015) observed no alterations in certain selected fecal bacterial groups before and after a dietary intervention with healthy human volunteers consuming 300 g lean red meat per day for four weeks.

When using animal feeding studies, often high levels of protein are administered (Nielsen et al., 2019; Van Hecke et al., 2019a) or isolated meat compounds, such as heme-iron or extracted meat proteins (IJssennagger et al., 2015; Zhu et al., 2015) are used. Some animal studies use semi-purified diets designed to mimic the nutrient loads in current Westernized diets, but whole foods are seldom included (Turner & Lloyd, 2017).

Fermentation of undigested foods in the colon results in the formation of various metabolites, including volatile organic compounds. The assessment of these small endogenous molecules is important for metabolome analysis (Zhang & Davies, 2016), and are complementary with metabolomic techniques to assess the impact of nutrition (Vernocchi et al., 2012). The assessment of volatile metabolites has been proposed as one of the approaches for investigating the biochemical characterization of metabolic changes triggered by the gut microbial community and dietary variation (Di Cagno et al., 2011; Vitali et al., 2010). Many microbial volatile organic compounds have biogenic actions on mammalian cells, including cytotoxic and inflammatory effects (Thorn & Greenman, 2012). The effects of long-term exposure of the colonic mucosa to these metabolites remain unclear, and it is possible that the impact of protein fermentation is overshadowed by other dietary or lifestyle factors (Windey et al., 2012). The most abundant end products of protein fermentation are short chain fatty acids (Oliphant & Allen-Vercoe, 2019), which are also derived from carbohydrates. A continually emerging body of evidence supports the role of short chain fatty acids as key mediators of cell function in a range of local, intermediary and peripheral tissues (Morrison & Preston, 2016). Also, branched-chain fatty acids, such as isovalerate and isobutyrate, can be produced as end-products from amino acids, but little is actually known about their impact on host health (Oliphant & Allen-Vercoe, 2019). The bacterial metabolism of tryptophan in the colon results in the production of a wide range of indolic compounds, whereas phenolic compounds, such as phenol and cresol, are generated by the partial breakdown of tyrosine and phenylalanine by a wide range of intestinal obligate or

facultative anaerobes (Evenepoel et al., 2009). These aromatic amino acid degradation products can act as toxins or neurotransmitters (Oliphant & Allen-Vercoe, 2019).

Volatile sulfur compounds are a family of gases that can be toxic to tissues at high concentrations, however, some of these compounds, such as dimethyl sulfide, methanethiol and dimethyl disulfide, may be produced by methylation of hydrogen sulfide as a detoxification mechanism by mucosal enzymes (Garner et al., 2007). Likewise, carbon disulfide may be produced by carbonation of hydrogen sulfide as a detoxification mechanism, exerted by colonic bacteria (Vitali et al., 2010). Low hydrogen sulfide concentrations in the colon improve intestinal health by maintaining the integrity of the mucus layer and alleviating inflammation (Blachier et al., 2019; Motta et al., 2015). In contrast, high concentrations of colonic hydrogen sulfide may be detrimental for health, since it reduces disulfide bonds of the colonic mucus layer, hereby breaking the intestinal mucus barrier, resulting in exposure of the epithelium to bacteria and toxins, leading to inflammation (IJssennagger et al., 2016). In addition, hydrogen sulfide may induce direct radical-associated DNA damage (Attene-Ramos et al. 2007). Yet, little is known about the role of meat consumption on the production of hydrogen sulfide and volatile sulfur compounds.

The purpose of this study was to investigate the effect of meat, consumed in representative quantities and combined with realistic dietary patterns, on the microbial community and colonic volatile organic compounds using a pig model as described in Chapter II.

4.2 Materials and methods

4.2.1 Study design

The diet formulation and study design of the pig feeding trial were described in Chapter II (2.1-2.4). For this particular chapter, the large intestinal content was collected and gently homogenized. The pH was measured using a Knick portamess 654 (Knick Elektronische Messgeräte GmbH, Berlin, Germany) with Schott N5800A electrode (Schott Instruments, Mainz, Germany) and the samples were stored in aliquots at −80 °C until further analysis. Eight animals were not included in the present trial (i.e., two, one, three and two pigs fed respectively chicken-prudent, red&processed-prudent, chicken-Western, and red&processed-Western), because piglets presented diarrhea, or looser stool (<15 g dry matter/100 g colon contents).

4.2.2 Microbial community analysis

The DNA extraction was carried out as described by (Vilchez-Vargas et al., 2013). The quality of the DNA extracts was validated through agarose gel electrophoresis and

via PCR, using the protocol of (Boon et al., 2002) with the bacterial primers P338 and P518r (Muyzer et al., 1993). The quality of the PCR product was determined with agarose gel electrophoresis to ensure that no inhibition of the PCR took place. The DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany) for Illumina sequencing on the MiSeq platform. Amplicon sequencing and data processing were carried out as follows.

The primers 341F (5'- NNNNNNNNNTCCTACGGGNGGCWGCAG) and 785R (5'-NNNNNNNNNNTGACTACHVGGGTATCTAAKCC) that target the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013) were used to target total bacteria. The PCR mix had a volume of 20 µL MyTaq buffer, and contained 50 pg of DNA, 15 pmol of each primer, 1.5 units of MyTaq DNA polymerase (Bioline London, UK) and 2 µL of BioStabII PCR optimizer (Sigma-Aldrich, St. Louis, MO). The PCR program consisted of an initial predenaturation step of 2 min at 96°C after which 35 cycles of 15 s at 96°C, 30 s at 50°C and 90 s at 70°C were implemented. The DNA concentration of the amplicons was determined by gel electrophoresis. The PCR products were pooled, and purified using the Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) to remove primer dimers and other small mispriming products. An additional purification step was performed with the MinElute PCR Purification Kit (Qiagen, Venlo, The Netherlands). Following purification, the amplicon pools were used to generate Illumina libraries by adaptor ligation using the Ovation Rapid DR Multiplex System 1-96 (NuGEN, San Carlos, CA). The Illumina libraries were pooled, and size selected by gel electrophoresis. Sequencing was carried out on an Illumina MiSeq, using V3 chemistry (Illumina).

The Mothur software package (v.1.39.3), and guidelines developed by (Schloss et al., 2009) were used to process the raw Illumina data on a GNU/Linux 3.16.0-46-generic x86_64 system. Forward and reverse reads were assembled into contigs by a heuristic approach, taking the Phred quality scores into account. After removing ambiguous contigs or with unsatisfying overlap, the remaining sequences were aligned to the mothur formatted silva seed v128 database. Sequences not aligning within the region targeted by the primer set or sequences with homopolymer stretches with a length higher 12 were removed. The sequences were pre-clustered, allowing 1 difference for every 100 bp of sequence. Chimeric sequences were removed with UCHIME. Classification of the sequences was carried out by a naïve Bayesian classifier, using the RDP 16S rRNA gene training set, release 14 with an 85% cut-off for the pseudobootstrap confidence score. Taxa that were annotated as Chloroplast, Mitochondria, unknown, Archaea or Eukarya at the kingdom level were excluded. Sequences were clustered into OTUs (operational taxonomic units) with an average linkage, and at a 97% sequence identity. Representative sequences were picked for each OTU as the most abundant sequence within that OTU.

A table containing the relative abundance of different OTUs, together with their taxonomic assignments for each sample was generated. The OTUs were delineated at the 97% similarity level. The raw fastq files that were used to create the OTU table that served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information database (Accession number SRP185872). The total bacterial count in the colon was analyzed by means of real-time PCR, as previously described (De Vrieze et al., 2015) and expressed as mean ± standard error of the mean.

A microbial community can be characterized by the total number of organisms (abundance), by the number of different species (species richness) or by the combination of abundance and the evenness of their abundances (diversity). Dependent on weight given to rare versus abundant species, several diversity indices have been proposed (Hill, 1973). Higher values of order-based Hill numbers indicate a higher species richness or bacterial diversity. Hill diversity order H0 corresponds with the number of OTUs (species richness), whereas Hill diversity orders H1 and H2 represent the exponential value of the Shannon index and the inverse Simpson index respectively, with H2 giving more weight to abundant species compared to H1.

4.2.3 Statistical analyses of the microbial community

The R Studio version 3.2.3. software (http://www.r-project.org, R Development Core Team, 2013) was used for statistical analysis. The packages phyloseq (McMurdie & Holmes, 2013) and vegan (Oksanen et al., 2016) were used for microbial community analysis. The community composition of the biological replicates was statistically compared by means of ANOVA (aov function) to validate that the bacterial community did not significantly differ between the biological replicates. Heatmaps were generated using the weighted average values of the biological replicates by means of the 'pheatmap' package. Differences in order-based Hill's numbers (Hill, 1973) between the four treatments were defined via ANOVA and the post-hoc Dunn's Test of Multiple Comparisons (dunn.test package) with Benjamini-Hochberg correction. Separate non-metric multidimensional scaling (NMDS) plots of the bacterial community data were created based on Bray-Curtis distance measures. Significant differences in community composition between treatments were identified by means of pairwise Permutational ANOVA (PERMANOVA) with Bonferroni correction, using the adonis function (vegan). The effect of 'dietary pattern', 'meat type' and 'treatment' on the total bacterial counts in the colon were analyzed using one-way ANOVA, after testing the normality and homogeneity of variance of the data. Results were considered significantly different when $p < 0.05$.

To identify characteristic bacterial groups, linear discriminant analysis (LDA) effective size (LEfSe) was performed using the online interface for the method, provided at http://huttenhower.sph.harvard.edu/lefse/ (Segata et al., 2011). Analyses were performed at different taxonomic levels combining the corresponding OTUs. To investigate the effect of 'dietary pattern', 'meat type' and 'treatment', the factorial Kruskal-Wallis test and the pairwise Wilcoxon test were run three times. The non-negative threshold for the logarithmic LDA score for discriminative features was set on two, the strategy for multi-class analysis was set as 'all against all' and the alpha-values among subclasses was set at 0.05.

4.2.4 Colonic volatile organic compounds analyses

The volatile organic compounds were extracted from the headspace of the colon samples using solid phase micro-extraction (SPME) with a carboxen-polydimethylsiloxane coated fiber (85 µm) and the Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). The fiber was conditioned prior to analysis, following the instructions of the manufacturer. Before sampling, the fiber was exposed to the GC inlet for 20 min for thermal desorption at 250 °C. One gram of colonic sample was placed into 10 mL glass vials, and 4-methyl-2-pentanone (final concentration of 2 µg/g colon) was added as the internal standard. The SPME fiber was exposed to each sample for 40 min at 50 °C. The fiber was then inserted into the injection port of the GC for sample desorption for 20 min. As control, an empty glass vial was stored under similar conditions to the sample vials and analyzed using the same method. The GC–MS analyses were carried out on a Trace DSQ II (Thermo, Finnigan) that operated at 70 eV (EI + mode) with Xcalibur software (version 1.4 SR1) for data acquisition and processing. A fused silica capillary column (SLB-IL60 column) of 30 m × 0.25 mm × 0.2 µm was used (Supelco, Bellefonte, PA, USA). The temperature program was 40 °C for 5 min, 4.5 °C/min to 65 °C for 1 min and 10 °C/min to 250 °C, which was held for 10 min (Di Cagno et al., 2011). Interface and ion source temperatures were 250 and 230 °C, respectively. The mass-to-charge ratio interval was 35–350 a.m.u. at 3.0 scans per second. Injections were carried out in splitless mode, and helium (1 mL/min) was used as carrier gas. Compounds were putatively identified by comparison with the National Institute of Standards and Technology (version 2005) mass spectral library, followed by manual visual inspection, and retention time matching of selected standards (ethanol, propanoic acid, cresol, acidic acid, nonanal, heptanal, 1-propanol, methyl acetate, ethyl acetate, and 1-butanol). A dichotomous variable was assigned based on the presence and absence of the volatile compound: i.e., 1 if the volatile was present in both analytical replicates with a 50% or higher probability of a match to library standards and 0 if the the probability was lower than 50% in at least one analytical replicate.

Semi-quantitative data of three protein degradation products and two methylated sulfides (cresol, indole, x-methylindole, methanethiol and dimethyl disulfide, present in all pigs) were obtained by expressing the relative area of specific quantification ions (respectively *m/z* 108, 117, 130, 48 and 94) versus the internal standard area (quantification ion *m/z* 100).

Additionally, the short chain fatty acids and ammonia were quantified according to (Gadeyne et al., 2016). Five mL of 10% formic acid containing the internal standard (1 mg 2-ethylbutanoic acid) were added to 1 mL colon content, and after 15 min centrifugation (22,000 g at 4 °C), the supernatant was filtered and an aliquot transferred into a 1.5 mL glass vial. The short chain fatty acids were measured by GC (HP 7890A, Agilent Technologies, Diegem, Belgium), equipped with a flame ionization detector and a Supelco Nukol capillary column (30 m × 0.25 mm × 0.25 µm, Sigma-Aldrich, Diegem, Belgium). Results are expressed as µmol/g colon content and limits of quantification (LOQs) per g colon content for acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate and caproate are respectively 2 µmol/g, 1.2 µmol/g, 0.9 µmol/g, 1.7 µmol/g, 0.7 µmol/g, 0.6 µmol/g and 0.7 µmol/g. Ammonia was measured using the same supernatants of the short chain fatty acid analysis (Chaney & Marbach, 1962; Gadeyne et al., 2016). Briefly, 2 mL of supernatant was taken and acidified using 2 mL 0.2 N HCl. Samples were shaken, centrifuged (1700 g, 20 min) and 1 mL supernatant was collected in an experimental tube. Next, 4.5 mL of a solution containing 10 g/L C_6H_5OH (Merck, Darmstadt, Germany) and 0.05 g/l $Na_2[Fe(CN)_5NO]\cdot 2H_2O$ (Sigma-Aldrich, Diegem, Belgium), and 4.5 mL of a solution containing 5 g/L NaOH and 4.2 mL 10% NaClO (Sigma-Aldrich, Diegem, Belgium), were added, vortexed and left at room temperature for 1 h. The absorbance was measured at 625 nm and ammonia was quantified using an external standard (0–20 mg/L NH_4Cl). LOQ for ammonia detection by this method is 3 µmol/g colon content.

4.2.5 Statistical analysis of the colonic volatile organic compounds

For the (semi-)quantified metabolites, normality was checked by the Shapiro-Wilk test and the homogeneity of variance was verified by the Levene's test. For all parameters, with exception of acetic acid and propionic acid, at least one of the conditions was not fulfilled. Therefore, the non-parametric Kruskal-Wallis test was used. The fixed effects ‘dietary pattern’, ‘meat type’ and ‘treatment’ were analysed separately. The results are reported as mean ± standard error of the mean. The binomial data were analyzed using Firth’s logistic regression at a confidence level of 95% with ‘dietary pattern’, ‘meat type’ and ‘treatment’ as the fixed effect. Statistical analyses were performed using SPSS Statistics version 24 (IBM SPSS, Armonk, NY) with ‘pig’ considered the experimental unit. Results were found significantly different when $p < 0.05$.

4.3 Results

4.3.1 Microbial community

The total bacterial counts were significantly ($p < 0.05$) higher in the colon of pigs fed a prudent diet (8.88 ± 0.07 log copies 16S rRNA gene/µl; mean ± standard error of the mean) compared to a Western diet (8.53 ± 0.13), whereas no significant effect of 'meat type' and 'treatment' were observed ($p > 0.05$).

After 16S rRNA gene amplicon sequencing, a total of 285,322 usable raw reads were obtained from all samples, with 11,888 ± 6856 (mean ± SD) reads per sample. The total number of OTU was 4259 with 476 ± 101 OTU per sample.

No significant differences were observed for the Hill diversity orders H0 (richness, number of OTUs after correction for singletons) and H1 (exponential value of the Shannon index) between the four treatments (Figure 4.1). The H2 diversity (inverse Simpson index) of the pigs receiving the prudent diet with red and processed meat was significantly lower compared to the pigs receiving a Western diet with either chicken or red and processed meat ($p = 0.033$ and 0.032, respectively).

The clustering between the dietary patterns was more pronounced than between chicken and red and processed meat (Figure 4.2). A significant difference in community composition was observed between the red&processed-Western diet and the red&processed-prudent diet ($p = 0.002$, using PERMANOVA). No significant differences were observed among the other treatments. The relative abundances of the colonic bacteria at phylum and family level are presented in Figure 4.3. At the phylum level, Firmicutes and Bacteroidetes were the most predominant phyla in all four treatments, with their relative abundance varying among treatments between 15 and 74% for Firmicutes and 17 and 83% for Bacteroidetes. The phyla Proteobacteria, Tenericutes and Spirocheates were present to a lesser extent (<5%). No significant differences at the phylum level were observed.

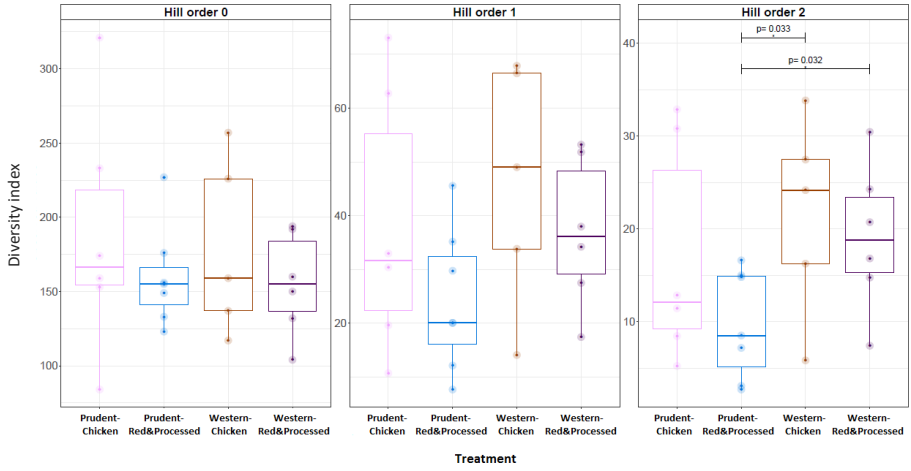

Figure 4.1 The α-diversity of the colon microbial community, calculated by the three Hill orders: H0 (richness, number of OTUs), H1 (exponential value of the Shannon index) and H2 (inverse Simpson index) according to Hill (1973). Pigs received different treatments representing human diets, varying in dietary pattern and meat type. The number of pigs are 6, 7, 5 and 6 for prudent-chicken, prudent-red&processed, Western-chicken and Western-red&processed, respectively. Only significant p-values are reported in the figures.

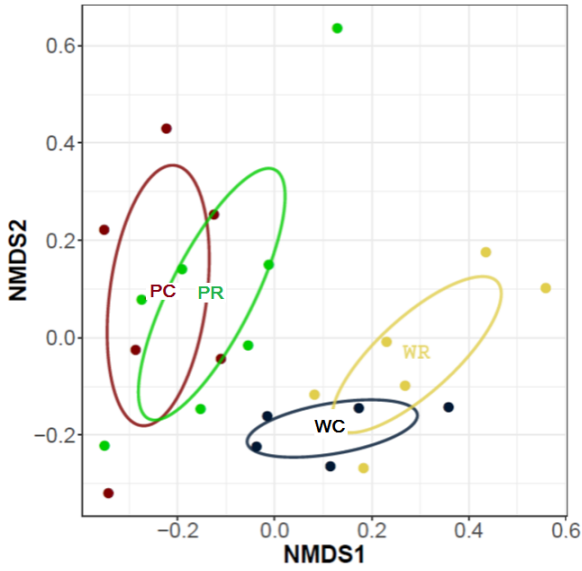

Figure 4.2 Non-metric multidimensional scaling (NMDS) analysis of the bacterial community at OTU level, based on the Bray-Curtis distance measures. Pigs received different treatments representing human diets, varying in dietary pattern and meat type. PC = prudent-chicken; PR = prudent-red&processed; WC = Western-chicken; WR = Western-red&processed meat. Only the bacterial composition of PR and WR are significantly different ($p < 0.05$).

The results concerning the LEfSe analyses are presented in Figure 4.4. Several differential features with an LDA score higher than 2.0 were observed between the prudent and Western dietary pattern (Figure 4.4A). Listed in order of LDA score, higher relative abundances were observed for Prevotellaceae, *Prevotella*, *Butyrivibrio*, *Anaerovibrio*, Sutterellaceae, *Desulfovibrio*, Burkholderiales, Clostridiaceae_1, Betaproteobacteria, *Dorea* Bacteroides, Bacteroidaceae, *Clostridium_sensu_stricto*, Enterobacteriales, *Escherichia*/*Shigella*, *Pyramidobacter*, Synergistales, Synergistaceae, Synergistia, Synergistetes and Enterobacteriaceae in the colon of animals fed a Western dietary pattern, compared to the prudent dietary pattern. *Paraprevotella*, *Mitsuokella*, *Megaspaera* and *Dialister* were relatively more abundant in the colon of animals fed a prudent dietary pattern, compared to the Western dietary pattern.

Campylobacter and Campylobacteraceae were characteristic for chicken meat, whereas *Clostridium* cluster XIVa was found to be characteristic for red and processed meat (Figure 4.4B) consumption. Figure 4.4C presents the differential features for the four treatments separately. *Butyrivibrio* was characteristic for the treatment combining red and processed meat with a Western dietary pattern. Regarding the treatment with chicken combined with a Western dietary pattern, Enterobacteriaceae, Bacteroides, Bacteroidaceae, *Escherichia*/*Shigella*, Enterobacteriales, *Anaevibrio*, and *Desulfovibrio* were characteristic. The treatment with red and processed meat combined with the prudent dietary pattern had significantly higher relative abundances for Paraprevotella compared to the others ($p < 0.05$). The treatment with chicken and a prudent dietary pattern had higher relative abundances of Lactobacillales, Lactobacillaceae, *Lactobacillus*, Bacilli and *Mitsuokella* compared to the other three treatments.

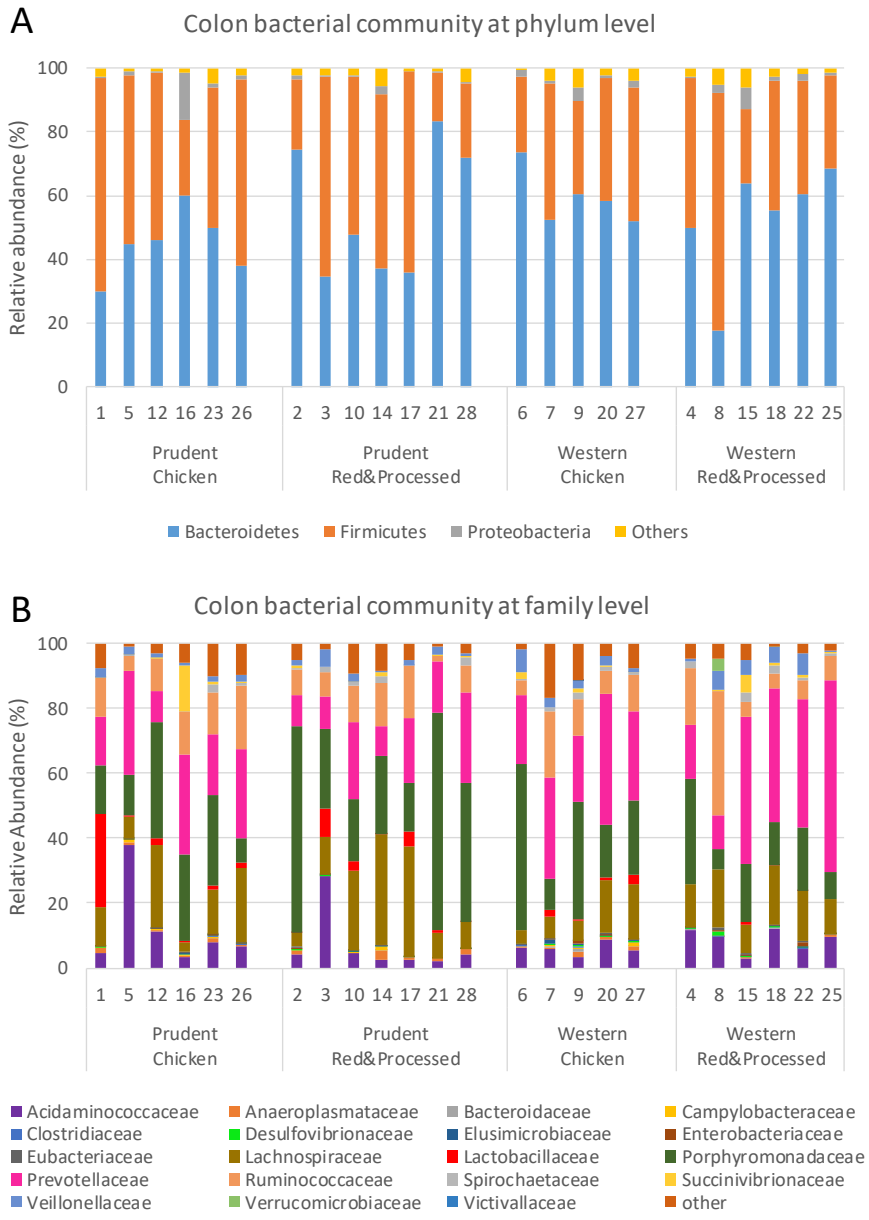

Figure 4.3 The relative abundances of colonic bacteria at the phylum (A) and family level (B). Pigs received different treatments representing human diets, varying in dietary pattern and meat type. Each column represents one animal.

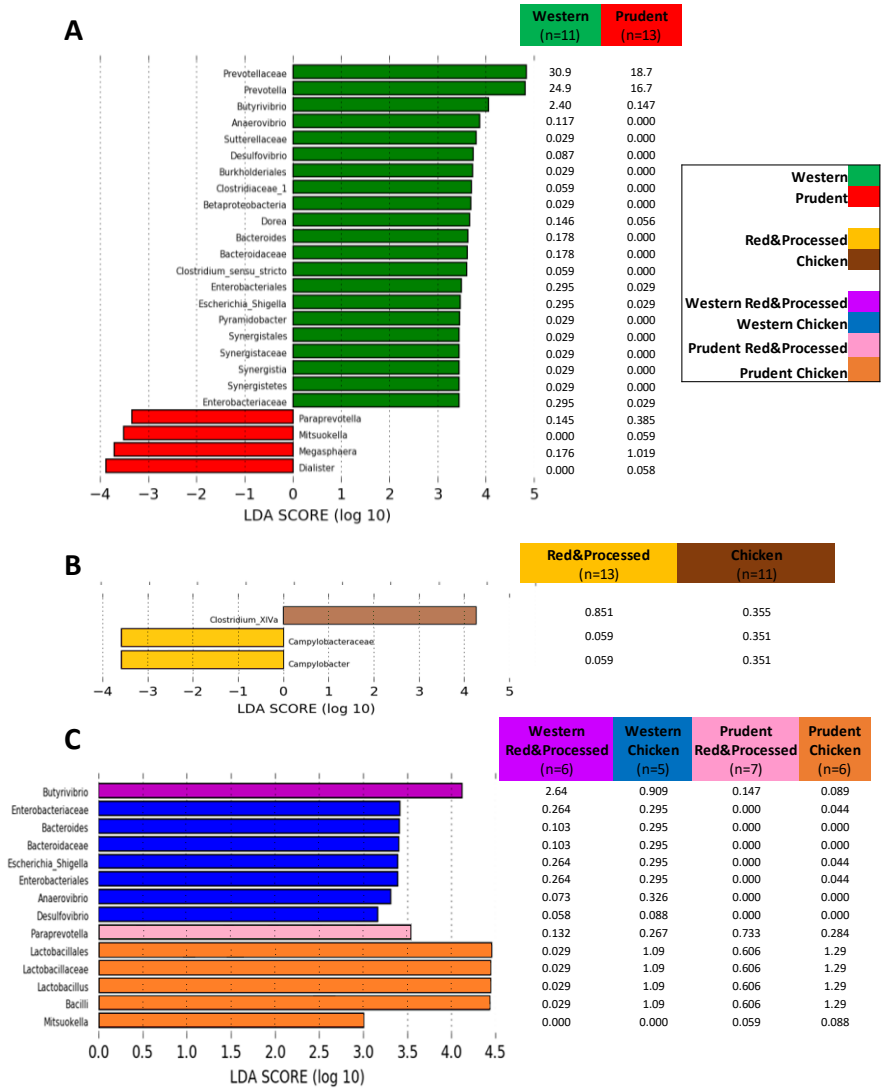

Figure 4.4 Comparisons of colonic bacteria at different taxonomic levels using linear discriminant analysis (LDA) effective size (LEfSe) with α = 0.05. LEfSe scores can be interpreted as the degree of consistent difference in relative abundance. The histograms show the LDA scores for bacterial communities that were differentially abundant between A) dietary pattern, B) meat type and C) whole dietary treatments. The tables show the median relative abundances of the different OTUs.

4.3.2 Colonic volatile organic compounds

The quantified volatile organic compounds are presented in Table 4.1. The factor 'meat type' and 'treatment' had no effect (all $p > 0.05$), whereas for the dietary pattern significantly higher concentrations were only observed for butyrate, with higher concentrations when the prudent dietary pattern was fed compared to the Western dietary pattern ($p < 0.05$). In addition to the volatile compounds reported in Table 4.1, another 54 volatile organic compounds were detected (Table 4.2). Although some compounds were ubiquitously or commonly present, most of them were found infrequently. Compared to the prudent dietary pattern, phenol was significantly less common in pigs receiving a Western dietary pattern, whereas 3-methylbutanal was observed more frequently in the latter, regardless of the meat type ($p < 0.05$). Ethyl pentanoate was detected significantly more in pigs fed red and processed meat compared to chicken, regardless of the dietary pattern. The compound 1-(methylthio)-propane was observed in certain pigs fed red and processed meat, whereas this compounds was absent when chicken was fed. Ethylene glycol was present in half of the pigs fed red and processed meat within a Western dietary pattern, whereas it was not observed in the other three treatments.

Table 4.1. Short chain fatty acids and ammonia (expressed as µmol/g colon), protein fermentation products and volatile sulfur organic compounds (expressed as % area under the curve vs. the internal standard) present in all samples. Results are reported as mean ± standard error of the mean. P values ≤ 0.05 are highlighted in boldface, and P values between 0.05 and 0.10 are highlighted in italics.

	Prudent Chicken	**Prudent Red&Processed**	**Western Chicken**	**Western Red&Processed**	Pdiet	Pmeat
acetate	68.5 ± 4.36	59.4 ± 2.61	55.4 ± 3.46	55.6 ± 4.80	0.077	0.311
propionate	28.4 ± 2.82	25.8 ± 2.070	24.0 ± 3.27	27.1 ± 4.00	0.706	0.931
iso-butyrate	2.11 ± 0.306	1.97 ± 0.241	1.70 ± 0.132	1.51 ± 0.102	0.192	0.434
butyrate	17.6 ± 1.55	17.7 ± 2.136	12.3 ± 1.79	13.1 ± 2.36	**0.022**	0.977
iso-valerate	3.23 ± 0.508	3.02 ± 0.389	2.72 ± 0.216	2.32 ± 0.142	0.259	0.434
valerate	4.80 ± 0.359	4.44 ± 0.332	3.99 ± 0.704	5.40 ± 1.05	0.622	0.622
caproate	1.42 ± 0.193	1.52 ± 0.177	1.31 ± 0.263	1.30 ± 0.205	0.311	0.622
ammonia	90.6 ± 14.9	64.5 ± 10.92	76.4 ± 1.682	66.1 ± 4.07	0.311	0.156
cresol	112 ± 49.1	110 ± 40.4	46.7 ± 8.82	59.4 ± 10.5	0.469	0.839
indole	10.1 ± 4.30	6.81 ± 2.27	4.42 ± 1.97	2.37 ± 0.694	0.087	0.339
x-methylindole	25.6 ± 10.6	24.8 ± 7.27	10.9 ± 3.57	21.3 ± 4.96	0.434	0.339
methanethiol	2.98±0.618	4.28±1.46	1.85±0.709	2.79±0.738	0.252	0.392
dimethyl disulfide	12.8±3.77	14.0±3.08	15.7±4.40	14.6±3.47	0.531	0.776

Parameters were analysed using the non-parametric Kruskal-Wallis test comparing distributions across two groups. The fixed effects 'diet' and 'meat' were analysed separately. The effect 'dietary treatment' was not significant (all p>0.05). The number of pigs are 6, 7, 5 and 6 for prudent-chicken, prudent-red&processed, Western-chicken and Western-red&processed, respectively.

Table 4.2 Prevalence of volatile organic compounds observed in the colon content of pigs fed diets varying in dietary pattern and meat type. Listed in order of prevalence.

Volatile organic compounds	Prudent Chicken (n = 6)	Prudent Red&Processed (n = 7)	Western Chicken (n = 5)	Western Red&Processed (n = 6)
carbon dioxide	100	100	100	100
benzaldehyde	100	100	100	100
ethanol	100	100	100	100
2-methylbutanoic acid	100	100	100	100
propan-1-ol	83	86	100	100
butan-1-ol	83	86	100	83
phenol (*)	**100**	**100**	80	50
2-phenylethanol	100	71	80	67
(methyltrisulfanyl)methane	100	86	60	67
acetone	50	86	80	67
ethyl acetate	83	86	60	50
ethyl butyrate	83	71	60	50
3-methylbutanal (*)	33	29	100	83
allyl isothiocyanate	67	71	40	50
ethyl pentanoate (¥)	50	86	20	67
carbon disulfide	33	57	40	67
dimethyl sulfide	50	57	40	50
propyl butyrate	50	43	40	33
pentan-1-ol	50	14	60	33
3-methylbutan-1-ol	33	29	60	33
pentan-2-one	17	71	40	17
2-nitroethyl propanoate	17	29	60	33
1-(7-acetyl-1,8-dihydroxy-3,6-dimethylnaphthalen-2-yl)ethanone	50	43	20	0
6-methylhept-5-en-2-one	33	57	20	0
2-methylpropan-1-ol	17	14	20	50
3-tert-butylfuran-2,5-dione	17	57	20	0
heptan-2-one	17	29	40	0
ethyl hexanoate	17	29	20	17
1-(methylthio)-propane (¥)	0	57	0	17
butan-2-one	0	29	20	17
pentane-2,3-dione	17	0	20	17
ethylene glycol	0	0	0	50
nonan-2-one	17	0	0	33
propyl acetate	0	14	0	33
hexan-2-one	0	14	20	0
dimethyl sulfoxide	0	14	20	0
acetaldehyde	0	0	0	33
propyl propionate	0	0	0	33
piperidin-2-one	17	0	0	17
trimethylene oxide	0	0	0	33
propyl valerate	0	0	0	33
methyl propionate	17	14	0	0
ethyl 2-methylbutyrate	0	0	0	17
propyl 2-methylbutyrate	0	0	0	17
propyl 3-methylbutyrate	0	0	0	17
butyl butyrate	0	0	0	17
methyl caproate	17	0	0	0
4-amino-pentan-1-ol	0	0	0	17
propyl caproate	0	0	0	17
nitrosomethane	17	0	0	0
ethyl propionate	0	0	0	17
3-(methylthio)propyl isothiocyanate	0	14	0	0
methyl acetate	17	0	0	0
2-methylheptanoic acid	17	0	0	0

%Prevalence

<25% **25-49%** **50-75%** **>75%**

%Prevalence = (number of pigs in which the volatile was observed)/(total amount of pigs per treatment)*100
* Significant effect of dietary pattern ($p<0.05$)
¥ Significant effect of meat type ($p<0.05$)
The effect 'dietary treatment' was not significant (all $p>0.05$).

4.4 Discussion

This study investigates the impact of meat type on the colon microbial community and volatile organic compounds within two different dietary patterns. A higher number of significant differences was found between the dietary patterns than between meat types. This outcome was expected, as the composition of the dietary patterns is more contrasting compared to the two animal protein sources. In general, the gut microbial community is relatively stable over time and can be resistant to change (Winglee & Fodor, 2015), however, microbiota changes within just 24 h of diet initiation have also been reported (Singh et al., 2017b). Like in humans, the pigs' colon in present study was dominated by a mix of Bacteroidetes and Firmicutes. However, an increase of Firmicutes with a prudent dietary pattern or an increase of Bacteroidetes, due to the high fat content of the Western dietary pattern, was not observed (Winglee & Fodor, 2015). Also no effect of meat type was observed on these phyla, although it has been shown that, in a Western dietary model, heme-iron supplementation increased the relative abundances of Bacteroidetes and decreased Firmicutes in colonic contents of mice (IJssennagger et al., 2012a). Yet, in a similar experiment, the same authors did not find changes in the relative abundances of Firmicutes (IJssennagger et al., 2015).

Several studies investigating the impact of dietary protein on gut microbial composition showed that protein consumption positively correlates with overall microbial diversity (Singh et al., 2017b). Also a healthier dietary pattern is usually associated with a higher microbial community diversity (Doré & Blottière, 2015), but in present study little effect herein was observed. In contrast, slightly higher values were obtained for the H2 diversity order after consumption of the Western diets compared to the prudent diet in combination with red and processed meat. A higher total bacterial count and higher concentrations of butyrate, which are associated with individuals consuming a plant-rich diet (Kau et al., 2011), were measured in the pigs receiving a prudent dietary pattern. Although this difference in bacterial count was statistically significant, results should be interpreted with care since no mock community was included in the analysis to calculate the error rate of the amplicon sequencing. Furthermore, the biological relevance of this statistically significant difference can be questioned. Butyrate is considered the most preferred source of energy and it improves the integrity of host intestinal epithelial and exhibits anti-inflammatory effects (Louis & Flint, 2017; Oliphant & Allen-Vercoe, 2019). This short chain fatty acid is produced predominantly by *Clostridium* clusters XIVa and IV of the phylum Firmicutes (Vipperla & O'Keefe, 2013), with the former being characteristic for red and processed meat consumption in present study. Bacteria from the *Clostridium* cluster XIVa, are significantly more abundant in feces of omnivores compared to vegetarians (Kabeerdoss et al., 2012; Kim et al., 2013) and present study demonstrates that (processed) red meat, rather than chicken meat, is responsible for this difference. *Butyrivibrio*, belonging to the *Clostridium* cluster XIVa

(Devillard et al., 2007), are also predominantly present in the rumen of beef cattle (Durso et al., 2013), and *Campylobacter* is frequently found in poultry (Newell & Fearnley, 2003). Similarly, bacteria associated with cheese and cured meats (*Lactococcus lactis*, *Pediococcus acidilactici* and *Staphylococcus*) became significantly more prevalent in human fecal samples after consumption of an animal-based diet, compared to a plant-based diet, indicating that bacteria found in foods can reach the gut at abundances above the detection limit (David et al., 2014). In addition to resident microbial communities that shift in response to the diet, it seems that the differences between the chicken and red and processed meat treatments of present study is directly linked to the meat source.
Interestingly, *Lactobacilli* were characteristic for a prudent dietary pattern combined with chicken, whereas their presence was considerably lower in the diet combining red and processed meat with a Western dietary pattern. Likewise, Van Hecke et al. (2019a) reported that the *Lactobacillus* genus was the most discriminative for rats fed a diet containing chicken and starch compared to, amongst others, a diet with beef and sucrose. Zhu et al. (2015) found that *Lactobacillus* was higher in rats fed white meat proteins (chicken and fish) compared to red meat (beef and pork) or non-meat protein (soy and casein) groups. Reductions in *Lactobacillus* were also reported in several mouse studies fed high fat diets (Tachon et al., 2014). Some species of the *Lactobacillus* genus have been shown to play a role in the gut mucosal immune and barrier function (Miranda et al., 2018) and a higher abundance of *Lactobacillus* may reduce the antigen load from gut bacteria to the host and alleviate certain inflammation responses and metabolic syndromes (Zhu et al., 2015).

Prevotella are frequently associated with plant-rich diets (Ley, 2016), but in present study were found to be characteristic for the Western dietary pattern. Yet, *Prevotella* are commonly encountered in the Western gut microbiome (Johnson et al., 2017), and have also been linked with inflammatory conditions (Ley, 2016). *Paraprevotella*, characteristic for the prudent dietary pattern, has been recognized relatively recently, and little is known about its clinical significance (Tan et al., 2018). It has been postulated that the shifts in microbial populations driven by fat and the associated increases in gut bacteria, such as *Escherichia coli* and Enterobacteriaceae (characteristic for present Western dietary pattern), are linked with poor health outcomes (Conlon & Bird, 2015). Of course, many characteristic bacterial groups identified in this study are commonly present in the intestinal tract and not *per se* harmful, which is why additional insight on the presence of specific metabolites is needed. For example, members of the Sutterellaceae family are frequently found in the intestinal tract of humans, but have also been reported to cause gastrointestinal infections (Morotomi, 2014). The phylum Synergistetes consists of anaerobic Gram-negative bacteria, and although the significance of *Pyramidobacter* in fecal microbial community is yet unknown, the oral isolate *Pyramidobacter piscolens* is known to produce hydrogen sulfide (Lankelma et al., 2017). Likewise, *Desulfovibrio* are known

for their sulfate-reducing capacities, hereby producing hydrogen sulfide (IJssennagger et al., 2016). In a previous study, red meat consumption has been associated with an outgrowth of *Desulfovibrionaceae* in rats, with median relative abundances of 23% (Van Hecke et al., 2019a), which is more than hundred-fold higher compared to those observed in present study. As these bacteria were only observed in present study in pigs receiving a Western dietary pattern, it should be elucidated whether the meat type, the dietary pattern or their interaction is responsible for the outgrowth of *Desulfovibrionaceae*. It was hypothesized that meat, containing relatively high amounts of dietary sulfur, would promote the growth of sulfate-reducing bacteria or other bacteria involved in pathways of colonic sulfur metabolism such as *Clostridium* spp., *Enterobacter* spp., and *Campylobacter* (Carbonero et al., 2012) and that these bacteria would affect the presence and concentration of toxic sulfur compounds. In addition, fermented foodstuffs, such as fermented sausages, contain a wide array of volatile sulfur compounds (Landaud et al., 2008), which could also increase the concentrations of these compounds in the colon. Although this latter hypothesis was not confirmed in the present study, effects of meat on the production of toxic sulfur compounds should be further explored.

According to Raman et al., 2013, there is a core group of volatile organic compounds that reflect essential functions of the resident colonic microbial community irrespective of day-to-day dietary influences. Some volatiles may originate directly from dietary sources, and variations in the remaining volatile organic compounds are most likely related to individual dietary practices and distinctions of the microbial community. No distinction between meat types or between dietary patterns could be made in present study for the investigated protein degradation products, except for phenol. The diets consisted of comparable crude protein levels and the amino acid pattern of poultry and mammal muscle tissues are comparable (Beach et al., 1943), which may be the reason why little effect of meat type and dietary pattern on the protein degradation products was observed. Most of the volatile organic compounds observed in present study have been described in the colon of humans (de Lacy Costello et al., 2014; De Preter et al., 2009; el Manouni el Hassani et al., 2018; Garner et al., 2007; Raman et al., 2013). Therefore, only the compounds of which the prevalence significantly differed between treatments will be discussed here.

The organic ester ethyl pentanoate was more present when red and processed meat was consumed, regardless of the dietary pattern and has been reported in feces of both healthy and unhealthy subjects (Garner et al., 2007; Raman et al., 2013; Saa et al., 2014). Ester syntheses, using different alcohols and fatty acids as substrates, may be mediated by bacteria, such as in *E. coli*, or by enzymes derived from the intestinal mucosa (Garner et al., 2007), and ester volatile organic compounds may have injurious effects on barrier or local immune functions in the gut (Raman et al., 2013).

However, no differences among the other esters were observed in present study, and ethyl pentanoate, commonly present in fruits, is approved as a flavour additive by the Joint FAO/WHO Expert Committee on Food additives (JECFA), so little harmful effects of this specific compound are expected. Likewise, for 3-methylbutanal, 'no safety concern at current levels of intake when used as a flavouring agent' has been reported by JECFA. This aldehyde has been found mainly in the treatments with a Western dietary pattern in present study. It is a key flavour component in semi-hard cheeses, and is commonly found in (dry cured) meat (Ruiz et al., 1999). Aldehydes are produced during inflammatory processes as a result of lipid peroxidation and oxidative stress (Fritz & Petersen, 2013), but for 3-methylbutanal there is no certainty about its reactivity (Estévez, 2011). The compound 3-methylbutanal can either be formed by Strecker degradation of leucine or during cholesterol thermoxidation (Cardenia et al., 2015). Cardenia et al., 2015 showed that 3-methylbutanal can be used as volatile marker of cholesterol oxidation in egg yolk, which is in accordance with the high cholesterol content of the Western dietary pattern. Cholesterol oxidation products are known to be potentially involved in the initiation and progression of various chronic diseases (Otaegui-Arrazola et al., 2010). It needs to be elucidated whether the occurrence of 3-methylbutanal is mainly a direct result of the dietary composition or because of reactions that occurred during digestion (or both).

The compound 1-(methylthio)-propane, in present study characteristic for red and processed meat and especially when combined with a prudent dietary pattern, has not been evaluated by JECFA. It is found in dry cured loin and fresh beef as being a potent aroma compound originating from sulfur containing amino acids (Muriel et al., 2004; Raes et al., 2003), and *Microbacterium foliorum* was able to produce this compound during ripening in a cheese model medium (Deetae et al., 2009). This fits with the composition of the red and processed meat with a prudent dietary pattern containing fresh beef, dry cured meat products and considerable amounts of cheese. The compound has been detected in feces of healthy volunteers, but not in patients with ulcerative colitis, *Clostridium difficile* or *Campylobacter jejuni* infections (Garner et al., 2007). To the best of our knowledge, this compound has not yet been associated with specific bacteria in the colon or with the detoxification of hydrogen sulfide, and its importance in this context should be further explored.

Ethylene glycol is a colourless, odourless and soluble chemical, mainly used in automobile anti-freeze, brake solution and as an industrial solvent. Metabolites of ethylene glycol are toxic compounds (Leth & Gregersen, 2005; Seo et al., 2012), and Khattab (2007) showed histological changes in the cells of the gastric mucosa, including disruption of surface epithelium or mucosal erosions in rats administered a sublethal dose of ethylene glycol. Moreover, Aye et al. (2010) reported that ethylene glycol can induce DNA strand-breaks and chromosome mutations in

Chinese Hamster Ovary cell lines. Furthermore, this compound has been reported in culture media and/or human specimens infected with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Sohrabi et al., 2014). As ethylene glycol was solely detected in the colon of pigs fed a Western dietary pattern combined with red and processed meat, it certainly deserves further investigation.

4.5 Concluding remarks

The consumption of either chicken or red and processed meat, combined with two different dietary patterns, resulted in distinct modifications of the colon microbial community of the pigs. Characteristic bacterial groups related to a specific meat type were identified, with *Campylobacter* for chicken and *Clostridium XIVa* for red & processed meat consumption. The abundances of *Lactobacilli* and *Desulfovibrio*, both related to important metabolic functions in health and disease, were altered due to the combined effect of meat type and dietary pattern. Specific volatile organic compounds, such as 1-(methylthio)-propane and ethyl pentanoate were observed at higher concentrations after the consumption of red and processed meat, whereas 3-methylbutanal was higher in the piglets subjected to the Western diets. The cause and implication of these findings should be further investigated.

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Chapter V

In vitro gastrointestinal digests of commercial luncheon and deep-fried meat products contain more protein- but less lipid- oxidation products compared to fresh pork

Adapted from:

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Own contribution: input in the study design, responsible for the *in vitro* digestion experiment, carrying out most of the laboratory analyses, interpretation of results and writing the manuscript with input of all co-authors.

Chapter V - *In vitro* gastrointestinal digests of commercial luncheon and deep-fried meat products contain more protein- but less lipid- oxidation products compared to fresh pork

Abstract

Because of the large diversity in processed meat products and the potential involvement of oxidation processes in the association between red and processed meat consumption and chronic diseases, the concentration of oxidation products after gastrointestinal digestion of a broad spectrum of commercial meat products was investigated. In a first experiment, twenty-four ready-to-eat luncheon meat products, displaying large variation in macro- and micronutrient composition and processing procedures, were digested *in vitro* by simulating digestion fluids of the human gastrointestinal tract. Lipid and protein oxidation were assessed in the meat products before digestion and in the corresponding digests by measurement of free malondialdehyde, 4-hydroxy-2-nonenal, hexanal and protein carbonyl compounds. In a second experiment, lipid and protein oxidation products were evaluated in the digests of five different deep-fried meat products that were fried in either tallow or sunflower oil, which were used either as a fresh or reused frying medium. Compared to unprocessed cooked pork mince, which was included as a reference in both digestion experiments, levels of lipid oxidation products were low in the digests of most meat products. Only the digests of Parma ham had slightly higher or comparable levels as the reference pork. In contrast, protein carbonyl compounds were comparable or up to 6 times higher in the digests of the meat products compared to the reference pork. Although some differences in oxidation between the digests of the 5 types of deep-fried meat products and some small effects related to the type of frying oil and number of frying cycles were observed, these effects were limited compared to the differences between digests of the meat products and the reference pork. In conclusion, digests of most luncheon meat products and deep-fried meat products contained lower amounts of free lipid oxidation products, but more protein carbonyl compounds compared to the reference pork.

5.1 Introduction

Over the last decades, high red and/or processed meat consumption has been linked with chronic diseases such as colorectal cancer, type 2 diabetes and cardiovascular diseases, with a consistently higher relative risk for processed meat than for red meat (Chan et al., 2011; Ekmekcioglu et al., 2018; Micha et al., 2012). Conclusions on meat and the associated disease risks are primarily based on epidemiological studies and meta-analyses that generally only make a distinction between red and processed meat (Chan et al., 2011; Ekmekcioglu et al., 2018; Micha et al., 2012). Unfortunately, the enormous variety of processed meat products and the varying terms used in food frequency questionnaires, make it difficult to segregate different categories within the broad group of processed meat products and the associated disease risk. However, specific curing processes (the addition of salt, nitrite, or other additives), drying, smoking, cooking or other preserving or food preparation processes might induce differential health hazards within this broad group of meat products. According to the treatment of raw materials and the applied processing steps, a classification of processed meat products was proposed by the FAO in the following broad groups: fresh processed meat products (e.g. hamburgers, chicken nuggets), cured meat pieces-raw (e.g. raw cured ham), cured meat pieces-cooked (e.g. cooked ham), raw-cooked products (e.g. Frankfurter, meat loaf), precooked-cooked products (e.g. liver sausage, blood sausage), raw (dry)-fermented sausages (e.g. salami) and dried meat (e.g. beef jerky, meat floss) (Heinz & Hautzinger, 2007). Despite the negative health associations, consumption of meat still delivers high-quality protein, fat and essential micronutrients such as B-vitamins, iron and zinc (McAfee et al., 2010). Therefore, more insight in the underlying mechanisms explaining the link between meat consumption and chronic diseases is of paramount importance to understand the health effects of different meat types and to develop strategies to improve the composition and/or processing of meat products (De Smet & Vossen, 2016).

Potential underlying mechanisms have been discussed in several reviews (Demeyer et al., 2016; Hammerling et al., 2016; Wolk, 2017), and although the specific mechanisms are not fully elucidated yet, the involvement of lipid and/or protein oxidation products is one of the proposed mechanisms. Oxidation processes are an inherent part of the maturation process of meat products and are involved in the formation of volatile compounds that contribute to the typical aroma of meat products. For example, a typical marker for lipid oxidation, hexanal, can be associated with a pleasant and grassy, a fatty, or a rancid aroma, dependent on the concentration of the aldehyde (García-González et al., 2013). Indeed, oxidation reactions are also considered one of the main factors affecting shelf life and quality deterioration, due to adverse changes in color, flavor, texture and nutritional value.

In addition, potentially harmful lipid oxidation products, such as MDA and 4-HNE, and protein oxidation products are hypothesized to be involved in the positive epidemiological association between high red meat consumption and the higher risk to develop chronic diseases, as some lipid oxidation products may exert cyto- and/or genotoxic properties (Bastide et al., 2015; Esterbauer, 1993; Van Hecke et al., 2017a). The potential health risk attributed to oxidized protein intake was recently reviewed by Estévez & Luna (2017). Oxidation products can be formed in the muscle meat itself during storage and preparation, but several *in vitro* digestion studies demonstrated a considerable increase in oxidation products during digestion of meat or meat components (Kanner & Lapidot, 2001; Larsson et al., 2012; Nieva-Echevarría et al., 2018; Van Hecke et al., 2015). The effects of meat composition and some processing techniques on oxidation have been investigated and showed that levels of heme-Fe, PUFA and heating processes act as pro-oxidants, whereas the addition of nitrite, herbs and spices mostly prevented or limited lipid oxidation during digestion (Steppeler et al., 2016; Van Hecke et al., 2014a; 2017b). Consumption of blood sausage as a high heme-Fe meat source in an animal feeding study and a randomized crossover human intervention study consistently resulted in higher excretion of 1,4-dihydroxynonane mercapturic acid, the major urinary metabolite of 4-HNE, compared to the consumption of other meat-based diets (Pierre et al., 2006). In addition to the increase in lipid peroxidation markers in urine or fecal water, experimental diets containing high levels of ham, blood sausage and cured meat models resulted in an increase in the number of preneoplastic lesions in the colon of rats (Pierre et al., 2004; 2010; Santarelli et al., 2010). This potentially promoting effect of processed meat products on colorectal carcinogenesis was suggested to be related to the cytotoxicity of fecal water due to lipid peroxidation and levels of NOCs.

Despite these observations and given the large variability in composition and processing parameters among meat products, oxidation during the digestion of different processed meat products is yet scarcely investigated. On the one hand, meat products are subjected to several processes considered to promote oxidation such as aging, comminuting, slicing, cooking and fat addition (Soladoye et al., 2015), whereas on the other hand nitrite and ascorbate are mostly added to offer antioxidant protection and to extend the shelf life (Honikel, 2008; Van Hecke et al., 2014a). However, the efficacy of antioxidants in a food matrix depends on several factors including their concentration and solubility and the synergetic interactions between other components in the food matrix (Estévez & Cava, 2006; Vossen et al., 2012; Wang et al., 2011). Moreover, the addition of antioxidants inhibiting lipid oxidation do not necessarily limit protein oxidation or vice versa in meat products (Lund et al., 2007; Souza et al., 2013; Villaverde et al., 2014a). Also cooking procedures such as duration, temperature and cooking method (grilling, roasting, frying, deep-frying, sous-vide, microwave cooking...) influence oxidation processes

(Silva et al., 2016; Soladoye et al., 2017; Van Hecke et al., 2015). In particular during deep-frying, a complex process of heat and mass transfer between the frying medium and the food occurs at high temperatures, leading to physical and chemical changes in both the frying medium and the food product. Due to the intense heat, oxygen and water exposure, chemical reactions such as hydrolysis, oxidation, isomerization, cyclization and polymerization reactions take place, resulting in the formation of, amongst others, free fatty acids, dimers, trimers, cyclic compounds, polymers, alcohols, aldehydes and epoxides (Bordin et al., 2013; Zhang et al., 2012). The changes in frying medium and food product depend on the characteristics of the food product, type of frying fat, surface/volume ratio, types of breading or battering materials, time of immersion, temperature etc. Since the oxidative susceptibility and the rate of fragmentation of lipid hydroperoxides into secondary oxidation products increases with the number of double bonds in the fatty acid chains, the composition of the frying medium is of paramount importance (Choe & Min, 2006; Frankel, 1983). Whereas most of the volatile components evaporate in the atmosphere, the non-volatile polar compounds such as dimers and polymers are the major decomposition products in frying fat. During extended exposure of the fat to these frying conditions, the oil quality deteriorates by the accumulation of these decomposition products, whereby oil viscosity, foam formation and oil absorption to foods increases whereas heat transfer reduces (Choe & Min, 2007).

In the present study, two *in vitro* gastrointestinal digestion experiments were conducted to evaluate lipid and protein oxidation products in a broad spectrum of commercial meat products. In the first experiment, ready-to-eat luncheon meat products (n = 24) from different brands and subjected to different processing technologies were selected in order to gain more insight in the variation in oxidation products within similar meat products or in the effects of particular processing steps on oxidation. Lipid (MDA, 4-HNE and hexanal) and protein oxidation (PCC) were assessed before and following digestion of the luncheon meat products. In the second experiment, the variation of oxidation within the *in vitro* digests of five typical deep-fried meat products was investigated, as well as the impact of the frying medium (tallow versus sunflower oil) and amount of frying cycles (fresh ,T1) versus reused fat (10 frying cycles, T10). To situate the levels of oxidation products in the meat products across fresh red meat, (minced and vacuum cooked) pork neck was included in both experiments as a reference since many meat products are based on pork.

5.2 Materials and methods

5.2.1 Meat

For the first experiment, twenty-four ready-to-eat luncheon meat products based on pork from different brands (B1, B2, B3, B4) and processing technologies were purchased in a local supermarket: Parma ham (n = 2), back bacon (n = 1), Filet de Saxe (n = 2), Black Forest ham (n = 1), cooked ham (n = 3), salami (n = 4), bologna sausage (n = 3), meat loaf (n = 2), Frankfurter sausage (n = 1), liver pâté (n = 3) and blood sausage (n = 2) (Table 5.1 and Figure 5.1). The classification according to processing technologies was a selection of the categories proposed by the FAO as mentioned in the introduction (Heinz & Hautzinger, 2007). The meat products were purchased well below expiry date and kept for one day at 4°C before mincing and storage at -80°C.

For the second experiment, five types of typical deep-fried meat products (chicken nuggets, frikandel, brochette, turkey schnitzel and beef croquette) were selected (Table 5.2). Deep-fried meat products usually consist of raw meat, fat tissue, spices, common salt, binders, and extenders or fillers for volume extension and are frequently preheated as last step in the manufacturing process. The deep-fried meat products were purchased frozen (-20°C) in a local supermarket and stored for maximum one month at -20°C before being fried. Two types of frying medium, tallow and sunflower oil suited for domestic frying, were also purchased in a local supermarket (Table 5.2). The experiment was performed within a month after buying the meat products and the frying media. Although quality parameters of the frying oil were not measured, it can be assumed that the frying media mimic normal domestic frying conditions and that the frying media meet the reference values of frying media such as a free fatty acid content below 2.5 % and a peroxide value below 1.0 meq O_2/kg. Two domestic deep-fat fryers filled with 4 L of tallow or sunflower oil were heated until 175°C. The meat products (± 300 g) were fried for 4 min with a rest and cooling period of 6-20h between each frying cycle. The meat products that were fried in fresh frying medium (T1), and the meat products that were fried in a frying medium that had been used earlier in nine frying cycles with the same type and amount of meat product (T10) were collected after removal of excess oil with a tissue.

Table 5.1 List of luncheon meats and their corresponding ingredient list as indicated on the package label. For several types of luncheon meats, products from different brands (B1, B2, B3 and B4) and price categories were selected.

Luncheon meat products	Price (€/kg)	Ingredients
Cured meat pieces-raw		
Parma ham (B1)	31.19	pork ham, salt
Parma ham (B2)	70.71	pork ham, sea salt
Back bacon	14.33	pork, salt, glucose syrup, sugar, spices (mace, pepper, paprika), sodium ascorbate, sodium citrate, nitrite, smoke
Filet de Saxe (B1)	13.50	pork (96%), salt, corn syrup, extracts of spices and herbs, spices, sodium ascorbate, sodium nitrite, potassium nitrate, sodium acetate, smoke
Filet de Saxe (B2)	30.11	pork, salt, dextrose, aroma, potassium sorbate, sodium ascorbate, sodium nitrite, potassium nitrate, ferments, smoke
Black Forest ham	8.95	pork, salt, corn syrup, dextrose, extracts of spices, spices, sodium nitrite, potassium nitrate, smoke
Cured meat pieces-cooked		
Cooked ham (B1)	25.00	pork ham (98%), iodinated salt, sugar, corn syrup, salt, natural flavor, sodium ascorbate, sodium nitrite
Cooked ham (B2)	9.38	pork (91%), water, salt, dextrose, pork broth, flavors, sodium erythorbate, sodium nitrite
Cooked ham (B3)	13.75	pork (93%), salt, natural flavors, sugar, extract of rosemary, sodium ascorbate, sodium nitrite
Raw-fermented sausages		
Salami (B1)	6.25	pork, turkey, salt, pork rind, dextrose, spices, sugar, carmine, extract of rosemary, sodium nitrite, wood smoke
Salami (B2)	19.50	pork, salt, lactose, herbs and spices (pepper, garlic, nutmeg), corn syrup, wine, dextrose, sodium erythorbate, potassium nitrate, ferments
Salami (B3)	21.92	pork, salt, milk proteins, dextrose, spices, milk powder, natural flavor, aroma, glucose syrup, betanin, carmine, extract of rosemary, sodium ascorbate, sodium nitrite, potassium nitrate, ferments, smoke
Salami (B4)	18.80	pork (87%), pork fat (4%), salt, dextrose, spices, flavor, stabilizer (diphosphates), ascorbic acid, extract of rosemary, sodium nitrite, potassium nitrate, ferments, smoke
Raw-cooked products		
Bologna sausage (B1)	6.25	pork and pork fat (82%), water, iodinated salt, herbs and spices (ginger, pepper, coriander, nutmeg, onion, garlic, lovage, parsnip), dextrose, spice extracts (nutmeg, chili, lovage, tamarind), sodium ascorbate, trisodium citrate, sodium nitrite
Bologna sausage (B2)	2.97	pork (66%), water, potato starch, salt, spices, extracts of spices, dextrose, diphosphates, carmine, sodium ascorbate, sodium nitrite
Bologna sausage (B3)	11.93	pork (70%), water, pork fat, potato starch, salt, natural flavors, milk protein, dextrose, spices, extracts of spices, potassium chloride, bamboo fibers, carmine, diphosphates, sodium ascorbate, sodium nitrite
Meat loaf (B1)	10.60	pork (80%), milk, breadcrumbs (wheat flour, salt, yeast), onions, pork fat, iodinated salt, pork proteins, white pepper, mace, natural flavor, extracts of spices, ascorbic acid, sodium ascorbate, sodium nitrite
Meat loaf (B2)	19.58	pork (86%), water, breadcrumbs (wheat flour, salt, yeast), iodized salt, wheat starch, corn starch, spices, dextrose, flavors, glucono delta-lactone, diphosphates, ascorbic acid, sodium ascorbate, citric acid, sodium nitrite
Frankfurter sausage	6.71	pork, water, pork fat, whey, salt, milk proteins (0.8%), yeast, wheat fibers, corn dextrose, aromas (celery, gluten), pea protein, carmine, pepper extract, sodium isoascorbate, sodium nitrite, ferments, smoke
Precooked-cooked products		
Liver pâté (B1)	8.33	pig liver 34%, pork fat, pork 9%, connective tissue, water, onion, eggs, salt, wheat starch, dextrose, tomato concentrate, spices and herbs (marjoram, coriander, white pepper, nutmeg, ginger, cardamom, mace), sodium ascorbate, ascorbic acid, sodium nitrite
Liver pâté (B2)	8.33	pork (41.5%), pork liver (21.5%), pork fat, mushrooms 8.5%, water, onion, eggs, wheat starch, salt, spices and herbs in varying proportions (pepper, nutmeg, ginger, coriander, mace, cardamom, cayenne pepper, marjoram, allspice, caraway, turmeric, paprika), dietary fiber, garlic, dextrose, extracts of spices, mustard seeds, ascorbic acid, sodium nitrite
Liver pâté (B3)	10.40	pig liver (35%), pork fat, pork (18%), water, rice starch, salt, spice extracts, onion, chicken egg, milk powder, dextrose, lutein, ascorbic acid, sodium nitrite
Blood sausage (B2)	6.95	bacon, blood, onion, bread (wheat flour, water, yeast, salt), salt, sugar, spices, monosodium glutamate
Blood sausage (B1)	8.45	pig blood (38%), pork (24%), onions, bread (wheat flour, water, yeast, salt), milk, breadcrumbs (wheat flour, salt), binder (wheat and corn starch), diphosphates, brown sugar, spices, sodium acetates, nitrite salt

In both experiments, minced and vacuum cooked (uncured) pork neck was used as a reference. In the first experiment, pork neck was purchased as fresh as possible from a meat retailer, whereas in the second experiment, pork neck was purchased frozen (-20°C) in a local supermarket. The pork neck was manually chopped and minced in a grinder (Omega T-12) equipped with a 10 mm plate and again grinded using a 3.5 mm plate. The minced meat was heated in a warm water bath at 70°C for 70 min. The cooked pork neck, all luncheon meats, and samples from the deep-fried meat products, both unfried and fried samples, were homogenized using a food processor (Moulinex DP700) and vacuum packed in different aliquots. The aliquots were stored at -80°C until analysis and *in vitro* digestion.

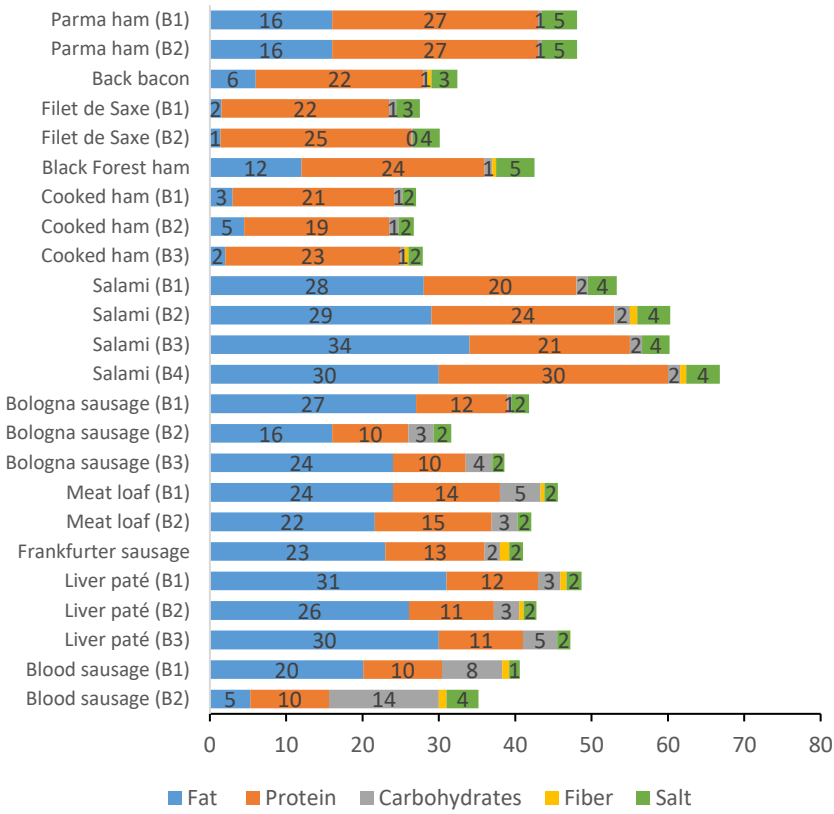

Figure 5.1 Nutrient composition (g/100 g) of the 24 luncheon meat products as indicated on the package label.

Table 5.2 List of deep-fried meat products and frying media and their corresponding ingredient list as indicated on the package label.

Deep-fried meat products	Ingredients
Chicken nuggets	47% chicken meat, water, wheat flower, vegetables oils (sunflower oil, rapeseed oil), corn flower, modified starch, salt, wheat gluten, raising agents (E450, E500, E341), dextrose, wheat starch, spices
Frikandel	66% mechanically recovered chicken, water, breadcrumbs (wheat flower, yeast, salt), 5% pork bacon, salt, herbs and spices, vegetable powder (onion, garlic), dextrose, antioxidant (E300), stabilisers (E451, E450) soy protein
Brochette	59% pork, onions, water, maltodextrin, salt, spices (contains mustard), sunflower oil, flavour enhancer (E621), starch, acids (E262, E325, E331), acidity regulator (E501), dried glucose syrup, spice extract, stabiliser (E407), garlic, preservative (E202), smoke flavour
Turkey schnitzel	68% turkey, water, 13% breadcrumbs (wheat flower, salt, yeast, pigment (E160b), curcuma), flower mix (wheat flower, corn flower), modified wheat starch, salt, spices (0.082% coriander, 0.025% paprika, 0.004% clove), pepper aroma, starch, dextrose, whey powder, wheat gluten, stabilizer (E450)
Beef croquette	water, 14% beef, wheat flower, vegetables oils (palm oil, sunflower oil, turnip oil), breadcrumbs (wheat), egg-white, gelatine, salt, natural aroma (celery), beef collagen, wheat gluten, onion, thickener: guar gum, wheat starch, garlic, dextrose, herbs and spices, parsley, emulator: soy-lecithin, pigment: E150d
Frying medium	
Tallow	beef tallow, antifoaming agent (E900)
Sunflower oil	vegetable oils 99.9% (sunflower oil and high oleic sunflower oil), antioxidant (E307), natural aroma, antifoaming agent (E900)

5.2.2 Meat and frying medium characteristics

Dry matter, crude protein and crude fat in the cooked pork neck, luncheon meats, and the unfried meat products were analyzed according to ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods respectively. Residual nitrite contents were measured in the pork neck and luncheon meats according to ISO 2918-1975. Fatty acids were measured in the cooked pork neck, luncheon meats, both the unfried and fried meat products, and in the frying fats by GC according to (Raes et al., 2001). Briefly, lipids were extracted using chloroform/methanol (2/1; v/v) followed by methylation and GC analysis using a fused silica SP-2560 capillary column to separate the fatty acid methyl esters. α-Tocopherol in all meats and frying media was extracted with ethanol and hexane and quantified by HPLC (Supelcosil LC18 column, 25 cm × 4.6 mm × 5 µm) and UV detection at 292 nm (Claeys et al., 2016). Concentrations of α-tocopherol were determined by comparing the peak areas with a standard curve of α-tocopherol (0 – 15 µg/ml). Hematin in all meat samples was extracted with a mixture of aceton, water and HCl (12 M) and was

spectrophotometrically (640 nm) determined according to the method of (Hornsey, 1956). The hematin concentrations were converted to heme-Fe contents using their molecular weight. Extraction of carnosine and anserine in the cooked pork neck, luncheon meats, and the unfried meat products was carried out in a 0.01 M phosphate buffer (pH 7.4) followed by the addition of acetonitrile (phosphate buffered extract/acetonitrile, 2/1, v/v) as previously described (Zdanowska-Sąsiadek et al., 2018). The dipeptides were measured by HPLC (EC250/4.6 Nucleosil 120-7 NH_2 column) and UV detection at 210 nm and quantified (mg/g meat, FW) using standard curves of anserine and carnosine (0.02 - 0.10 mg/ml). All meat characteristics were analyzed in duplicate. Results are expressed on fresh weight basis (FW) of meat or as % of fatty acid methyl esters (FAME). LOQs for the micronutrient composition parameters in meat are 2 µg residual nitrite/g FW, 0.06 mg heme-Fe/100 g FW, 0.92 µg α-tocopherol/g FW, 0.04 mg carnosine/g FW and 0.1 mg anserine/g FW. The LOQ for α-tocopherol in frying fat is 0.92 mg/100 g fat.

5.2.3 In vitro digestion

Two *in vitro* digestion experiments were conducted according to the same *in vitro* digestion model used in Chapter II, simulating human mouth, stomach and duodenum (Van Hecke, 2014b). The first experiment consisted of the luncheon meats and reference pork neck, whereas the second experiment included the unfried and fried meat products and reference pork neck. The reference pork neck in both experiments came from different suppliers. Briefly, meat samples (4.5 g) were consecutively incubated at 37°C with several enzymatic digestion buffers: 6 mL saliva (5 min), 12 mL gastric juice (2 h), followed by 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile juice (2 h). After this last incubation step, samples were homogenized with an Ultra Turrax apparatus (9500 rpm) and aliquots were stored immediately at -80°C. The digestions were performed in quadruplicate. Within each experiment, the meats were digested in one digestion run to exclude any day-effects.

5.2.4 Oxidation products in meat and in vitro digests

Lipid oxidation products (MDA, 4-HNE, hexanal) and PCC were measured in duplicate in the meat products before digestion as well as in the *in vitro* digests. Concentrations of free MDA were determined involving extraction with trichloroacetic acid (7.5%), derivatization with DNPH, HPLC separation (Supelcosil LC18 column, 25 cm × 4.6 mm × 5 µm) and UV/VIS detection (310 nm) (Mendes et al., 2009). Standard solutions (0 – 20 nmol/ml) were made with 1,1,3,3-tetramethoxypropane and results were expressed as nmol MDA/g (digested) meat. Levels of free reactive 4-HNE and hexanal were analyzed after formation of their

fluorescent derivatives with 1,3-cyclohexanedione through HPLC (Supelcosil LC18 column, 25 cm × 4.6 mm × 5 µm) based on the method of Holley et al. (Holley, Walker, Cheeseman, & Slater, 1993). Concentrations were determined by comparing the peak areas with those obtained from the standard curves of 4-HNE (0 – 2 nmol/mL) and hexanal (0 – 40 nmol/mL) and results were provided in nmol/g (digested) meat. LOQs for the quantification of the lipid oxidation products in meat are 0.21 nmol MDA/g FW, 0.08 nmol 4-HNE/g FW and 0.45 nmol hexanal/g FW. This corresponds with LOQs in the digests of 1.95 nmol MDA/g digested meat, 0.79 nmol 4-HNE/g digested meat and 4.3 nmol hexanal/g digested meat. The extent of protein oxidation was evaluated by PCC after derivatization with DNPH and detection with a spectrophotometer (Ganhão et al., 2010). The PCC content (nmol DNPH/mg protein) was calculated from the protein concentration and concentration of protein hydrazones, measured by the absorbance at 280 nm and 370 nm, respectively (Levine et al., 1994). The PCC content expressed per g meat was calculated from the PCC content per mg protein and the protein content of the luncheon meat products. Results were expressed per milligram protein and/or per gram of meat.

5.2.5 Statistical analysis

The results for each meat product were presented as means ± SD. For experiment 1, the meat products were grouped according to processing technology and analyzed using one-way ANOVA with 'processing technology' (cured meat pieces-raw, cured meat pieces-cooked, raw-cooked products, precooked-cooked products, raw fermented sausages) as fixed factor and pairwise comparisons were studied using Tukey HSD test (SAS enterprise guide 7). If the assumptions for ANOVA (verified by Kolmogorov-Smirnov and modified-Levene test) were violated, the non-parametric independent-samples Kruskal-Wallis test with pairwise multiple comparisons was performed using SPSS Statistics 25. Spearman correlations were calculated using SAS Enterprise Guide 7. For experiment 2, a linear model ANOVA procedure was used to compare the digests of the fried meat samples with the fixed effects of 'meat product', 'frying medium, 'frying cycle', and their interaction terms. In addition, differences between the fatty acid composition of the meat products were investigated using a mixed model ANOVA procedure with 'frying' (unfried versus fried, and unfried versus tallowT1 versus tallowT10 versus sunflowerT1 versus sunflowerT10) as fixed factor and 'meat product' as random factor, whereas the comparison between the digests of the different fried meat products and the reference pork neck was investigated by applying a linear model ANOVA procedure with 'meat type' as fixed factor. Changes in the frying medium were also analyzed with a linear model ANOVA with the fixed factors 'frying medium', 'frying cycle' and their interaction term. Tukey-adjusted post hoc tests were performed for all pairwise comparisons and P-values ≤ 0.05 were considered statistically significant.

5.3 Results

5.3.1 Experiment 1

5.3.1.1 Macronutrient composition

A large variation of luncheon meats was obtained according to the ingredients list and the analyzed macronutrient composition. The analyzed crude fat, crude protein and dry matter contents of the different meat products are presented in Table S5.1, along with the carbohydrate and salt contents that were indicated on the packaging labels. The macronutrient and fatty acid composition, grouped according to processing category and with the values for the reference pork indicated as a dotted line, are illustrated in Figure 5.2, whereas the concentrations per individual meat product are illustrated in Figure S5.1 and S5.2. Compared to the majority of luncheon meats, the reference pork neck had a lower dry matter content, average protein content, lower fat percentage, and accordingly also lower SFA, MUFA, n-6 and n-3 PUFA levels. Among the luncheon meats, crude fat, crude protein and dry matter contents ranged from 1-35%, 9-27% and 27-69%, respectively. SFA, MUFA, n-6 and n-3 PUFA levels in the meat products reflect the levels of the crude fat content. The n-6/n-3 PUFA ratio ranged from 6.9-19.3. Cooked ham and Filet de Saxe contained the lowest fat percentages (1-3%), whereas salami and liver pâté contained between 25-35% fat. Protein percentages were significantly lower in raw-cooked and precooked-cooked products (9-15%) compared to cured meat pieces, raw fermented sausages and the reference pork neck (18-27%). As illustrated in Figure 5.2, the macronutrient composition varied greatly among the different processing categories, but the variability within the same processing category was rather small.

5.3.1.2 Residual nitrite, heme-Fe and endogenous antioxidants

Concentrations of residual nitrite, heme-Fe, α-tocopherol, carnosine and anserine are presented in Figure 5.3 (grouped per category) and Figure S5.3 (individual values). Since nitrite was not added to these products, the reference pork (and the two types of Parma ham and blood sausage B1), contained very low levels of residual nitrite (< LOQ, 2 µg/g FW). Among the different luncheon meats, bologna sausages had the highest levels of residual nitrite (43-57 µg/g FW), whereas blood sausages and liver pâté contained the highest amounts of heme-Fe (1.5-16.2 mg/100g FW). Levels of α-tocopherol ranged from values below 0.92 µg/g FW (LOQ) until 10.6 µg/g FW, with higher levels in the raw-fermented sausages and lower levels in the cured meat products. The large variation in α-tocopherol levels within the precooked-cooked products originates from the higher levels detected in liver pâté (6.5-9.6 µg/g FW) and the low levels in blood sausage (0.5-0.7 µg/g FW). The concentrations of the dipeptides anserine and carnosine showed a similar pattern as the protein content.

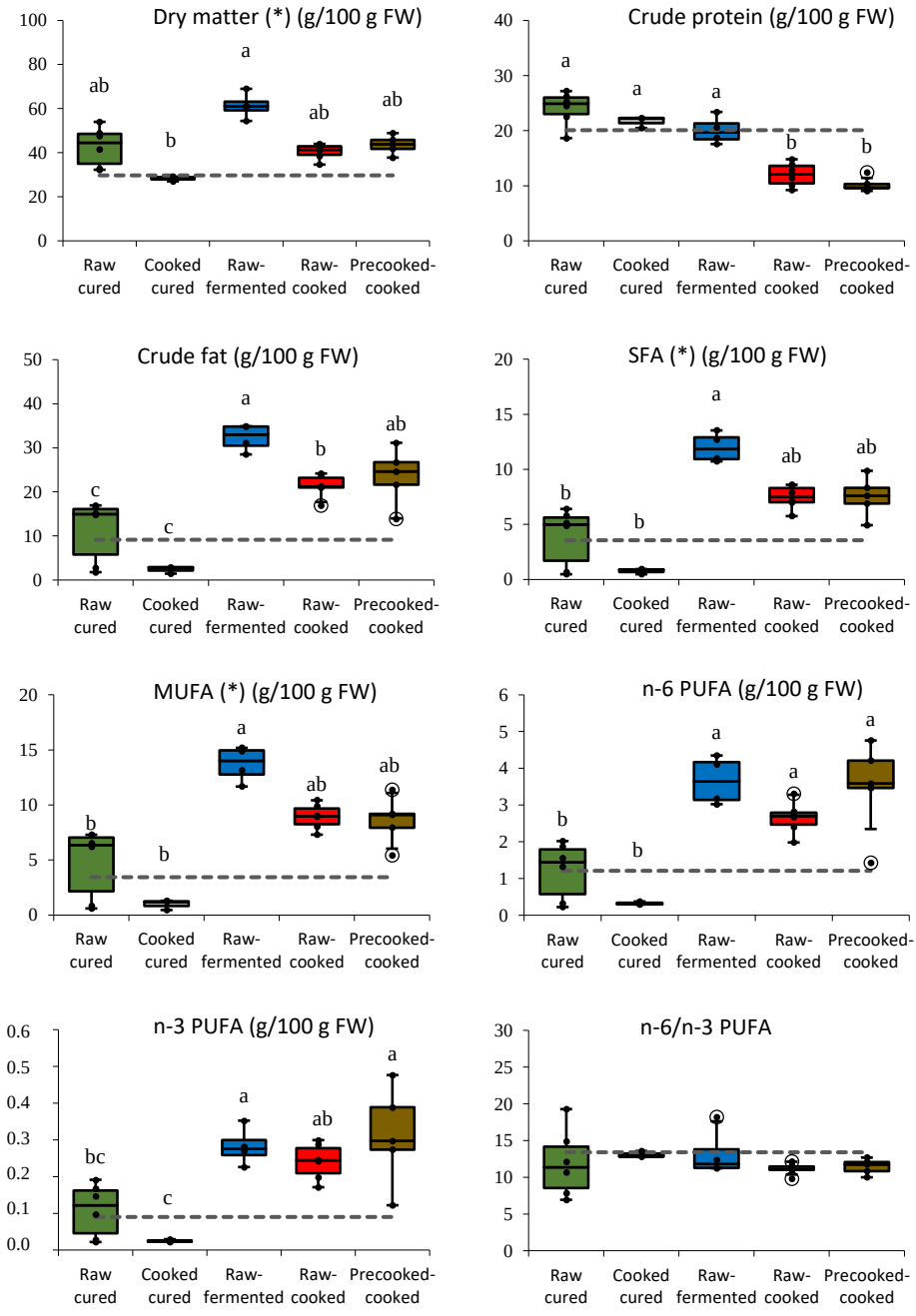

Figure 5.2. Macronutrient and fatty acid composition of the meat products classified according to processing category. The dotted horizontal line in the figures represents the value of the reference vacuum cooked pork. Parameters with an asterix (*) were analyzed using non-parametric statistical tests (Kruskal-Wallis test with pairwise multiple comparisons), others were analyzed using one-way ANOVA and the Tukey HSD test. Concentrations in box plots with different superscripts show significant differences (P-values ≤ 0.05).

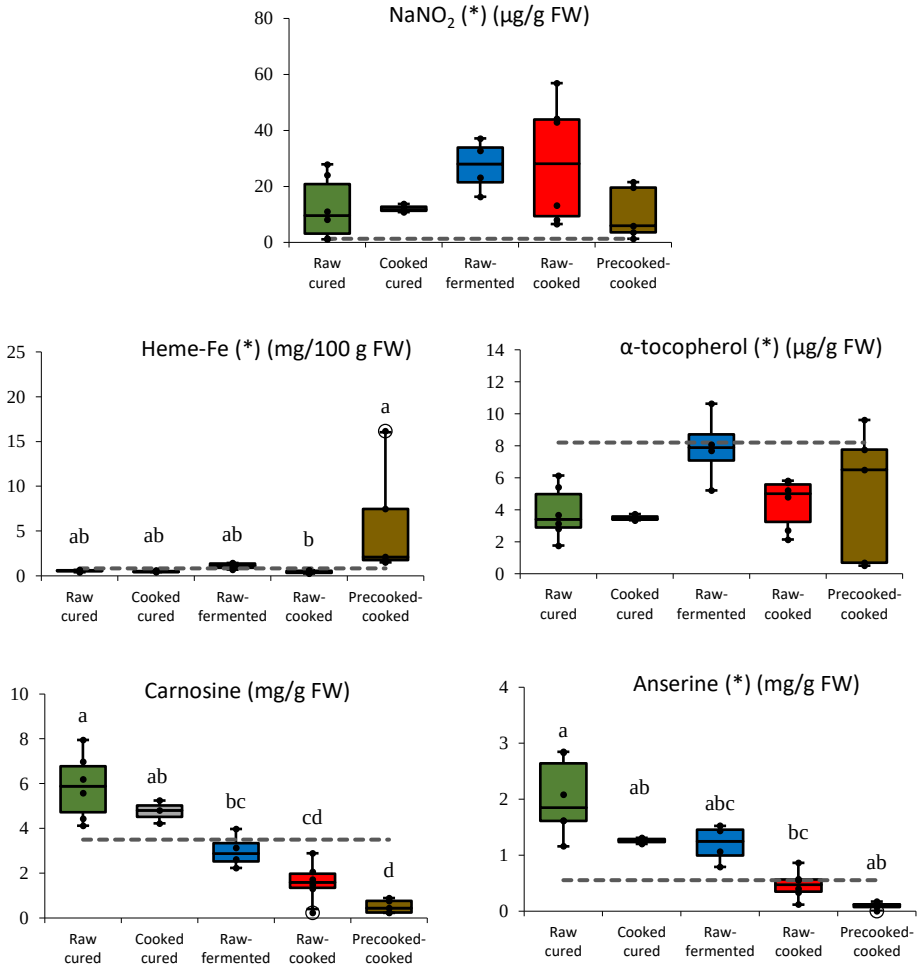

Figure 5.3 Residual nitrite, heme-Fe and the endogenous antioxidants α-tocopherol, carnosine and anserine in the meat products classified according to processing category. The dotted horizontal line in the figures represents the value of the reference vacuum cooked pork. Parameters with an asterix (*) were analyzed using non-parametric statistical tests (Kruskal-Wallis test with pairwise multiple comparisons), others were analyzed using one-way ANOVA and the Tukey HSD test. Concentrations in box plots with different superscripts show significant differences (P-values ≤ 0.05).

The highest concentrations of the dipeptides were detected in the cured meat pieces, followed by intermediate levels in the raw fermented sausages and the lowest concentrations in the raw-cooked and precooked-cooked meat products. The concentration of α-tocopherol was rather high in the reference pork (8.2 µg/g FW), compared to the majority of luncheon meats, whereas intermediate levels for carnosine (3.5 mg/g FW), anserine (0.6 mg/g FW) and heme-Fe (0.8 mg/100 g FW) were noted.

5.3.1.3 Lipid oxidation

Compared to the reference pork, lipid oxidation products in most meat products were low in concentrations or even not detected, except for blood sausage, Parma ham and liver pâté (Figure 5.4). Especially in blood sausage (B1), 4-HNE was more than tenfold higher compared to the reference pork (1.40 nmol/g FW versus 0.12 nmol/g FW). However, all digests of the luncheon meats contained lower levels of lipid oxidation products in comparison with the reference pork digests, except for the Parma ham (B2) digests.

5.3.1.4 Protein oxidation

In contrast to lipid oxidation products, the levels of PCC in the meat and digests were situated within the same range or up to 6-fold higher compared to the reference pork meat (Figure 5.5A-5.5B). Particularly meat products classified within the raw-cooked and precooked-cooked meat products (meat loaf, bologna sausage, Frankfurter sausage, liver pâté and blood sausage) had higher PCC concentrations compared to the reference pork meat and other meat products (Figure 5.5B). However, when PCC concentrations are expressed per gram of meat, instead of per milligram protein, no significant differences in PCC between the meat products of different processing categories were found (Figure 5.5C).

5.3.1.5 Correlations between protein oxidation products and meat characteristics

Since concentrations of lipid oxidation products were not detected or low, especially compared to the reference pork, only Spearman correlations between PCC (in meat and digests, both expressed per mg protein and per g meat) and meat characteristics were calculated (Table 5.3). Significantly negative correlations were found between PCC in meat and digests expressed in nmol 2,4-DNPH fixed/mg protein and the crude protein content, and anserine and carnosine levels ($-0.54 < r < -0.60$). Anserine, carnosine, and protein levels were also mutually associated with each other ($0.91 < r < 0.92$, $P < 0.001$). In addition, carbohydrate levels, n-6 and n-3 PUFA content and the fat to protein ratio were significantly positively correlated with PCC ($0.44 < r < 0.65$). A positive correlation was found between PCC (expressed in nmol 2,4-DNPH fixed/mg protein) and hexanal levels in the luncheon meats and digests ($r = 0.48$). However, when the PCC in meat are expressed in nmol 2,4-DNPH fixed/g meat, almost no significant correlations were found.

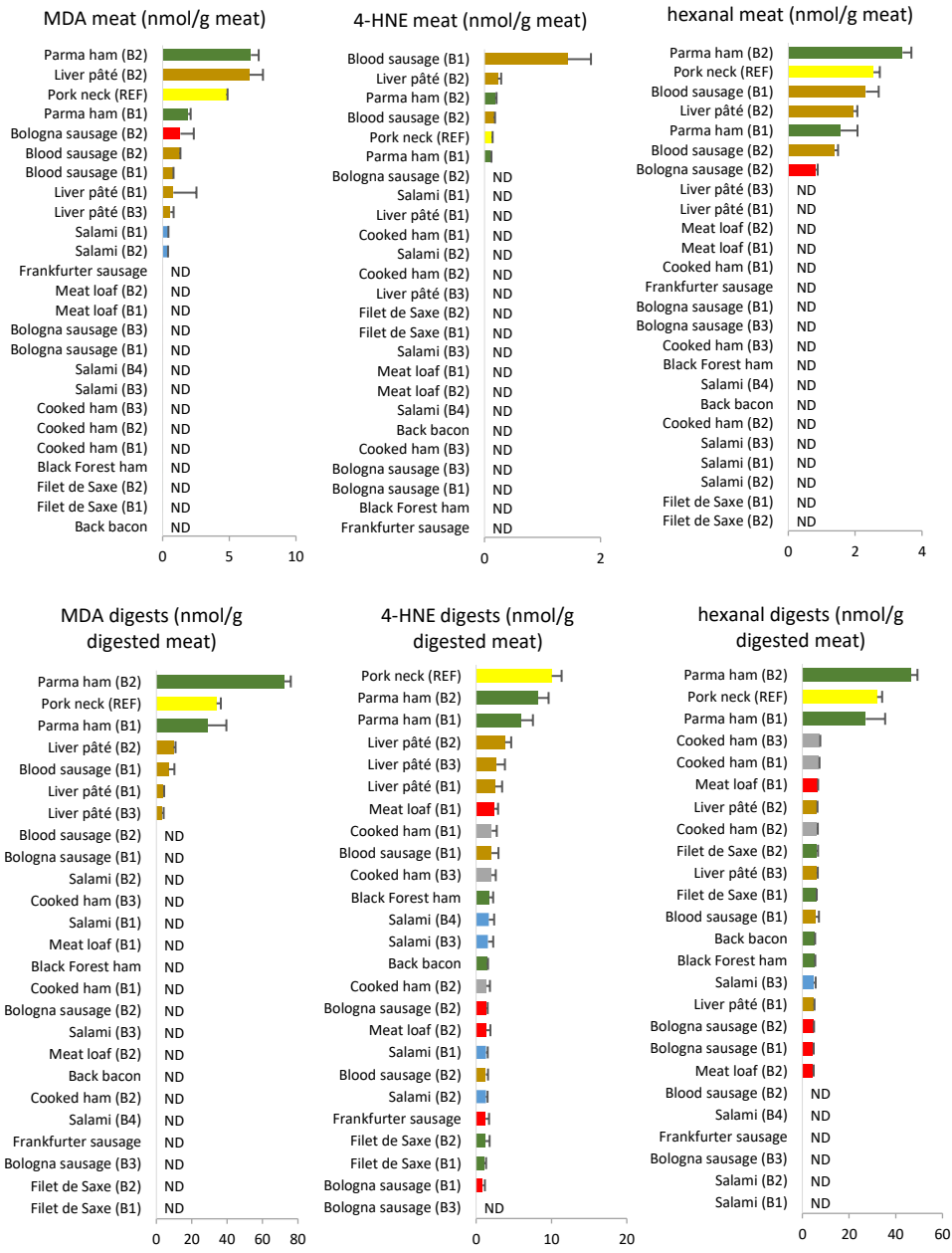

Figure 5.4 Concentrations of malondialdehyde, 4-hydroxy-2-nonenal and hexanal as measures of lipid oxidation in the luncheon meat products expressed per g meat on fresh weight basis (above) and the *in vitro* digests of the meat products expressed per g digested meat (below). Meat products belonging to the same processing category were indicated in a same color (cured meat pieces-raw = green; cured meat pieces-cooked = grey; raw-fermented sausages = blue; raw-cooked products = red; precooked-cooked products = brown). Results were expressed as mean ± SD.

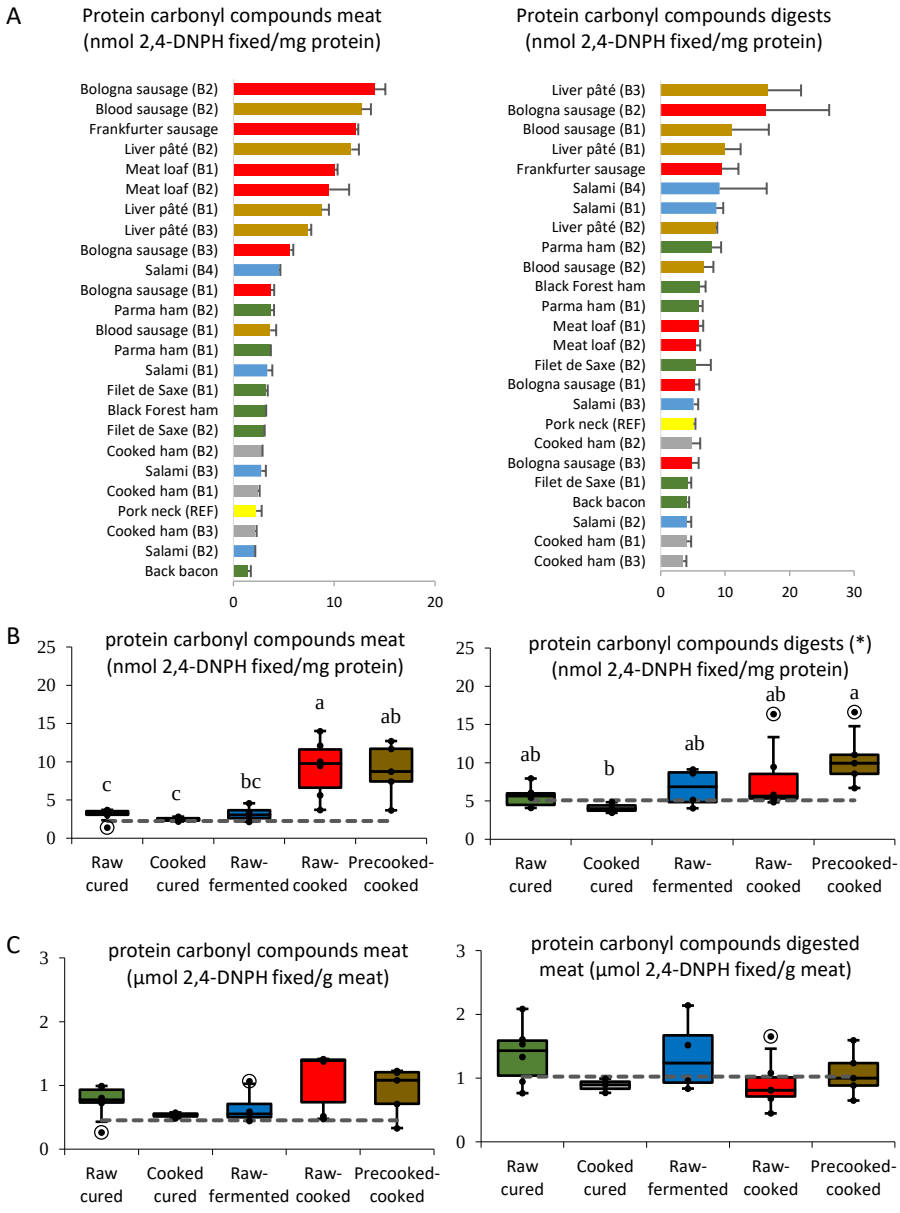

Figure 5.5 Concentrations of protein carbonyl compounds in the luncheon meat products (left) and the *in vitro* digests of the meat products (right) per individual meat product (A) and classified according to processing category, expressed per mg protein (B) and per g meat (C). Meat products belonging to the same processing category were indicated in a same color (cured meat pieces-raw = green; cured meat pieces-cooked = grey; raw-fermented sausages = blue; raw-cooked products = red; precooked-cooked products = brown). The dotted horizontal line in the figures represents the value of the reference vacuum cooked pork. Parameters with an asterix (*) were analyzed using non-parametric statistical tests. Concentrations in box plots with different superscripts differ significantly. Results were expressed as mean ± SD.

Table 5.3 Spearman correlations among protein carbonyl compounds in meats and digests and their correlation with the meat characteristics and other oxidation products.

	unit	protein carbonyl compounds meat	protein carbonyl compounds digests	protein carbonyl compounds meat	protein carbonyl compounds digests
		nmol 2,4-DNPH/mg protein	nmol 2,4-DNPH/mg protein	nmol 2,4-DNPH/g meat	nmol 2,4-DNPH/g digested meat
dry matter	g/100 g FW	-	**0.43**	-	-
crude protein	g/100 g FW	**-0.58***	**-0.41**	-	-
carnosine	mg/g FW	**-0.60***	**-0.50**	-	-
anserine	mg/g FW	**-0.54***	**-0.48**	-	-
crude fat	g/100 g FW	-	**0.46**	-	-
SFA	g/100 g FW	-	-	-	-
MUFA	g/100 g FW	-	0.43	-	-
n-6 PUFA	g/100 g FW	**0.44**	**0.59***	-	-
n-3 PUFA	g/100 g FW	**0.52***	**0.64***	-	-
n-6/n-3	-	-	-	-	-
carbohydrates	g/100 g FW	**0.65***	**0.47**	-	-
crude fat/crude protein	-	**0.56***	**0.60***	-	-
heme-Fe	mg/100 g FW	-	-	-	-
α-tocopherol	µg/g FW	-	-	-	-
$NaNO_2$	µg/g FW	-	-	-	-
MDA meat	nmol/g meat	-	**0.49**	-	-
MDA digests	nmol/g digested meat	-	-	-	-
4-HNE meat	nmol/g meat	-	-	-	-
4-HNE digests	nmol/g digested meat	-	-	-	**0.41**
hexanal meat	nmol/g meat	**0.48**	**0.47**	-	-
hexanal digests	nmol/g digested meat	-	-	-	-
protein carbonyl compounds meat	nmol 2,4-DNPH/ mg protein	/	**0.72****	**0.80****	**0.54***
protein carbonyl compounds digests	nmol 2,4-DNPH/ mg protein	/	/	/	**0.61***

MDA = malondialdehyde; 4-HNE = 4-hydroxy-2-nonenal; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; DNPH = 2,4-dinitrophenylhydrazine; n-6 and n-3 PUFA = n-6 and n-3 polyunsaturated fatty acids. Correlations in bold are significant ($P < 0.05$), * indicates $P < 0.01$; ** indicates $P < 0.001$, - indicates no significant correlation.

5.3.2 Experiment 2

5.3.2.1 Meat characteristics in meat products prior to frying

The meat products that contained meat from different animal species encompassed a large variation, both in macro- and micronutrient composition (Figure S5.4). Dry matter, fat and protein content ranged from 22-44%, 1.5-11.9% and 7.6-20.3% respectively. Brochette and turkey schnitzel had the lowest fat content before frying. Heme-Fe concentrations were at least two-fold higher in pork neck and frikandel compared to the other meat products. Alpha-tocopherol levels were highest in chicken nuggets, followed by meat croquette. The concentration of carnosine in the meat products was the highest in the meat products containing mammal meat (brochette and pork neck), whereas anserine was the highest in turkey schnitzel.

5.3.2.2 *Changes in fatty acid profiles and α-tocopherol content in frying fats during frying*

Tallow was characterized by high amounts of SFA (52%), whereas sunflower oil contained high levels of n-6 PUFA (46%) (Figure 5.7). Relative MUFA contents were comparable in the two types of frying medium, but slightly higher in sunflower oil than in tallow. Levels of n-3 PUFA were low in both frying fats. Frying the meat products once, or ten times in the frying medium did not result in pronounced changes in the fatty acid profile of the frying fats, although some significant effects were observed. Pairwise comparisons showed slightly higher MUFA levels in sunflower oil after ten frying cycles compared to one frying cycle (P=0.014), whereas the levels of n-6 PUFA in sunflower oil slightly decreased when comparing one and ten frying cycles (P=0.021). Sunflower oil contained significantly more α-tocopherol compared to tallow, however, the α-tocopherol levels in sunflower oil decreased during frying (P=0.026).

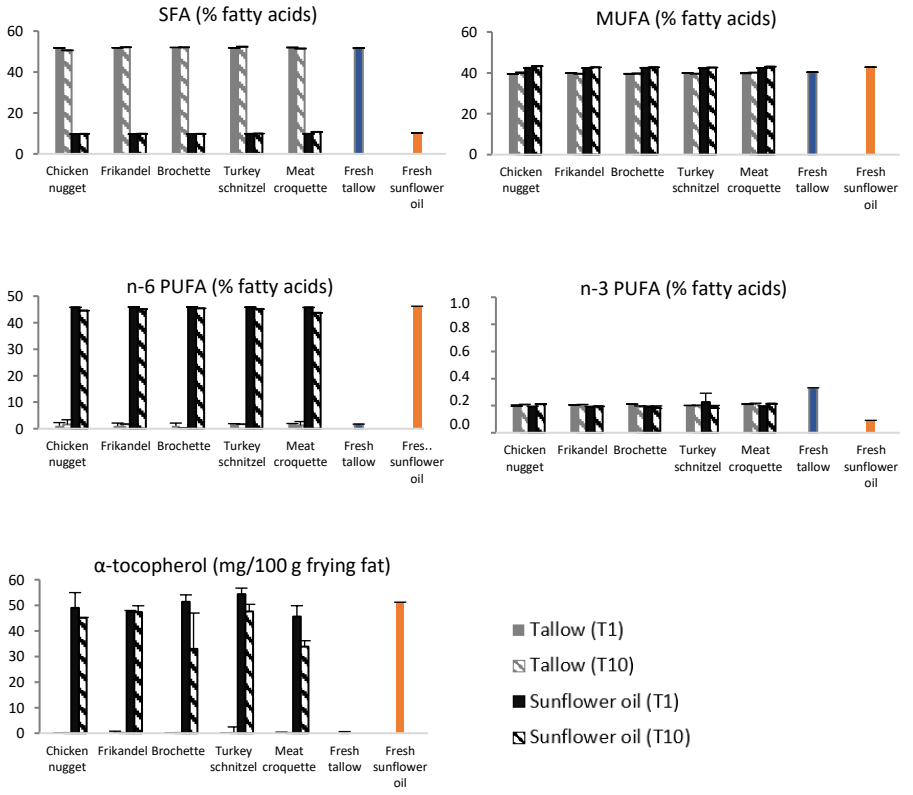

Figure 5.7 Fatty acid profiles and α-tocopherol content in the frying medium before frying, and after one (T1) and 10 frying cycles (T10) for every deep-fried meat product. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; n-6 and n-3 PUFA = n-6 and n-3 polyunsaturated fatty acids.

5.3.2.3 *Changes in fat content, fatty acid profiles and α-tocopherol content in meat products during frying*

The fat content of the meat products significantly increased during frying (P<0.001). During frying in both frying media, relative amounts of n-3 PUFA in the meat products decreased (P<0.001). The relative amounts of SFA increased in the meat products after frying in tallow, whereas relative amounts of n-6 PUFA and levels of α-tocopherol increased after frying in sunflower oil.

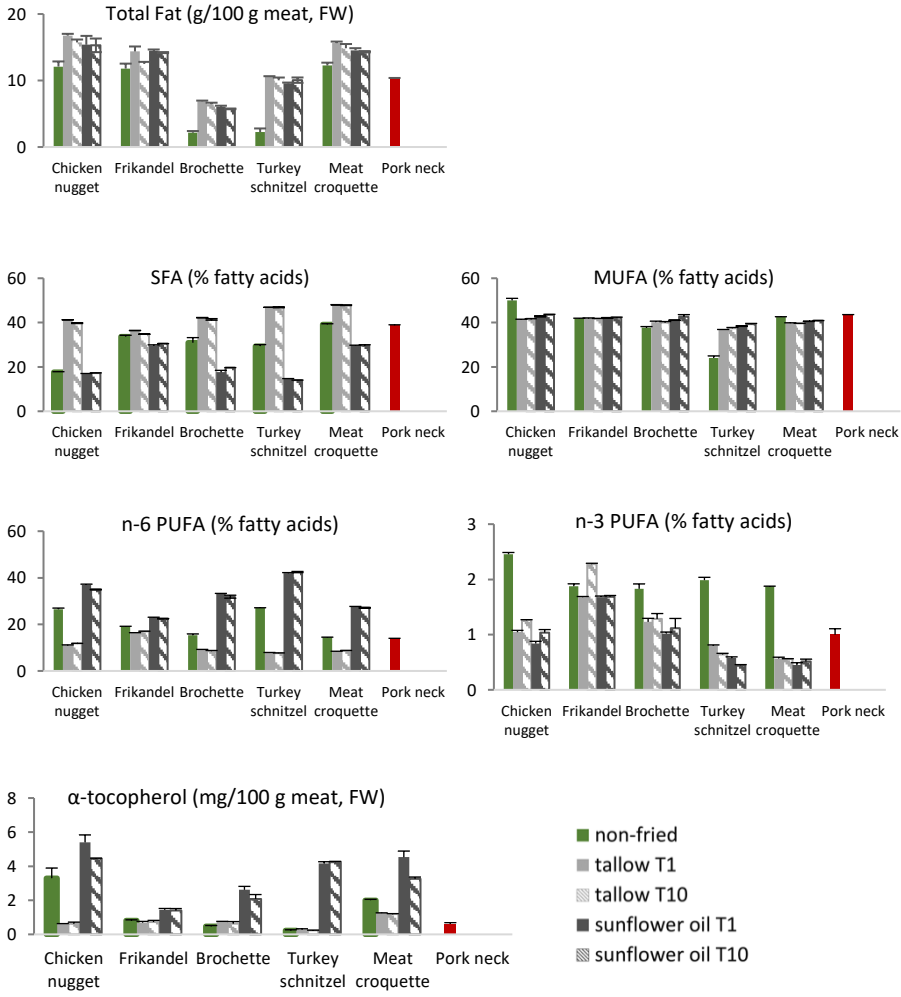

Figure 5.8 Fatty acid composition and α-tocopherol content in meat products before frying, and in the deep-fried meat products that were fried in a fresh frying medium (T1), or a frying medium that had previously been used in nine frying cycles (T10). SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; n-6 and n-3 PUFA = n-6 and n-3 polyunsaturated fatty acids.

5.3.2.4 Lipid and protein oxidation

Levels of free lipid oxidation products in the digests of the deep-fried meat products were low compared to the reference pork (Figure 5.9). In contrast, levels of PCC were generally higher or similar in the digests of the meat products compared to the reference pork. Some significant differences related to the frying medium and/or number of frying cycles were observed for the different oxidation parameters. However, these differences were relatively small compared to the differences between the deep-fried meat products and the reference pork.

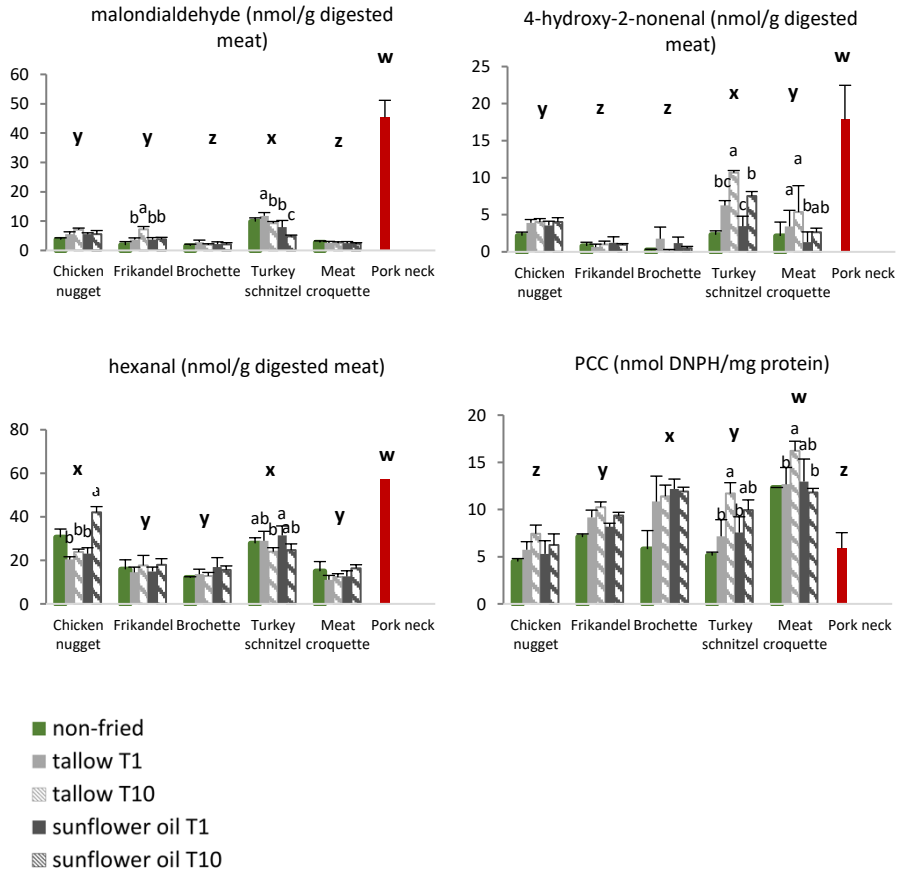

Figure 5.9 Concentrations of lipid and protein oxidation products in the *in vitro* digests of meat products before frying, and in the meat products that were fried in a fresh frying medium (T1), or a frying medium that had previously been used in nine frying cycles (T10). Bars with different superscripts (a, b, c) within the same type of deep-fried meat product differed significantly. Different superscripts (w, x, y, z) correspond with significant differences between the averages of the different deep-fried meat products and the reference pork. PCC = protein carbonyl compounds; DNPH = 2,4-dinitrophenylhydrazine

Due to a technical problem with the HPLC when analyzing MDA levels in the deep-fried meat products, and because of the main interest in the levels of oxidation products after meat digestion, only results of the oxidation parameters in the digests are shown for the second experiment. Based on the preliminary results obtained, it can be mentioned that the levels of oxidation products (MDA, 4-HNE, hexanal and PCC) after the *in vitro* digestion reflect the relative differences that were already present in the deep-fried meat before digestion with higher levels of lipid oxidation products in the reference pork compared to most of the deep-fried meat products and only limited effects of type of frying medium. Similar to the results obtained after digestion, PCC levels were generally higher or similar in the deep-fried meat products compared to the reference pork, with meat croquette and brochette having the highest levels of PCC. Although the relative initial differences in the meat were reflected in the digests, a general strong increase of oxidation products (up to 10-fold) was observed for lipid oxidation products, whereas levels of PCC expressed per mg protein were situated within the same range.

5.4 Discussion

Processed meat is an overarching term covering many heterogenous products with potentially different oxidation profiles. However, despite the large compositional differences, this study showed that the majority of luncheon and deep-fried meat products had low concentrations of free lipid oxidation products, especially in comparison to fresh pork neck. Since this fresh (but minced and vacuum cooked) pork neck has been shown to contain average or low levels of oxidation products in comparison with digests of a wide range of similarly prepared mammal, poultry or fish muscles (Van Hecke et al., 2019b), it can be concluded that free lipid oxidation products in most luncheon and deep-fried meat were lower compared to most fresh muscle meats. In contrast, protein oxidation products, which can however result from reactions of proteins with lipid oxidation products, were higher for some types of luncheon and deep-fried meat products compared to the reference pork.

The lower concentrations of lipid oxidation products in the digests of meat products compared to the reference meat in the present study were somewhat unexpected based on previous results in literature. However, only a limited number of studies directly compared the level of oxidation products in different luncheon meat products, especially in combination with subsequent *in vitro* gastrointestinal digestion. The study of Armenteros et al. (2009) compared levels of MDA-equivalents and hexanal in dry-cured meat, cooked sausages, liver pâté and raw meat. In contrast with our results, levels of lipid oxidation products in the meat products were higher or similar compared to the unprocessed raw meat, whereas in our study, lipid oxidation was generally lower in the meat products compared to the unprocessed

(but cooked) reference pork. An important difference is that cooked meat was used as a reference in the present study, whereas the study of Armenteros et al. used raw ground meat. Considering the prooxidative effect of heating, as illustrated by Van Hecke et al. (2015), this could have influenced the final outcome and conclusions, however other factors could also be involved. Higher lipid oxidation products could have been expected in the blood sausages and corresponding digests due to the high heme-Fe content. Indeed, Pierre et al. (2004; 2006) reported a 23-fold increase in MDA-equivalents in fecal water of rats and very high levels of urinary 1,4-dihydroxynonane mercapturic acid excretion after consumption of a blood sausage-based diet, compared to chicken and beef-based diets. However, the diets in the studies of Pierre et al. (2004; 2006) included 5% safflower oil and freeze-dried samples of (raw) chicken, (raw) beef and blood sausage. Other studies have demonstrated the oxidation promoting potential of addition of high amounts of n-6 PUFA, and of cooking and freeze-drying of meat (Gasc et al., 2007; Steppeler et al., 2016; Van Hecke, et al., 2019b). Furthermore, the study of Gasc et al. (2007) emphasized the inter-batch variability, since the 4-HNE content differed more than 15-fold between two blood sausage samples coming from different suppliers.

Among the tested luncheon meat products and digests in this study, Parma ham resulted in the highest levels of most lipid oxidation products, followed by liver pâté and blood sausage. Similar to our findings, among the processed meat products in the study of Armenteros et al., the dry-cured products contained the highest levels of MDA-equivalents, whereas the dry-cured meat products and cooked sausages had higher levels of hexanal compared to liver pâté. Dry-cured meat products such as Parma ham are typically exposed to extensive salting, dehydration and continuous exposure to air and ambient temperature during the long ripening and maturation process and the absence of nitrite, which are factors known to promote oxidation (Amaral et al., 2018; Koutina et al., 2012). The biochemical reactions taking place during maturation of Parma ham such as proteolysis, lipolysis and oxidation are considered to play an important role in the aroma of dry cured hams (Buscailhon, et al., 1994; Ventanas et al., 2007). Therefore, considerable levels of lipid oxidation products are present in dry-cured meat products. On the other hand, by studying the lean and fat parts of Parma ham separately, the study of Vestergaard et al. (1999) demonstrated that only the external adipose tissue part of the ham contained higher levels of lipid oxidation products, whereas the lean muscle part remained rather unaffected, even after long ageing times. Several studies investigated the kinetics of oxidation products during Parma ham and Iberian ham production and liver pâté storage. Considerable differences in oxidation levels at different time points were noticed. First, an increase in lipid oxidation products was observed, followed by a subsequent decrease, probably related to a decreased water activity, lowering reaction rates and to the low stability of lipid oxidation products and/or further

reactions with protein residues leading to PCC (Cava et al. 1999; Koutina et al., 2012; Munekata et al., 2017). Furthermore, the degradation of MDA in protein solutions was shown to depend on the protein concentrations (Vandemoortele & De Meulenaer, 2015). In the present study, oxidation products in luncheon meats were only measured at one time point, representing the timeframe of user consumption. Therefore, it could be hypothesized that the lower concentrations of free lipid oxidation products in the processed meat products compared to the reference meat are not necessarily due to a lower formation, but maybe due to the decline of reactive lipid oxidation products as a result of longer ripening times or closer interactions with proteins in processed meat products. The relatively higher concentrations of lipid oxidation products in liver pâté and blood sausage could be related to the high heme-Fe and PUFA levels, and intense mincing and cooking procedures. However, many other pro- or antioxidative factors could also be involved, but the overall low levels of lipid oxidation products impede to investigate specific pro- or antioxidant effects related to compositional differences or processing conditions. In fresh muscle meats, strongest correlations were found between lipid oxidation products and the fatty acid composition (Van Hecke et al., 2019b), however, in the commercial meat products in the present study, the overall antioxidant effect seemed to predominate the potential influence due to compositional differences. Similarly, Soladoye et al. (2017) could not find a significant correlation between MDA-equivalents and the fatty acid profiles of bacon. In the latter study, the lack of correlation was proposed to be related to the greater overall effect of antioxidants and effects of production compared to the effects of the fatty acid composition.

Unlike numerous studies that studied chemical and physical changes in frying media by measurements of oil degradation parameters such as the fatty acid composition, acid value, anisidine value, polymer content etc., the second experiment in this study focused on the impact of frying medium on formation of oxidation products following the *in vitro* digestion of the deep-fried meat products. Although a large number of volatile aldehydes that are formed during frying probably end up in the atmosphere, some meat- or frying medium-derived aldehydes are probably still present or absorbed by the meat. The importance of the fatty acid profile of the frying medium and the number of frying cycles on the aldehyde content in fried food was demonstrated by Moumtaz et al. (2019) in potato chips. Furthermore, as Van Hecke et al. (2019b) demonstrated that the fatty acid composition of mammal, poultry and fish muscles play an important role in the protein and lipid oxidation profiles following gastrointestinal digestion, frying-induced changes in the fatty acid composition of the meat could influence oxidation as well. The increase of saturated fatty acids, and n-6 PUFA and α-tocopherol in the meat products after frying in respectively tallow and sunflower oil demonstrated the significant amount of fat

absorption during the frying process. This increase was particularly pronounced in the deep-fried meat products with a breaded coat and a high surface to volume ratio. The decrease in the relative amount of n-3 PUFA in the meat products after frying is particularly due to the relative increase of SFA and n-6 PUFA, but also thermal degradation reactions or the exchange of fatty acids from the food product with the frying medium could play a role. The high susceptibility of n-3 PUFA to thermo-oxidative degradation have led to the regulation in Belgium and France to limit linolenate content of frying oils to 2% (w/w) (Firestone, 1993). Similar to the study of Quiles et al. (2002), no pronounced changes in the fatty acid profile of the frying media was observed during this short-time and low-impact frying procedure, compared to some other studies investigating more extreme frying conditions (Park & Kim, 2016; Tyagi & Vasishtha, 1996). The decrease in the α-tocopherol levels in the sunflower oil during repeated frying and in the corresponding deep-fried meat products is in agreement with numerous studies and suggests thermal degradation and/or chemical consumption of α-tocopherol by lipid peroxyl radical during frying processes (Chung et al., 2004; Moumtaz et al., 2019; Quiles et al., 2002). Despite the considerable altered fatty acid profiles, that were largely determined by the fatty acid profile of the frying medium, and the differences in α-tocopherol in the deep-fried meat products, no pronounced effects of frying medium nor number of frying cycles was observed in the lipid oxidation product concentrations in the digests of the deep-fried meat products.

Most of the luncheon and deep-fried meat products had similar or higher levels of PCC compared to the reference pork neck. Also in the study of Armenteros et al. (2009), the unprocessed (raw) meat had the lowest PCC levels compared to the other processed meat products. The luncheon meat products with the highest PCC levels in the present study, namely the raw-cooked and precooked-cooked meat products, were more prone to intense lipid-protein interactions due to structure disruption and/or subjected to oxidation promoting factors such as mincing and cooking (Soladoye et al., 2015). Furthermore, these luncheon meat products also had the lowest protein and dipeptide contents and higher fat to protein ratios. The negative correlations between the PCC expressed per mg protein and the protein and dipeptide contents of the luncheon meat products could indicate that meat products with a lower protein content are more susceptible to protein oxidation. Furthermore, the antioxidant capacity of meat is frequently ascribed to different amino acids and dipeptides such as anserine, carnosine and GSH through potential pH-buffering, carbonyl scavenging and metal ion chelation capacities (Colzani et al., 2015; Wu et al., 2003). Therefore, the higher amounts of dipeptides could also have contributed to the lower PCC levels. In line with the negative correlation with protein content, a positive correlation between the fat to protein ratio and PCC levels expressed per mg protein was found. Although limited amounts of free lipid

oxidation products were detected in the meat products, it cannot be excluded that previously formed lipid oxidation products had reacted with proteins and contributed to the formation of PCC. However, when PCC concentrations are expressed per g meat, instead of per mg protein, no correlation with protein content, fat to protein ratio or significant differences in PCC between the meat products subjected to a different processing technology were found. This implicates that eating an equivalent amount of meat corresponds with the intake of similar amounts of PCC.

Another observation is the positive correlation between the labeled carbohydrate content and PCC (expressed per mg protein). This is interesting, since Maillard products originating from reducing sugars have been proposed to be involved in the oxidative de-amination of amino group residues in proteins, leading to the formation of PCC through a Maillard-mediated pathway (Akagawa et al., 2002; Luna & Estévez, 2018). Therefore, the use of reducing sugars as additives in processed meat (Kraybill, 1955) may have contributed to the higher PCC levels in some of the luncheon meats compared to the reference pork. Also, during deep-fat frying, Maillard-mediated formation of protein carbonyls is a very plausible reaction pathway due to the presence of proteins, reducing sugars and heat. Maillard reaction products such as melanoidins, have been shown to inhibit lipid oxidation i meat by radical scavenging or metal chelation (Bedinghaus & Ockerman, 1995; Miranda et al., 2012).

Protein oxidation was evaluated by the widely applied DNPH-based spectrophotometric method which involves the measurement of dinitrophenyl hydrazone per mg precipitated protein. Although this DNPH method is frequently used in meat and meat products, this method does not provide information through which pathways the PCC were formed. It was described that PCC can be formed via different pathways such as (i) direct oxidation of amino acid side chains, (ii) covalent binding of non-protein carbonyls, such as reactive lipid oxidation products, (iii) Maillard-mediated pathway with reducing sugars (Akagawa et al., 2002), and (iv) fragmentation of the protein backbone through α-amidation reactions or oxidation of glutamyl side chains (Akagawa et al., 2002; Stadtman & Levine, 2003; Zhang et al., 2013). However, also protein-phenol interactions have been suggested to react with DNPH, leading to an overestimation of lipid- or protein- derived carbonyls (Rysman et al., 2016). Both oxidation of proteins and the binding with phenol moieties can reduce protein digestibility by affecting the exposure of proteins to proteases in the digestive juices, or the amino acid chain length as such (Kroll et al., 2000; Santé-Lhoutellier et al., 2007). Since the DNPH method measures carbonyl moieties in proteins yielding molecular weights that are high enough to be precipitated by TCA, differences in the relative concentrations of oxidized proteins in the protein pellet versus the oxidized protein residues in de supernatant could be possible.

Next to differences in reaction mechanisms and stability of lipid versus protein oxidation products, the different action of antioxidants towards lipids and proteins should also be considered. Studies on antioxidants in meat have been shown to limit lipid oxidation in most cases, whereas their effect on protein oxidation is often less pronounced or even pro-oxidative. For example, numerous papers have reported the protective effect of nitrite salt against lipid oxidation in cured meat and during digestion, whereas its effect on protein oxidation is less consistent (Berardo et al., 2016; Van Hecke et al., 2014b; Villaverde et al., 2014a; 2014b; Vossen & De Smet, 2015). Also the addition of ascorbic acid and its salt sodium ascorbate has led to varying results. A rosemary extract and a mixture of ascorbate/citrate (1:1) was found to inhibit lipid oxidation in beef patties, whereas the ascorbate/citrate mixture promoted protein oxidation and the rosemary extract had no effect on protein oxidation (Lund et al., 2007). Other authors illustrated the importance of (i) the concentration of the antioxidant and of (ii) the product characteristics on the oxidative stability in frankfurters. The addition of rosemary oil inhibited lipid and protein oxidation in frankfurters from Iberian pigs, but had anti- or prooxidative effects in frankfurters from white pigs dependent on the concentration of the essential oil (Estévez & Cava, 2006). The authors suggested that the activity of rosemary oil could be affected by the levels of polyphenols and α-tocopherol. This illustrates that the final effect of a combination of different antioxidants in different matrices can deviate from the expected individual effects. Although most of the processing steps in meat production such as mincing, heating and ripening are considered to enhance oxidation (Soladoye et al., 2015), the formation of bioactive protein hydrolysates during enzymatic hydrolysis, gastrointestinal digestion, and/or food processing, can act as antioxidants by scavenging free radicals and/or inhibit free radical formation (Broncano et al., 2012; Gómez-Guillén et al., 2011). However, since this study compared ready-to-eat commercial meat products and deep-fried meat products containing several antioxidants, herbs, spices and extracts, no effect can be directly attributed to one of the specific antioxidants, nutrients or processing steps.

In conclusion, *in vitro* digestion of a wide range of commercial luncheon and deep-fried meat products resulted in lower levels of free lipid oxidation products compared to unprocessed cooked pork. As the relative risk ratio regarding meat consumption and chronic diseases is higher for processed meat compared to red meat (Chan et al., 2011; Ekmekcioglu et al., 2018; Micha et al., 2012), the results of the present study suggest that the contribution of free lipid oxidation products in this epidemiological link is probably rather limited. Protein oxidation products, in contrast, were higher for most of the meat products compared to the reference pork. The higher levels of protein oxidation products in the raw-cooked and precooked-cooked meat products compared to the other luncheon meats were possibly related

to a low protein content, high fat to protein ratio and/or intense mincing and cooking procedures during processing. The results emphasize the need for further investigating the impact of protein oxidation and the different reaction mechanisms between lipid and protein oxidation in meat products.

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5.6 Supporting information

Table S5.1 Nutrient composition of the 24 luncheon meat products and the reference pork neck
Figure S5.1 Macronutrient composition of the luncheon meats and reference pork.
Figure S5.2 Fatty acid composition of the luncheon meats and reference pork.
Figure S5.3 Residual nitrite, heme-Fe and the endogenous antioxidants α-tocopherol, carnosine and anserine of the luncheon meats and reference pork.
Figure S5.4 Macronutrient composition, and levels of heme-Fe and endogenous antioxidants in meat products prior to frying and in the reference pork neck

Table S5.1 Nutrient composition of the 24 luncheon meat products and the reference pork neck (obtained from the package label and/or analyzed).

Meat product	Fat (g/100 g FW)		SFA	MUFA	n-3 PUFA	n-6 PUFA	Ratio n-6/n-3	Carbohydrates (g/100 g FW)	Fibre (g/100 g FW)	Protein (g/100 g FW)		Salt (g/100g FW)	Dry matter (g/100 g FW)
	label	analysed	analysed fatty acid composition (g/100 g meat, FW)					label	label	label	analysed	label	analysed
Cured meat pieces-raw													
Parma ham (B1)	16	17	5.8	7.3	0.2	2.0	12.1	<0.5	0	27	27	4.6	54
Parma ham (B2)	16	15	4.9	6.5	0.1	1.9	19.3	<0.5	0	27	26	4.6	49
Back bacon	6	16	6.4	6.2	0.1	1.6	10.6	<0.5	<0.5	22	19	3.4	42
Filet de Saxe (B1)	2	3	0.7	0.8	0.0	0.3	14.9	0.9	0	22	23	3.1	32
Filet de Saxe (B2)	1	2	0.5	0.6	0.0	0.2	7.8	0.2	0	25	24	3.5	33
Black Forest ham	12	15	5.1	7.2	0.2	1.3	6.9	1	<0.5	24	25	5	48
Cured meat pieces-cooked													
Cooked ham (B1)	3	3	0.9	1.3	0.0	0.3	13.6	1	0	21	22	1.8	29
Cooked ham (B2)	4	1	0.5	0.5	0.0	0.3	12.9	1.2	0	19	20	2	27
Cooked ham (B3)	2	3	0.9	1.2	0.0	0.4	12.8	<0.5	<0.5	23	22	1.9	29
Raw-fermented sausages													
Salami (B1)	28	29	10.7	11.7	0.3	3.2	11.3	1.5	0	20	18	3.8	54
Salami (B2)	29	31	11.0	13.1	0.3	3.0	11.2	2	1	24	21	4.3	61
Salami (B3)	34	35	12.7	14.9	0.2	4.1	18.2	1.6	0	21	19	3.6	61
Salami (B4)	30	35	13.5	15.2	0.4	4.3	12.3	1.6	0.8	30	23	4.4	69
Raw-cooked products													
Bologna sausage (B1)	27	24	8.6	10.4	0.2	2.4	12.1	<0.5	0	12	13	2.3	42
Bologna sausage (B2)	16	17	5.7	7.3	0.2	2.0	11.6	3.3	0	10	10	2.3	35
Bologna sausage (B3)	24	21	8.4	9.0	0.2	2.7	11.0	3.5	0	9	9	1.6	38
Meat loaf (B1)	24	21	7.0	8.0	0.3	3.3	11.1	5.3	<0.5	14	14	1.8	43
Meat loaf (B2)	22	21	7.1	8.9	0.2	2.7	11.1	3.4	0	15	15	1.8	44
Frankfurter sausage	23	24	7.9	9.9	0.3	2.8	9.8	2	1.2	13	11	1.8	41
Precooked-cooked products													
Liver pâté (B1)	31	27	8.3	9.2	0.4	4.2	10.8	2.9	0.8	12	12	2	46
Liver pâté (B2)	26	25	7.6	9.1	0.3	3.6	12.1	3.3	0.6	11	10	1.7	42
Liver pâté (B3)	30	31	9.9	11.4	0.5	4.8	10.0	4.5	0	11	10	1.7	49
Blood sausage (B1)	20	22	6.9	7.9	0.3	3.5	12.7	7.9	0.9	10	9	1.4	44
Blood sausage (B2)	5	14	4.9	5.4	0.1	1.4	11.7	14.4	1	10	10	4.2	38
Reference (unprocessed)													
Pork neck		9	3.6	3.4	0.1	1.2	13.4				20		30

SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, FW = fresh weight

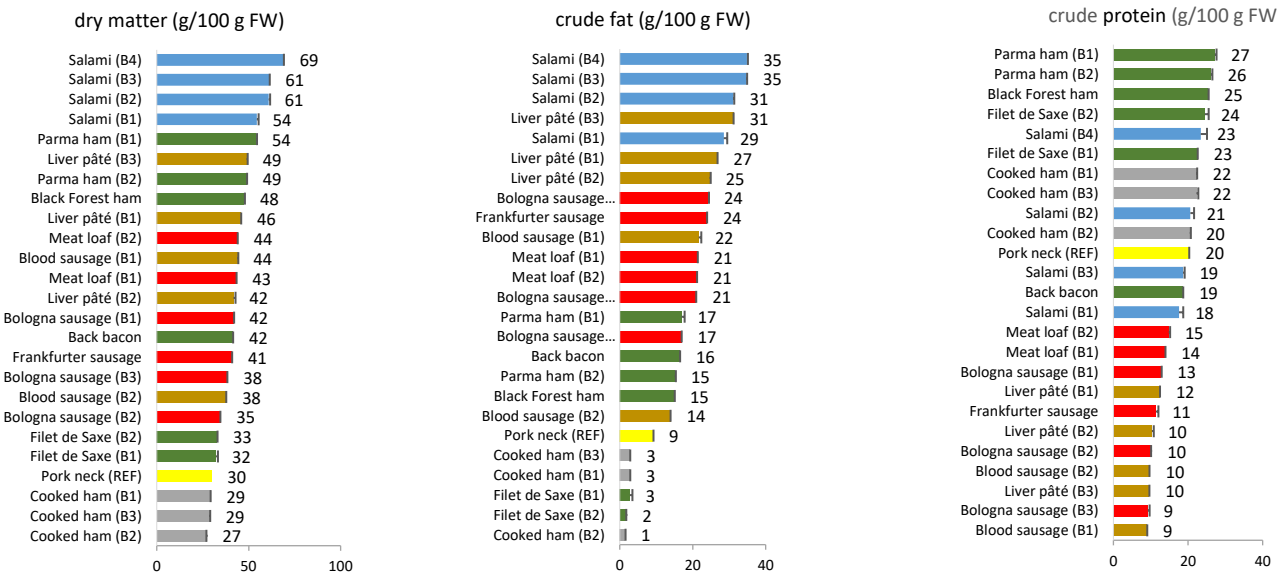

Figure S5.1 Macronutrient composition of the luncheon meats and reference pork. Meat products belonging to the same processing category were indicated in a same color (cured meat pieces-raw = green; cured meat pieces-cooked = grey; raw-fermented sausages = blue; raw-cooked products = red; precooked-cooked products = brown). Results were expressed as mean ± SD. FW = fresh weight

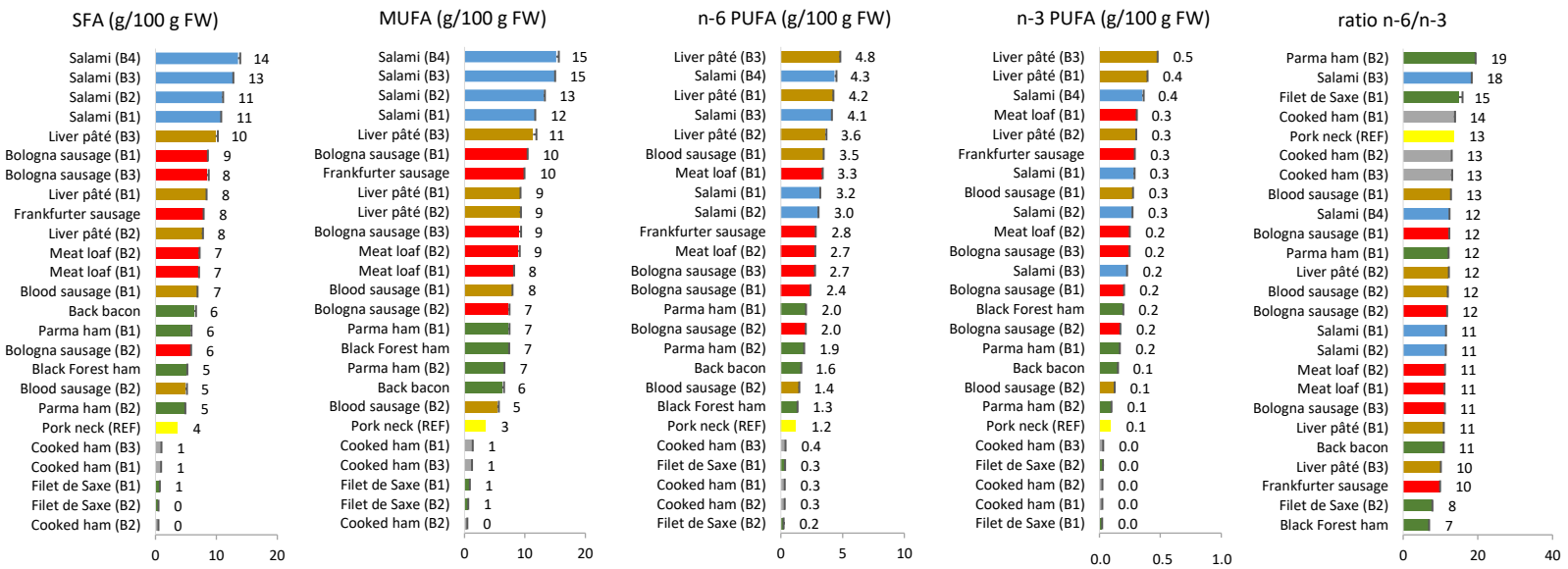

Figure S5.2 Fatty acid composition of the luncheon meats and reference pork expressed per 100g meat. Meat products belonging to the same processing category were indicated in a same color (cured meat pieces-raw = green; cured meat pieces-cooked = grey; raw-fermented sausages = blue; raw-cooked products = red; precooked-cooked products = brown). Results were expressed as mean ± SD. FW= fresh weight

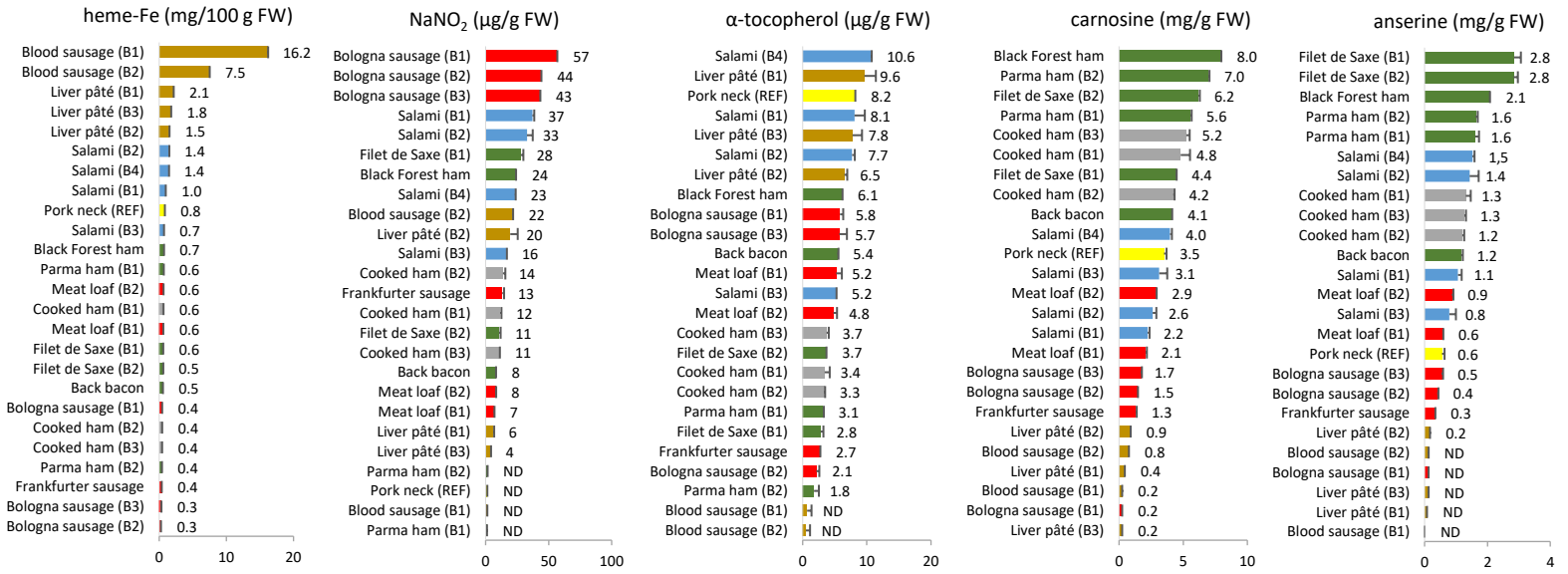

Figure S5.3 Residual nitrite, heme-Fe and the endogenous antioxidants α-tocopherol, carnosine and anserine of the luncheon meats and reference pork. Meat products belonging to the same processing category were indicated in a same color (cured meat pieces-raw = green; cured meat pieces-cooked = grey; raw-fermented sausages = blue; raw-cooked products = red; precooked-cooked products = brown). Results were expressed as mean ± SD.

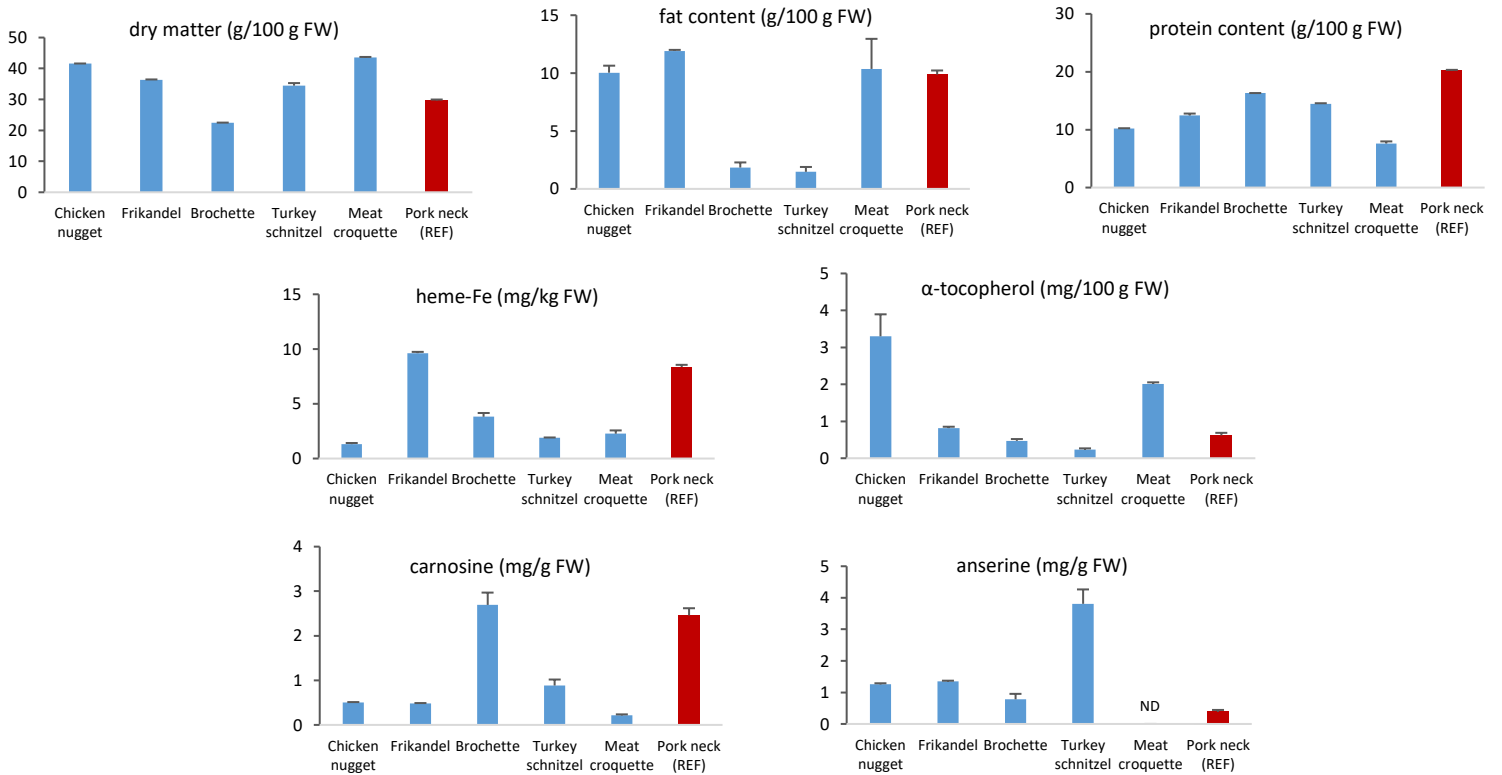

Figure S5.4 Macronutrient composition, and levels of heme-Fe and endogenous antioxidants in meat products prior to frying and the reference pork neck. FW = fresh weight

Chapter VI – General discussion and future perspectives

6.1 Evolution in and contribution of this PhD thesis to the hypotheses linking high meat consumption with chronic diseases

6.1.1 Targeted approach: lipid and protein oxidation

The rationale of this research was based on the epidemiological link between the consumption of red and processed meat and the risk for chronic diseases, with a specific focus on colorectal cancer development. At the start of this PhD, the review of Demeyer et al. entitled 'Mechanisms linking colorectal cancer to the consumption of (processed) red meat', was the most recent review discussing the hypotheses and mechanistic evidence underlying the link between colorectal cancer and red and processed meat consumption (Demeyer et al., 2016). Some hypotheses such as excess animal protein and fat, or the involvement of heterocyclic amines and polycyclic aromatic hydrocarbons were discussed but are very unlikely the solely contributors since they are not specific to red meat but also present in other foods such as poultry or fish, which are not associated with higher risks to develop chronic diseases. The hypothesis which has received most attention and support is called the 'heme hypothesis'. Both epidemiological and mechanistic studies indicate a role for heme-Fe in the red meat-colorectal cancer association through its catalytic effect of heme-Fe on the formation on NOCs and lipid oxidation products, and through a direct toxic effect of heme in the gut (Bastide et al., 2015; Corpet, 2011; Sesink et al., 1999). Furthermore, the involvement of lipid oxidation products in other red meat-associated chronic diseases, as reviewed by Van Hecke et al. (2017a), highlighted the need for more mechanistic research investigating the role of meat consumption as an external factor in the contribution of oxidative stress.

At first, the higher concentrations of lipid oxidation products in the chicken diets and digests compared to the red and processed meat diets and digests in Chapter II seemed to be in contrast with the hypothesis and with several observations in previous studies. However, the results do make sense if we take a closer look to the evolution in this research topic and the compiled evidence over the last two decades. The impact of meat consumption on postprandial oxidation has been studied at different levels (meat constituents, processing and additives, kinetics during digestion, meat or meat constituents in combination with reducing compounds or other food items,) as illustrated in Figure 6.1 and through different approaches (*in vitro* gastric and gastrointestinal digestion studies, *in vivo* animal studies, human interventions, and through the analysis of different endpoints). Some conflicting results emphasize the need to combine results from reductionistic approaches as well as from more top-down approaches and the need to measure multiple endpoints in various models before reliable conclusions can be drawn.

A complete literature overview is not provided in this discussion section, but a generalized overview of the different research approaches and important research groups that contributed to the current knowledge regarding the impact of meat consumption on postprandial oxidation will contribute to the interpretation of the present results.

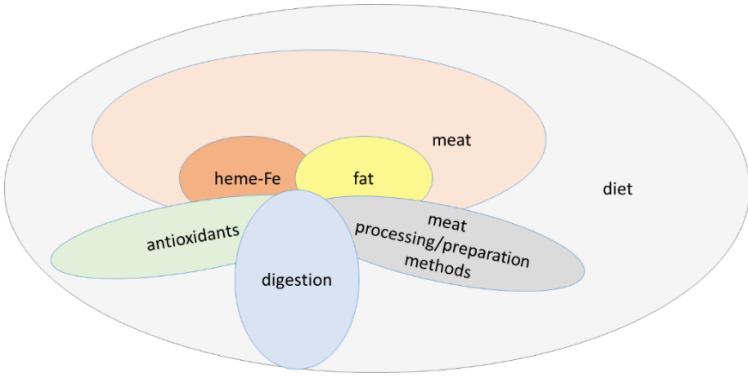

Figure 6.1 Simplified illustration of the different levels at which meat and lipid oxidation have been studied

Starting around 2000, several research groups focussed on the hyperproliferative and prooxidative effects of heme-Fe. The research groups of van der Meer and Pierre conducted multiple *in vivo* studies with mice or rats that were supplemented with different forms of isolated heme or non-heme-Fe such as hemin, protoporphyrin IX, ferric citrate, bilirubine or hemoglobin in which an increase in MDA-equivalents in fecal water of heme-fed animals was observed (Bastide et al., 2015; Gueraud et al., 2015; IJssennagger et al., 2013; Pierre et al., 2003; Sesink et al., 1999). The same research groups demonstrated that the supplementation of other nutrients such as chlorophyll, or even the influence of microbiota by antibiotic suppression, can inhibit or mitigate the prooxidative effect of heme (de Vogel et al., 2008; Martin et al., 2015). The catalytic effect of heme-Fe on lipid oxidation was also shown by our research group by comparing *in vitro* digests of chicken, pork and beef with different heme-Fe levels but with standardized fatty acid profiles to isolate the effect of heme-Fe (Van Hecke et al., 2014a). Another study of Pierre et al. (2006) studied varying concentrations of heme by the addition of freeze-dried chicken, beef, and blood sausage to a rat diet. At that time, the prooxidative effect of heme-Fe under controlled conditions was clearly illustrated. However, results from more recent experiments including more variables such as the pig trial in Chapter II and the recent *in vitro* studies of Steppeler et al. (2016), Kanner et al. (2017) and Van Hecke et al. (2019b) indicate that the level of heme-Fe is not the sole and probably not the most predominant factor in the formation of lipid oxidation products during digestion of meats or diets. The three latter studies showed that digests of several fish species

such as salmon and tuna contained approximately twice the concentration of MDA equivalents, and much more n-3 PUFA derived oxidation products compared to the beef digests. The studies also showed fewer gradual differences between beef, pork and chicken compared to previous studies, a high variability between different muscles from a same animal species (chicken thigh versus chicken breast), and large differences between different lipid oxidation products (MDA-equivalents, 4-HNE, 4-HHE, hexanal). In all three studies an additional experiment was performed, that illustrated the paramount importance of the fatty acid composition by altering the fatty acid profiles by addition of lard (Van Hecke et al., 2019b), fish oil (Steppeler et al., 2016) and n-3 PUFA supplement fish oil (Kanner et al., 2017). The subsequent *in vivo* study of Steppeler et al. (2017) confirmed these observations in a mouse model, reporting the highest concentrations of MDA-equivalents in fecal water of animals fed a salmon and a beef + safflower seed oil containing diet, and lower concentrations in samples of animals fed beef (with a same fat content, but lower unsaturated fatty acid proportion), pork and chicken diets. Similarly, Gueraud et al. (2015) and Tirosh et al. (2015) showed that the formation of lipid oxidation products upon digestion of a hemin enriched diet or red turkey meat strongly depended on the type of dietary oil. In the latter study, addition of olive oil and fish oil from tuna resulted in respectively 2.5-fold lower and 5-fold higher MDA equivalents compared to the control turkey meat digest (without oil addition) (Tirosh et al., 2015). Also the work of the research groups of Gomez-Guillen and Undeland demonstrated that fish, fish oil and vegetable oils with high n-6 and n-3 PUFA contents generated high concentrations of lipid oxidation products during gastrointestinal digestion (Larsson et al., 2012; 2016; Nieva-Echevarría, Goicoechea, & Guillén, 2017). In the studies of Larsson et al. (2012; 2016), the synergic effect of the fatty acid composition and heme-Fe was shown by the drastic increase of lipid oxidation products in an *in vitro* gastrointestinal model with cod liver oil emulsion fortified by haemoglobin. These current insights are important to re-evaluate the interpretation of previous studies in which heme-Fe was shown to increase MDA-equivalents in fecal water of heme-fed animals, since many of these diets contained high amounts of n-6 PUFA rich safflower seed oil (Bastide et al., 2015; Gueraud et al., 2015; IJssennagger et al., 2013; Pierre et al., 2003). This could indicate that not only heme-Fe, but also the PUFA content could have strongly enhanced the formation of lipid oxidation products in these studies.

Compilation of these findings support the hypothesis that the lower concentrations of lipid oxidation products in the intestinal contents of the pigs fed a mixture of red and processed meat compared to pigs fed chicken in Chapter II are related to the higher polyunsaturated fatty acid content in chicken meat, to which (a small proportion of) chicken skin was added. Moreover, a mixture of chicken breasts (lean meat) and thighs (higher fat content, relatively more PUFA and higher heme-Fe content) was used in Chapter II, whereas most previous studies demonstrating lower

lipid oxidation products in chicken compared to red meat muscles used chicken breasts or did not report the origin (Pierre et al., 2006; Van Hecke et al., 2014a). Another factor that could have played a role, and which is highly supported by the results from Chapter V, is the high proportion of processed meat in the red and processed meat-based diets. Indeed, about 1/3 of the red and processed meat mixture in Chapter II consisted out of ready-to-eat luncheon meat products such as salami, cooked ham, liver pâté, bacon, filet de Sax and blood sausage. In Chapter V, *in vitro* digestion of among others these luncheon meat products resulted in very low concentrations of lipid oxidation products. These concentrations were compared with minced and vacuum cooked pork neck, which was shown to contain average or low levels of oxidation products in comparison with digests of a wide range of similarly prepared mammal, poultry or fish muscles (Van Hecke et al., 2019b). Therefore, it can be concluded that free lipid oxidation products in most luncheon meats are lower compared to most fresh muscle meats. The use of these luncheon meats in Chapter II could thus considerably have diluted the amount of free lipid oxidation products in the mixture of red and processed meat. As discussed in Chapter V, the low levels of free lipid oxidation products in the luncheon meats are probably related to the action of antioxidants and/or further reactions of the formed lipid oxidation products with proteins (Vandemoortele & De Meulenaer, 2015) leading to PCC. Interestingly, levels of PCC after *in vitro* digestion of the diets in Chapter II were slightly higher in the digests of the red and processed meat-based diets compared to the chicken-based diets. It could thus be hypothesized that the extent of secondary reactions between reactive lipid oxidation products and proteins was higher in the red and processed meat mixture compared to chicken, resulting in relatively lower lipid oxidation products but higher PCC concentrations in the *in vitro* digests of the red and processed meat mixture. In the small intestinal content, PCC were highest in the piglets that consumed the red and processed meat in combination with the Western background diet, and significantly higher in comparison to the piglets that consumed the red and processed meat combined with the prudent background diet. This work suggests more research on the relative contribution of lipid oxidation in protein oxidation processes and on health consequences of protein oxidation products.

The effect of antioxidants added as isolated compounds or as whole foods on oxidation during meat digestion has been investigated by several research groups such as the research groups of Kanner, Santé-Lhoutellier and our group (Gobert et al., 2014; Gorelik et al., 2008a; Kanner & Lapidot, 2001; Lapidot et al., 2005; Lorrain et al., 2012; Sirota et al., 2013; Van Hecke et al., 2017b). In most cases, simultaneous digestion of meat and minor food components such as polyphenols resulted in remarkably lower levels of lipid oxidation products compared to the control meals without antioxidants. In some cases, also prooxidant effects were observed and compounds that inhibited or decreased lipid oxidation did not necessarily inhibit

protein oxidation (Berardo et al., 2016; Lund et al., 2007; Van Hecke et al., 2014b; Villaverde et al., 2014b; 2014c; Vossen & De Smet, 2015). The low level of lipid oxidation products after *in vitro* digestion of the deep-fat fried meat products, regardless of their fatty acid composition, in Chapter V suggests a considerably protection against lipid oxidation as a result of the presence of antioxidants, which were added as antioxidants or through herbs and spices. However, in this study, many other factors could also be involved. Causality was thus not proven, and a considerable proportion of lipid oxidation products probably ended up in the atmosphere as a result of their low boiling point compared to the frying temperature.

To conclude, based on the results of Chapter II and V and the available literature, it can be hypothesized that the presence of antioxidants is probably the predominating factor in the final concentration of lipid oxidation products formed during meat digestion. Next, the combination and the synergistic effect of the heme-Fe content and the fatty acid profile are likely more important than the fatty acid profile or the heme-Fe content as such.

6.1.2 Main research findings from untargeted approaches

Although many chronic diseases are closely linked with diet and lifestyle, their etiology and pathogenesis is very complex and can probably not be explained by one single factor. As illustrated in Figure 6.2, a balanced or disturbed metabolic phenotype is the result of reciprocal interactions between the host's response, environmental factors including lifestyle and diet, and the gut microbiome. Using pigs as a model for humans enables to control the three variable factors genetics, environmental conditions and diet to a large extent. As a result, studying the alterations in the gut microbiome and metabolic phenotype in function of different diets in a pig model, is an interesting untargeted approach to improve understanding of the impact of the diet on the metabolome and microbiome (Chapter III and IV).

6.1.2.1 Impact of chicken versus red and processed meat consumption in the context of a prudent and Western diet on the gut metabolome and microbiome

Clustering of the piglets according to dietary treatment in the PLS-DA score plots of the colon metabolome (Chapter III) and in the NMDS plot of the bacterial communities (Chapter IV) showed a larger impact of the background diet compared to the impact of the type of meat (Figure 6.3). Also, a higher number of significant differences in metabolites and bacterial taxa was found resulting from the different background diets as compared to two meat groups. This outcome is not surprising, since both the proportion and the differences in composition of the dietary patterns are larger than the differences between the two meat groups.

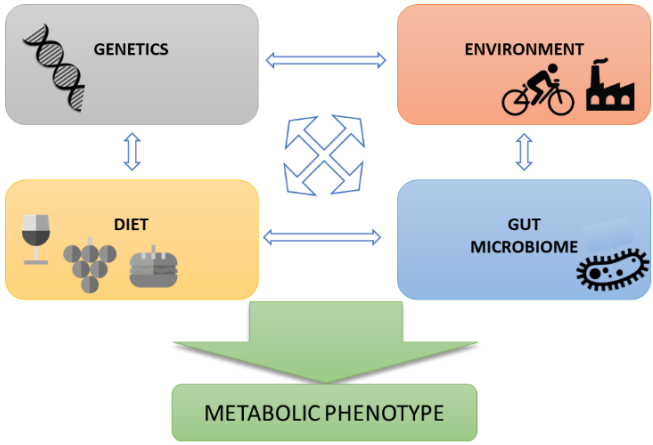

Figure 6.2 The metabolic phenotype is the result of the reciprocal interaction between the host, environmental factors, diet and the microbiome (adopted from Ussar et al. 2016)

Interestingly, both in the PLS-DA score plot of the colon metabolome and in the NMDS plot of the microbiome, a closer clustering between piglets subjected to the chicken-prudent and the red&processed-prudent dietary treatment was observed in comparison to the clustering between the chicken-Western and red&processed-Western fed pigs. Using a same selection criterium (adjusted p-values obtained in R package *limma* < 0.05 and VIP scores > 1 from OPLS-DA models) resulted in a higher number of differentiating metabolites comparing the pigs fed the red&processed-Western versus chicken-Western diet (84 significant metabolites) and the pigs fed the red&processed-prudent versus chicken-prudent diet (39 significant metabolites). This could indicate that the impact of the meat type on the colon metabolome and microbiome was more pronounced in combination with the Western background diet compared to the prudent background diet. This observation is difficult to explain at this stage, but it could be hypothesized that differences in transit time or absorption due to the background diets could influence the effect of meat on the microbiome and metabolome. The higher amount of dietary fiber in the prudent diets could increase stool bulk and decrease transit time (Cummings et al., 1976), which could limit the formation or absorption or reaction time of meat-specific metabolites. Multiple studies have reported a positive correlation between fiber intake, gut microbiota diversity and positive health effects (Claesson et al., 2012; Schnorr et al., 2014; Sonnenburg & Bäckhed, 2016). Therefore, it could be expected that the higher fiber content of the prudent diets could result in a more resilient microbial composition and protect against microbial changes caused by meat intake. However, in our experiment, no differences in the Hill diversity orders H_0 (species richness, determined by number of OTUs) and H_1 (species diversity, determined by the Shannon index) were observed between the four treatments (Chapter IV).

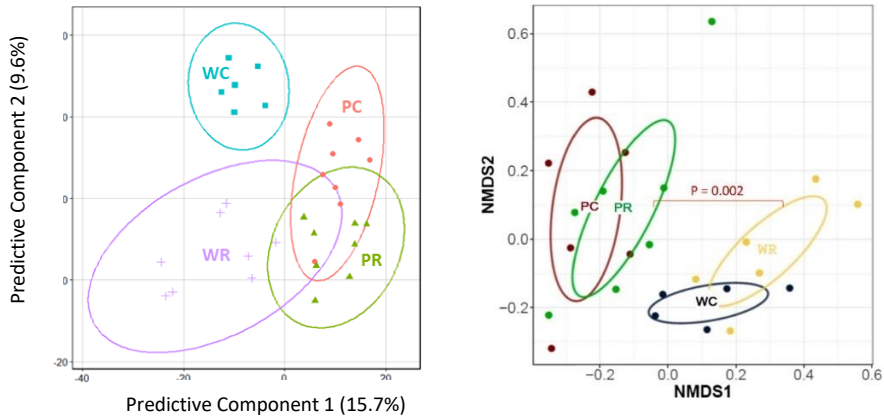

Figure 6.3 Similarities between the PLS-DA score plot of the colon metabolome (left) and NMDS plot of the gut bacterial community (right) of the pigs subjected to four dietary treatments: PC = prudent-chicken; PR = prudent-red&processed; WC = Western-chicken; WR = Western-red&processed

6.1.2.2 Role of carnitine metabolism

The potential proatherogenic effect of TMAO is one of the most recent proposed mechanisms to explain the link with meat intake and cardiovascular disease risk (Koeth et al., 2013), but also with colorectal cancer (Bae et al., 2014) and type 2 diabetes (Zhuang et al., 2019). Free TMAO is present in some dietary sources such as fish and crustaceae, whereas TMAO from meat is formed after microbial and endogenous metabolism of TMAO precursors including carnitine and choline. Particular gut bacterial species produce TMA and/or 3-dehydroxycarnitine out of these precursors. After absorption, TMA is oxidized in the liver to TMAO by flavin monooxygenase enzymes and excreted in urine.

One of the most pronounced outcomes of the untargeted metabolomics analysis is the detection of higher concentrations of carnitine related metabolites (carnitine, acylcarnitines and 3-dehydroxycarnitine) and lysophosphatidylcholine, another precursor of TMA, in the gut after the consumption of red and processed versus chicken meat (Chapter III). Although higher concentrations of mainly carnitine, acetylcarnitine and some other acylcarnitines in blood and/or urine upon meat intake have been reported in previous studies (Khodorova et al., 2019; O'Sullivan et al., 2010; Playdon et al., 2016; Schmedes et al., 2016; Schmidt et al., 2015; Stella et al., 2006; Wang et al., 2018), this high number of altered acylcarnitines and the impact of the dietary context had not been reported before. The higher abundances of long-chain acylcarnitines after the consumption of the red&processed-Western diet compared to the other dietary treatments is probably a reflection of dietary

intake of carnitine (highly present in red meat (Demarquoy et al., 2004)) and of the fatty acid profile of the Western background diet (Chapter II). This interaction effect of meat type and dietary context is not overly surprising, but it does emphasize the importance to consider a whole diet instead of (or in addition to) particular food items. Most of the differentiating acylcarnitines were detected in the luminal content of the small intestine, and only a smaller number in the colon. It is possible that these initial differences diminish during further digestion and absorption and have therefore not been detected to a same extent in blood or urine in previous metabolomics studies (Khodorova et al., 2019; O'Sullivan et al., 2010; Stella et al., 2006).

Despite the considerable differences in TMA precursors in the gut as a result of the dietary treatments, we did not detect differences in TMA or TMAO in the luminal contents of the gut. TMAO levels were additionally analysed in colon tissue (Rombouts et al. 2020 submitted) and urine (Thøgersen et al., 2020) of pigs from the same pig feeding trial. No TMAO was detected in the colon tissue samples. In contrasts, TMAO levels were detected by the same authors at higher concentrations in tissue of rats fed a high red meat dose (Rombouts et al. 2020 submitted). In urine, the highest concentrations of TMAO were detected in pigs fed the red&processed-Western diet (3.38 mM), followed by the chicken-Western (1.83 mM), the chicken-prudent (1.54 mM) and the red&processed-prudent diet (1.38 mM). However, the difference was only statistically significant between the highest and the lowest concentration, corresponding with the pigs fed red and processed meat in combination with the Western and prudent background diet, respectively (Thøgersen et al., 2020). The potential influence of the background diet, host and microbiome on the urinary TMAO concentrations are discussed in the paper of Thøgersen et al. (2020). It was concluded that the formation of TMA from dietary precursors is microbiota dependent, and potentially promoted by the microbiome of high-meat eaters compared to vegetarians or vegans (Koeth et al., 2013). Therefore, correlations between the urinary TMAO concentrations and the analysed microbiome (Chapter IV) were reported in the related paper of Thøgersen et al. (2020). Although some correlations and agreements with observations in literature were found, results should be interpreted with caution because correlations do not imply causation and only borderline statistically significant correlations were found.

6.1.3 Interpretation of the main results in terms of health impact

The present thesis describes the differences in oxidation products (Chapter II and V), metabolites (Chapter III and IV) and microbiota (Chapter IV) after the *in vitro* or *in vivo* digestion of meat or meat-based diets. Based on literature, several of these differences can be linked to a positive or negative health impact. However, the many variables in this top-down research approach do not allow an in-depth assessment

of causal relationships. It is difficult to conclude to what extent the observed differences in oxidation products, metabolites and microbiota are derived from the food itself, transformation of food-derived compounds, or from difference in host/microbiome response as a result of dietary intake. However, in all experimental Chapters, it seems that many of the differences observed in the *in vitro* and *in vivo* digests can be attributed to differences that were already present in the diets or originate directly from the dietary composition. Differences in the systemic oxidative status of the pigs could be an indicator of a health consequence of the dietary treatments. However, only marginal or no effects were found in the oxidative stress parameters in blood, organs or gut mucosa (Chapter II). Increased levels of oxidation products, TMAO, acylcarnitines and microbiome shifts have been found in patients suffering from chronic diseases and pathophysiological effects of those compounds have been described in mechanistic studies (Ceriello & Motz, 2004; Dambrova et al., 2016; Mihalik et al., 2010). However, it remains difficult to determine unambiguous cause and effect relationships. Furthermore, it is challenging to understand the biological relevance and critical concentrations of altered metabolites. For some metabolites, a 5% increase might be of tremendous importance, whereas for other metabolites, a 1000-fold increase might have no impact.

Regarding the current hypotheses in the link between meat consumption and chronic diseases and our main findings, there seem to be some conflicts between the epidemiological evidence and the proposed underlying mechanisms. Starting from the epidemiological observations, several questions can be raised showing that the main investigated mechanisms and outcomes in this thesis (namely the impact of oxidation products and L-carnitine metabolism) cannot (solely) explain the link between red and processed meat consumption and chronic diseases:

- Why is red meat but not white meat consumption associated with several chronic diseases?
- Why is the risk to develop several chronic diseases higher for processed meat consumption compared to red meat consumption?
- Why is only processed meat associated with an increased risk for cardiovascular diseases and not unprocessed red meat?
- Why is the consumption of fish generally considered to have beneficial effects on several chronic diseases?

Both heme-Fe and L-carnitine, which are considered important contributors in the formation of oxidation products and TMAO respectively, are present in higher concentrations in red meat compared to white meat, but they are generally lower in processed meat compared to red meat (Demarquoy et al., 2004; Demeyer et al., 2016). Furthermore, some muscle types of chicken and pork do not differ greatly in terms of heme-Fe content, which has been described before as the white meat

controversy (Demeyer et al., 2016; Lombardi-Boccia et al., 2002). In addition, both lipid oxidation products and TMAO are frequently present at higher levels in muscle tissue of fish compared to meat (Bjarne Landfald, 2017; Tang et al., 2001), and in *in vitro* digests (Kanner et al., 2017; Van Hecke et al., 2019b) or human biofluids following the consumption of fish compared to meat (Cuparencu et al., 2019b). These two apparent discrepancies are important to consider when evaluating meat metabolites and the link with disease risk. Since TMAO is present in fish as such, in contrast to TMAO from meat which is formed through microbial and hepatic conversion, it could be hypothesized that early absorption of TMAO following fish consumption differentially affects disease development compared to TMAO from meat that was formed in later stages during digestion (Rombouts et al. 2020 submitted). Another hypothesis is that red and processed meat could contain additional factors, that are absent in fish, which could enhance the toxic effect of TMAO or lipid oxidation products (or other metabolites), and that this combined effect increases the disease risk. Following the same train of thoughts, fish could also contain protective factors resulting from the different fatty acid profiles or certain micronutrients, which could counteract adverse effects caused by TMAO or oxidation products (or other metabolites). Furthermore, also the method of preparation of meat and fish and the dietary context could play a role and can be responsible for interaction and contrasting effects.

For many of the altered metabolites, both adverse and positive health effects have been described. Oxidation processes and the formation of ROS are the basis of aerobic metabolism and a variety of cellular processes. Adverse or beneficial physiological effects of 4-HNE have been reported to depend on its intracellular concentrations, with low concentrations exerting beneficial effects through its various actions as a signaling molecule (Csala et al., 2015; Nègre-Salvayre et al., 2017; Shoeb et al., 2014). In contrast to the potential proatherogenic effects of L-carnitine and TMAO, also cardioprotective effects have been described for L-carnitine and carnitine-derived metabolites (Amin & Nagy, 2009; Bene et al., 2018; Collins et al., 2016; Ferrari et al., 2004; Flanagan et al., 2010). For example, the study Collins et al. demonstrated that aortic lesion size in ApoE-/- mice was negatively correlated with increased TMAO levels as a result of L-carnitine administration, which indicates a protective instead of causative effect of TMAO on atherosclerosis development (Collins et al., 2016), in contrast to the observations in previous studies (Koeth et al., 2013). These conflicting effects emphasize that the causal link between altered metabolites upon meat consumption and disease pathogenesis should be further investigated.

6.2 Reflections on the adequacy of the study design of the pig trial to answer the research questions

The main goal of this thesis was to investigate the metabolites formed during red and processed meat digestion that could potentially be involved in the higher risk to develop chronic diseases based on the outcome of multiple epidemiological studies. Recurring comments on nutritional epidemiological associations are the relatively low relative risk values, the lack of evidence of causality and the difficulties with residual confounding due to self-reporting bias, lifestyle or other dietary factors (Leroy & Cofnas, 2019). Bias from confounding is minimized to a high extent by a careful statistical analysis and interpretation, by replications of findings across different populations with different ethnicities and dietary patterns, and by grouping of varying results from independent studies in meta-analysis. However, a certain extent of residual confounding cannot be completely ruled out (Larsson & Orsini, 2013; Flogelholm et al., 2015). The study of Flogelholm et al. (2015) investigated the confounding role of other dietary factors in the association between meat consumption and chronic diseases and addressed the different approaches that had been used to adjust for dietary confounders. It was concluded that the association between meat consumption and a lower-quality diet may complicate studies on meat and health. Furthermore, based on results of a large cohort study of French women, Bastide et al. (2016) concluded that the association between heme-Fe intake and colorectal adenoma incidence may depend on the ratio of heme-Fe to the dietary antioxidant capacity of the diet. The synergetic effect of different dietary factors such as the inhibition of formation of lipid oxidation products by addition of herbs and spices or polyphenols to meat has been shown in several *in vitro* studies (Van Hecke et al. 2017b, Gorelik et al., 2008a). Therefore, it can be suspected that red meat consumption is not an independent risk factor, but that the dietary context might influence the relation between meat consumption and health.

Most mechanistic studies test a well-defined hypothesis within a well-controlled experimental set-up. To investigate a specific hypothesis, a trial with red meat high or low in heme-Fe levels, or processed meat subjected to varying processing conditions could be executed. However, since none of the currently suggested hypotheses can fully explain these epidemiological associations, exploratory top-down approaches are recommended, in addition to the further investigation of current hypotheses. Based on the outcome of epidemiological studies and the lack of a positive association between consumption of chicken and chronic diseases, it is interesting to use a top-down approach, comparing the differences in metabolite formation during digestion of red and processed meat versus chicken, in which chicken may serve as a negative control. However, as chicken and red and processed meat differ in many aspects, this approach will result in more exploratory or

descriptive research instead of an in-depth assessment of causal relationships. Moreover, since there is no clear hypothesis on the link between meat consumption and chronic disease onset, it is also difficult to define study endpoints in a controlled short-term study.

In our experimental set-up, we aimed to combine an exploratory non-hypothesis-driven approach with a targeted approach investigating the heme-Fe hypothesis, by which higher levels of oxidation products following red and processed meat consumption compared to chicken meat consumption were expected. In addition, we also aimed to compare the effects of meat consumption in two different dietary contexts that are considered of higher and lower quality, by which the higher quality diet was hypothesized to protect against negative effects caused by meat consumption. The combination of this targeted and exploratory approach has led to a controlled experiment with complex diets. To comply with the exploratory nature of the experimental design, the diversity and complexity of the targeted dietary patterns was simulated as much as possible. Nevertheless, some important choices were made in the formulation of the diets and in the experimental set-up to obtain semi-controlled conditions to allow comparability between the dietary treatment groups and because of practical constraints.

Related to the meat component in the diets, we formulated the diets to obtain an equal (and relatively high) meat intake in all diets, even in the diets with a prudent background diet. An equal meat intake in all treatment groups was obtained by calculating the energy proportion of meat contributing to the total energy of the diets. Since the energy density of lean chicken is lower compared to the energy density of a mixture of red and processed meat, not adjusting the fat content would have resulted in different proportions of energy contribution in the diets, or different proportions of meat in g/kg diet. By adjusting the fat content of chicken meat, a similar energy proportion and similar proportion of meat in g/kg diet was realized. This adjustment resulted in an equal energy proportion of meat in the diet (21.5%) and in similar contributions of meat when comparing the two types of meat in combination with a same background diet (145 g meat/kg diet for both the chicken-prudent and red&processed-prudent diet, and 197 g meat/kg diet for the chicken-Western and red&processed-Western diet).

Also related to the composition of the background diets, crucial choices had to be made. Studying the interaction effect of meat with other dietary compounds could be conducted at different levels, by testing specific nutrients, individual food items, a combination of multiple food groups or a more complex dietary pattern. Less complex designs allow a better mechanistic understanding and generate more distinct conclusions, but conclusions are only valid for the specific conditions tested and potential interaction effect of food items or nutrients that are not included in

the experiment can be missed. Furthermore, because of the difficulties with extrapolation of results obtained under extreme conditions, and because of our interest in an exploratory approach under realistic conditions, we decided to simulate a lower and higher quality-considered diet based on Belgian consumption data and dietary guidelines. An exact formulation of a high or low quality diet does not exist and many more alternatives of dietary pattern formulations could have been applied. Nevertheless, as a compromise between realistic and contrasting diets, the choice for these two background diets can be justified. Three important considerations made during the formulation of the diets should be mentioned. First, several important differences between a prudent and a Western dietary pattern that were not simulated in our experimental design are the higher calorie intake and the higher levels of meat consumed in a Western dietary pattern. These aspects could not be simulated since growing piglets are not an optimal model to study effects of calorie intake in adults. Furthermore, we specifically intended to compare a same level of meat intake in all dietary treatments. Secondly, since we intended to isolate the effect of red and processed versus chicken meat, we decided not to include fish and crustaceans in the diets. Thirdly, important aspects of a dietary pattern such as portion size, temporal distribution of intake, food diversity etc. were not simulated. In contrast, all food items were mixed to avoid selection of food items and to obtain digest samples of the whole dietary pattern instead of one distinct meal. These limitations should be considered carefully. Nevertheless, this original approach is very interesting to obtain a general idea of the effect of meat in two contrasting dietary backgrounds on oxidation parameters, metabolome and microbiome. More specific strengths and limitations of the chosen study design are described under section 6.3.

Another difficulty of this unconventional approach is the determination of an adequate sample size because of the small anticipated findings due to realistic proportions of meat in the diets and because of the many variables measured and the untargeted character of the metabolomics and microbiome analysis. Because of the many variables of interest in the targeted approach and the unprecedented experimental diet formulation, no *a priori* exact power calculation or sample size estimation was performed. A minimal sample size to detect treatment effects was estimated by the combination of estimations of variance and estimations of effect size for different parameters in similar studies in literature. For example, the study of Gobert et al. (2014) reported a 3-fold and 5-fold postprandial increase compared to baseline levels of TBARS in the gastric digesta of cannulated minipigs after consumption of beef, either or not in combination with fruit and vegetables. The standard error of the mean obtained from the observation in 6 minipigs was estimated to be 10 times smaller compared to the effect size between the treatment groups with or without the addition of fruit and vegetables. Another interesting

study for sample size estimation was the human intervention study (n = 10) of Sirota et al. (2013) showing an 80% inhibition of postprandial plasma MDA absorption following the consumption of red meat in combination with roasted coffee. Also the study of Van Hecke et al. (2016) generated valuable insights in the variance and effect size estimation of multiple oxidative status parameters in rats (n = 6 per dietary treatment) that were fed chicken or red meat in a low or high fat dietary context.

A posteriori, a post-hoc power calculation or an adequate sample size could be calculated for the different oxidation related parameters. For example, for a desired significance level of $P < 0.05$ and 80% power, the results of the means and the variance within the treatment groups indicate that a total sample size of 10 piglets (5 per treatment group) would have been sufficient to denote the difference between the 4-HNE levels in the small intestinal content due to chicken versus red and processed meat consumption as significant. For the present study, we can thus conclude that the sample size was large enough to reliably detect significant differences in lipid oxidation products in the intestinal contents of the pigs as a result of meat consumption. For the other parameters or treatments, the numerical differences between the means among the treatment groups and the variance should be considered. If the means of a parameter of two treatment groups with a relatively high variance are numerically but not significantly different, a larger sample size could have resulted in statistically significant differences. On the other hand, parameters having similar means with low variances within the treatment groups would not profit from a larger sample size.

For the non-hypothesis-driven approaches, sample size calculation is even more difficult since no *a priori* known metabolite or species is targeted (Billoir et al., 2015) For these analyses, literature studies were also verified to consider an appropriate sample size. For microbiota analysis, the pig feeding study (n = 5 pigs per treatment) of Belobrajdic et al. (2012) investigating a high-protein diet comprising of human food items and meat found significant differences between the treatment groups for mid-colon microbiota composition and volatile organic compounds such as phenol, cresol and short chain fatty acids. The valid PLS-DA models in the metabolomics experiment show that the sample size was sufficient to retain metabolites that are different in the two meat groups and the two background diets. Also other metabolomics studies such as the randomised cross-over meal study and the independent meal intervention study of Cuparencu et al. (2019b) used a rather limited sample size (12 and 6 human volunteers) to reveal candidate biomarkers of general and red meat intake. The adequate sample size in untargeted multivariate analyses depend on the degree of control and whether an extensive metabolic characterization or a biomarker identification is aimed. In a more controlled setting, lower variability within a same group is suspected and smaller sample sizes are

required compared to population studies that require larger sample sizes. It is important to validate findings by traditional biological experiments or to replicate experiments in a different setting or population (Blaise, 2013).

6.3 Strengths and limitations of the PhD thesis

In addition to the adequacy of the study design to answer the research questions, more specific strengths and limitations of the study should be mentioned. One of the major strengths of the pig feeding trial is the use of a realistic proportion of meat in a complex dietary context. As addressed in the systematic review of Turner & Lloyd (2017), previous mechanistic studies investigating the link between meat consumption and colorectal cancer risk, used extremely high levels of meat or meat constituents, mostly combined with purified, or semi-purified diets that lack potential protective compounds present in whole foods. In this regard, our study did not only include whole foods, but specifically investigated the impact of two frequently described contrasting dietary patterns, namely a Western (high in sugars and fats) and a prudent (high in fruits and vegetables) dietary pattern. The assumptions and calculations proposed by Turner & Lloyd (2017) can be used to put the levels of heme-Fe used in the current pig feeding trial into perspective. The levels of heme-Fe in the diets of the pig feeding trial correspond to 4.1 µmol/kg (red&processed-Western diet), 3.0 µmol/kg (red&processed-prudent diet), 1.2 µmol/kg (chicken-Western diet) and 0.9 µmol/kg (chicken-prudent diet). Those levels are comparable with the levels of an average diet that contains approximately 2.75 µmol heme-Fe/kg diet, according to the assumptions of Turner & Lloyd (2017). In contrast, in the studies of IJssenagger et al. about 200 or 500 µmol heme was added per kg diet to study cytotoxic and hyperproliferative effects of dietary heme in rodent species (de Vogel et al., 2008; IJssennagger et al. 2012a; 2012b; 2012c; 2013). Although the use of high doses in mechanistic studies generates relevant information, extrapolation of results obtained from high doses in experimental animals (mostly rodent species) to predict the expected human risk at realistic exposures is not straightforward.

The implications of the choices made to formulate the background diets have been described in section 6.2. Another limitation related to the background diets, is the rather limited characterization of the background diets. Differences in the carbohydrate composition such as fiber content, natural and refined sugar content and differences in the antioxidant levels such as concentrations of vitamin C, polyphenols and carotenoids in the prudent versus Western background diets could have been expected to play an important role in the health effects of the background diets and in the interaction with meat. However, as the main differences in composition can be estimated from literature and since the interaction effects of the

background diets and the meats measured in this study were rather limited and not as pronounced as hypothesized, a detailed characterization of the background diets was not performed. Nevertheless, more mechanistic studies on the interactions between well-characterised food items would be very interesting as well as the influence of other diet quality aspects.

Although every model has its particular advantages and limitations, the use of rodents to study chronic diseases such as diabetes and cancer is increasingly receiving criticism, due to multiple failures in the translation of successful therapies in rodent models to human clinical trials (Akagi, 2004; Baker, 2008; Chandrasekera & Pippin, 2014; Lai et al., 2014; Roep et al., 2004). Rodents are the most frequently used experimental animals due to their small size, low cost, ease of handling, fast reproduction rate, the availability of various genetically modified models, and the possibility to compare the results with previous rodent studies in literature. However, multiple arguments can be raised to choose pigs over rodents as experimental animals to assess diet-microbiota-health interactions as reviewed by Guilloteau et al. (2010), Heinritz et al. (2013) and Roura et al. (2016). First, rodents are primarily caecal fermenters with higher feed intake relative to body size and much faster transit times, compared to pigs that share much more anatomical, metabolic and physiologic similarities with humans (Gandarillas & Bas, 2009). Although pigs also have a significant caecal fermentation, the predominant fermentation site in pigs (and humans) is the colon. Secondly, pigs and humans are both omnivorous animals with similar nutrient requirements. This enables to feed pigs with diets that are representative for human intake. Thirdly, the microbial similarities at phyla level between humans and pigs and especially the very close similarities between the microbiome of human-flora-associated pigs and the microbiome of its donor (Pang et al., 2007), make the pig an attractive model. In the study of Pang et al. (2007), human-flora-associated pigs were implemented by repeated oral administration of fecal material of a human subject to neonatal germfree animals. The bacterial composition of the human-flora-associated piglets were very close to that of the human donor, and different from that of conventional piglets (Pang et al., 2007; Vlasova et al., 2019; Wang & Donovan, 2015). In our study, we did not use human-flora-associated piglets. However, since diet is an important factor in shaping the microbiome of young animals, we can assume that the diet will have an important impact on the microbial composition. Furthermore, compared to human intervention trials, using animals has the advantage to allow strictly controlled feeding, to minimize individual variation due to better controlled genetics and environmental conditions, and to collect adequate samples of blood, tissues and gastrointestinal contents. However, despite the many similarities between humans and pigs and the superiority of the pig compared to other non-primate species, humans and pigs remain different animal species with own characteristics. Examples

of differences that could play a role in a different outcome are the lower gastric pH of humans compared to pigs, the ability of pigs (and not humans) to synthesize vitamin C endogenously, and the higher microbial density of the ileum in pigs compared to humans (Eberhard et al., 2007; Gasthuys et al., 2016; Sciascia et al., 2016).

Due to practical reasons, young and growing piglets were used instead of adult pigs or minipigs, which is a limitation of the present study. The piglets were weaned at 3 weeks old, 2 weeks before the start of the experiment. The post-weaning period is known to be very challenging for piglets due to the combination of their immature digestive and immune system and abrupt changes in feed and environment. The weaning period is often accompanied by reduced feed intake, reduced growth performance and increased incidence of diarrhoea (Jayaraman & Nyachoti, 2017). In our experiment, we included an adaptation period of 2 weeks before the effective start of the experimental feeding period. In this adaptation period, the pigs were first fed a commercial pre-starter diet followed by a gradual introduction of the dietary treatments. By the start of the experimental feeding period, the typical weaning diarrhoea seemed to have passed. However, in the second week of the experimental feeding period, the incidence of diarrhoea increased, which was accompanied by a drop in feed intake and stagnation of body weight gain, as illustrated in Chapter II. Compared to the balanced commercial pre-starter piglet diets, the composition of the experimental diets is not optimized for growing piglets, which may have influenced proliferation and absorption in intestinal epithelial cells and microbiome composition (Yang et al., 2016). The diarrhoea incidence was not specific for a particular dietary treatment, and most piglets recovered within 3-5 days. Although the gastrointestinal tract of young piglets is subjected to morphological, histological and microbial changes, the key processes of digestion are present and enable to use the piglet as a valuable model to compare the digestion of different diets.

In parallel with the *in vivo* pig feeding trial, the diets were also subjected to an *in vitro* digestion protocol that was used in previous studies by our research group (Van Hecke et al., 2014a; 2014b; 2015; 2017b; 2018; 2019b) and in Chapter V. Since the static *in vitro* model lacks several physiological aspects of an *in vivo* digestion process such as absorption and anaerobic conditions, differences between *in vitro* and *in vivo* obtained digest samples could be expected. Similar trends and results obtained in the *in vivo* and *in vitro* digests could confirm the validity of the *in vitro* digestion model. However, in this study, most of the observed relative differences in levels of oxidation were already present at the level of the diet before ingestion, although a considerable increase of oxidation products during digestion was observed (Chapter II and V). The intense interaction of the food items during preparation and storage is a limitation of our experimental design. Indeed, we chose to feed the piglets repeatedly the same complex homogenous mixtures of many food items instead of

feeding variable and distinct meals. In a dietary pattern, not only the dietary composition is important, but also portion size, temporal distribution of intake, variety etc. are important aspects of a dietary pattern (Eicher-Miller et al., 2016; Forslund et al., 2002). We decided to make homogenous diets, resulting in the concomitant intake of about 90 food items each feeding time, in order to allow representative sampling of digests of the whole diet instead of one specific meal and to avoid selection of particular food items by the piglets. Mixing all food items will have influenced food structure, increased potential chemical interactions during preparations and storage and will have affected digestibility and bioavailability. This approach has the advantage of strict controlled feeding and enables to compare the effects of the dietary composition without the presence of many other variables.

Since the previously proposed factors and mechanisms as such cannot fully explain the link between red and processed meat consumption and several chronic diseases, the untargeted metabolomics approach has the advantage to elucidate novel red meat-associated metabolites and potential involvement in disease development in a non-hypothesis driven manner. One of the main challenging steps in untargeted metabolomics is the identification and data interpretation. Only a fraction of the discriminating metabolites following red and processed versus chicken meat consumption could be identified (Chapter III), leaving multiple metabolites with a potential important biological impact unidentified. However, due to rapidly advancing analytical techniques, intelligent data acquisition strategies and enhanced *in silico* fragmentation modelling, metabolomic databases and libraries are exponentially growing. For example, the HMDB contained 2180 metabolites in 2007 and about 114000 (known, expected and predicted) metabolites in the latest version of the HMDB (version 4.0) (Wishart et al., 2018). The HMDB also includes the disposition of metabolites (route of exposure, origin of the metabolite and its location within an organism). The latest version contains 25424, 4225, 440, 1234, 90 and 1170 metabolites that were detected or expected in human serum, urine, cerebrospinal fluid, saliva, sweat and feces, respectively (Wishart et al., 2018). As most metabolomics studies focused on blood and urine, most data are available for these biological matrices in terms of number of metabolites and concentrations ranges, whereas gut metabolites remain largely unexplored. It can be expected that many metabolites in the gut have undergone microbial transformations, phase I or phase II reactions in the liver, or enzymatic reactions in the body, for which no authentic standards are available. For this type of metabolites, *in silico* metabolite prediction offers great opportunities (Djoumbou-Feunang et al., 2019). Upon metabolite identification, the next challenging step is the functional interpretation of the obtained data. Multiple pathway analysis and metabolic network modelling methods based on pathway libraries such as KEGG, MetaCyc and BioCyc have been developed to facilitate data interpretation (Caspi et al., 2016; Chong et al., 2019; King

et al., 2016; Zhou et al., 2019). Pathway enrichment analysis could indicate disturbances in the organism's metabolic regulation network and is very promising in e.g. the comparison of healthy and diseased subjects. However, in the case of gut metabolites in a dietary intervention trial, differential metabolites are not necessarily the result of altered cellular processes. As discussed in Chapter III, disturbances in the acylcarnitine profile could be due to a reduced or incomplete capacity for β-oxidation or to a decrease of mitochondrial CoA levels, but also other factors such as differences in dietary intake or intraluminal esterification could play a role. Therefore, and especially for gut metabolites, a careful interpretation in terms of cause and effect is warranted.

Another limitation of the metabolomics approach is the influence of the multiple choices during the metabolomics pipeline. As there exists no single analytical method to detect all metabolites, choices in sample preparation, instrumental conditions, solvent composition etc. will unavoidably influence the obtained results. Our analytical method was optimized to maximize metabolite coverage while considering linearity, repeatability, reproducibility, instrumental precision, recovery and detection capability (Vanden Bussche et al., 2015; De Paepe et al., 2018). However, the method of choice is not always the best option for some individual metabolites. As it is not possible to apply every possible analytical method to detect as many metabolites as possible, it is likely that some important metabolites are missed. Furthermore, also in the data processing and statistical analysis, multiple well-considered but arbitrary choices are made that lead to the best assessment of the majority of metabolites, but not of all individual metabolites.

6.4 Future perspectives

6.4.1 Further exploration of present findings

The potential involvement of lipid oxidation products in the relation between red and/or processed meat consumption and chronic diseases has been suggested many years ago in multiple studies and reviews (Bastide et al., 2011; Santarelli et al., 2008; Sesink et al., 1999). Multiple older studies in this research field mainly focussed on MDA, mostly measured as TBARS, as a primary marker for lipid oxidation. However, the diverse health implications of specific lipid oxidation products, the different origin of these oxidation products, and the importance of their concentration, urge a more specific evaluation of different lipid oxidation products.

Protein damage as a result of lipid oxidation products has always been acknowledged and the association between *in vivo* protein oxidation and age-related diseases is well established (Berlett & Stadtman, 1997; Liguori et al., 2018; Santos & Lindner, 2017). Nevertheless, the role of dietary protein oxidation products as red meat-

associated mechanisms contributing to increased chronic disease risk remains largely unexplored (Davies, 2003; Estévez & Luna, 2017; Santos & Lindner, 2017). The evolution of protein oxidation from 'poor cousin' to hot topic was outlined in the review of Estévez & Luna (2016), and the more recent review of Estévez and Xiong (2019) describes the current scientific evidence and hypotheses regarding the association between the dietary intake of oxidized proteins, oxidative stress and diseases. The present results also endorse more research in this direction since protein carbonyls, and not lipid oxidation products, were mostly higher in the *in vitro* digests of the processed meat products compared to the reference pork in Chapter V and because of the highest level of protein carbonyls found in the digests of the pigs fed the red&processed-Western diet in Chapter II. As discussed in these chapters, lipid oxidation could have contributed to the total amount of protein carbonyls, but also other mechanisms including Maillard-mediated formation and different actions of antioxidants on lipids versus proteins could be involved. Next to the frequently used DNPH method and other indicators of protein oxidation such as tryptophan degradation, fluorescent oxidation products, free thiol content, AAS and GGS, current developments in proteomic methodologies investigating post-translation modifications of proteins offer great opportunities in this research field (Domingues et al., 2013; Krisko & Radman, 2019; Mitra et al., 2018; Spickett & Pitt, 2019; Verrastro et al., 2015).

As mentioned under sections 6.1.2 and 6.1.3, the causative mechanisms related to red meat intake, carnitine-derived metabolites (3-dehydroxycarnitine and acylcarnitines) and the association with chronic diseases should be further investigated.

This study assessed one of multiple possibilities to evaluate the impact of the background diet. As discussed under sections 6.2 and 6.3, feeding normal meals with whole foods, instead of a mixture of many ingredients could have influenced the results. The Western background diet in the pig feeding trial reflected the average intake of the Belgian population and is therefore not an example of an extreme dietary background. Since even small amounts of antioxidants can inhibit the formation of oxidation products, the smaller fraction of fruits and vegetables in the Western background diet could have been adequate to attenuate adverse effects of a Western background diet. Likely, not every meal in an unhealthy dietary pattern contains protective compounds. Therefore, it would be interesting to assess the effects of a more extreme background diet without any fruits or vegetables, or to assess the impact of several extreme meals. Also, the comparison between a prudent and a Western diet either or not in combination with moderate amounts of meat would be an interesting set-up. It can be suspected that in some extreme diets, meat is one of the food items with the highest nutritional value, and that elimination of meat in these diets would result in adverse instead of beneficial health effects.

No significant effects of the dietary treatments were detected in the short-term SRB and GSH/GSSG assays in Caco-2 cells incubated with the colon supernatants in the preliminary cell culture experiments. However, multiple other tests and cell culture techniques could be applied in this research field. For example, a prolonged exposure time or other cell lines could be tested. Furthermore, instead of using colon supernatant samples, isolated compounds, such as the carnitine-derived metabolites (Chapter III) could be used. The effect of the digests or isolated compounds on molecular pathways could be tested by targeted molecular assays such as Western blotting or immunocytochemistry. Also the influence of the digestion samples or isolated compounds on epithelial barrier integrity by transepithelial electrical resistance measurements would be interesting to investigate.

6.4.2 Other potential red meat-associated mechanisms that were not addressed in the present thesis

Several other explanatory hypotheses involving the mammalian cell surface sialic acid Neu5Gc (Samraj et al., 2015), tryptophan catabolism (Rombouts et al., 2017) and NOCs (Mirvish et al., 2003) were not addressed. Humans lack the enzyme to catalyse the conversion of sialic acid N-acetylneuraminic acid (Neu5Ac) into Neu5Gc. However, Neu5Gc accumulates in the human body upon consumption of Neu5Gc-containing foods such as red meat and bovine milk (Byres et al., 2008). The low abundance or absence of this sialic acid in poultry and fish, and the production of anti-Neu5Gc antibodies leading to chronic inflammation suggest a role for this compound in the link between red meat consumption and the increased chronic disease risk. However, dairy products have not been linked with colorectal cancer, indicating that Neu5Gc as such is not the only potential cause (Demeyer et al., 2016).

A previous untargeted metabolomics study identified several oxidation products of tryptophan and derivatives including N'-formylkynurenine, kynurenine and kynurenic acid in the colonic digests following *in vitro* digestion of red compared to chicken meat (Rombouts et al., 2017). This finding was promising, since kynurenine metabolites were described to participate in the pathogenesis of numerous diseases including neurodegenerative disorders, cancer and cardiovascular diseases (Chen & Guillemin, 2009; Dehhaghi et al., 2019). Differences between the *in vitro* digestion in the study of Rombouts et al. (2017) and the pig feeding trial in this PhD thesis such as the influence of the background diets, absorption processes and the differences in microbial composition could contribute to the absence of differences in these metabolites in the pig feeding trial. Although these kynurenine metabolites were not identified in the present study, more knowledge concerning the formation and impact of these metabolites is warranted.

In addition to the catalysing effect of heme-Fe on the formation of lipid oxidation products, adverse health effects have been ascribed to heme-Fe through the stimulating role of the latter in the endogenous formation of genotoxic NOCs (Demeyer et al., 2016; Kuhnle & Bingham, 2007). NOCs can be analysed by the measurement of apparent total N-nitroso compounds (ATNC) (Mirvish et al., 2003), but in this method, no distinction is made between the toxic N-nitroso compounds and the non-toxic compounds such as S-nitrosothiols, O-nitroso compounds and nitrosyl-Fe (Hogg, 2007). Another approach to determine NOCs is the quantification of NOC-derived DNA-adducts such as O^6-methylguanine DNA adducts (Lewin et al., 2006; Van Hecke et al., 2014b; Vanden Bussche et al., 2014). The influence of meat characteristics, background diet and microbiome on NOC formation should be further investigated. However, the hypothesis of Corpet et al. (2011), presuming that nitrite-curing would enhance the formation of NOCs and consequently contribute to the higher relative risk for processed red meat compared to red meat was not supported by later studies (Van Hecke et al., 2014a; 2014b; Vanden Bussche et al., 2014). Moreover, nitrite is physiologically present in saliva and the levels of residual nitrite in meat products are negligible compared to the levels of nitrate/nitrite intake through vegetables (Temme et al., 2011).

6.4.3 Potential of metabolomics and multi-omics approaches

Metabolomic profiling of intestinal digest samples is very interesting to investigate the reciprocal interactions between diet, microbiome and the metabolic phenotype. However, insight in the origin and kinetics of the differentiating metabolites in Chapter III and IV is limited. A multi-matrix approach including blood, urine, intestinal and tissue samples would aid to reveal more insights in metabolite distribution, transformation, absorption and clearance effects. In addition, rapid developments in bioinformatics applications such as AMON (Annotation of Metabolite Origins via Networks) that integrates microbiome and metabolome data are very promising to increase the understanding of which metabolites in the metabolome could have been produced by the host or by microbial enzymes. The use of metabolic networks such as the KEGG database enables to capture the relationship between the genomes of the host and microbiome and the metabolites present in the sample (Shaffer et al., 2019). However, since many pathways and metabolic networks, and especially microbial metabolic processes are unknown or not fully known, the outcome depends on the knowledge on the metabolic networks and on the quality of the input data. For example, in the case study of Shaffer et al. (2019), in which the gut microbiome (16S rRNA) and blood metabolome (LC-MS) of HIV positive individuals and controls were correlated, only 471 out of 5971 compounds of the LC-MS data could be linked to enzymatic reactions in KEGG (Shaffer et al., 2019). In KEGG, multiple species are reduced to a single compound. Although more than 1000

phosphatidylcholine species exist, the glycerophospholipid pathway in KEGG includes only one single phosphatidylcholine lipid molecule. Since the number of acylcarnitine species in KEGG pathways is also limited and because these metabolites are among the most abundant differentiating metabolites found in Chapter III, improvements in KEGG could enhance the interpretation of the alterations in the acylcarnitine profile. Furthermore, AMON does not account for co-metabolism between host and microbiome and could not indicate that the production of TMAO depends on both the host and the microbiome (Shaffer et al., 2019). In addition to fundamental experimental research, the development of computationally derived genome-scale metabolic models highly contributes to move forward in the understanding of the metabolic capacity of the gut microbiome (Gu et al., 2019; Sen & Orešič, 2019), although many challenges are still present in this field. It should be emphasized that untargeted omics approaches and computational modelling are great exploratory tools, but targeted follow-up studies and final validation of the results are needed.

Another interesting perspective of metabolomics in nutrition science is its potential to identify metabolic phenotypes (metabotypes) and to develop personalized nutrition strategies (Tebani & Bekri, 2019). Currently, nutritional guidelines provide general population-based nutritional advice, with some specific guidelines related to age or gender. However, since the response to dietary interventions shows large inter-individual variation, it can be hypothesized that metabotype-specific dietary advice could largely improve disease prevention for people at risk. The selection of metabotypes could be based on multiple determinants including dietary intake, gut microbiota phenotypes (enterotypes), anthropometric, physiological, genetic and behavioral factors (Palmnäs et al., 2019). Since the metabolic profile provides a snapshot of the interaction of diet, host and microbiome, the use of metabolomics is very promising to discover different metabotypes and to identify corresponding biomarkers. Next to metabolomics, the integration of other omics technologies would increase the knowledge on the health status and its relationship with dietary intake. Nevertheless, in addition to many technical challenges, the willingness to adhere to dietary recommendations and the feasibility and cost-effectiveness of extensive metabotyping in preventing diseases will need to be determined (Lampe et al., 2013).

SUMMARY

Over the last decades, several epidemiological studies have reported that a high consumption of red and especially processed meat increases the risk to develop various chronic diseases such as colorectal cancer, cardiovascular diseases and diabetes. In contrast, no such associations have ever been reported for the consumption of white meat. Unfortunately, most of these studies only differentiate between red and processed meat, whereas these food groups both cover a wide range of meats, originating from different animal species and subjected to different processing procedures, that might induce differential health effects. Several potential factors that could be involved in the association between high red and processed meat consumption and colorectal cancer have been proposed. However, a complete understanding is still lacking, since many of these factors are not specific to red meat or cannot explain the higher epidemiological risk of processed meat compared to fresh red meat. Furthermore, there is probably not one single factor responsible for each of the associations, and a reciprocal interaction between the whole dietary pattern, microbiome and (epi)genetic factors is likely involved. More insight in the underlying mechanisms through mechanistic studies is of paramount importance in order to support a causal role of meat consumption in the etiology of chronic diseases, and to develop strategies to improve the composition and/or processing of meat.

Meat is mostly consumed within a meal, which contains many components that could interact with each other. Indeed, previous studies have demonstrated that the cumulative and interactive effects of multiple components in the diet may attenuate or exert stronger effects than the sum of the single components, and more consistent results are obtained when focusing on dietary patterns instead of single nutrients or foods in nutritional epidemiology. Therefore, in addition to the traditional nutrient approach, the complex relationship between diet and chronic diseases should be investigated at different levels, preferably using a 'top down' approach. In this 'top down' approach, the relationship between dietary patterns and health outcomes is first investigated, followed by the study of smaller units such as combinations of foods, food components, or nutrients to isolate the true causative agents and to improve the mechanistic understanding of the observed health outcomes. Typically, diets associated with a reduced risk of various chronic diseases, frequently called prudent diets, have several common elements such as high intake of fruits, vegetables, whole grains and nuts, and lower intake of red and processed meats, sugar-sweetened foods and drinks and refined grains. In contrast, a Western dietary pattern, frequently characterized by high consumption of fats and sweets, sugar-containing beverages, refined cereals, confectionaries and red and processed meat has been associated with higher risks to develop chronic diseases such as cardiovascular and metabolic diseases and colorectal cancer.

In this PhD thesis, the impact of meat consumption was evaluated in the context of a prudent and a Western dietary pattern. For this purpose, both a targeted and an untargeted approach was applied. The targeted approach (Chapter II and Chapter V) primarily focused on the role of meat consumption in the formation of cyto- and genotoxic oxidation products during digestion and postprandial oxidative stress, which is one of the most advanced hypotheses in the red meat-colorectal cancer association and also involved in other red meat-associated chronic diseases. Untargeted metabolomic approaches (Chapter III) and 16S rRNA gene sequencing (Chapter IV) were applied to investigate alterations in the gut metabolome and microbiome as a result of meat consumption in a complex dietary context to generate new insights and to confirm previous suggested mechanisms.

In **Chapter I**, first, the conceptual framework and aim of this work were formulated. As general introduction, the historical perspective of dietary patterns and nutrition research was briefly outlined. Next, both the importance of meat as nutritional source and the epidemiological associations with chronic diseases were summarized. The mechanism of oxidation in meat and during meat digestion, as one of the potential underlying mechanisms responsible for these associations, was explained. Furthermore, the basic principles of metabolomics and the use of this tool in nutrition research and meat biomarker discovery were described. Finally, different levels of nutrition research, including the introduction of *in vitro* models and the use of pigs as experimental animals in nutrition research, were briefly described.

A large part of this PhD thesis **(Chapter II, III and IV)**, resulted from an extensive pig feeding trial that was conducted as a digestion model for humans. A 2×2 factorial design with chicken meat versus a mixture of red and processed meat on the one hand, and a prudent and Western background diet on the other hand, was used the investigate the effects of meat consumption in a complex dietary context. After the four-week experimental period, the pigs (n = 8 per diet) were euthanized and blood, organ tissue and gastrointestinal digest samples were collected. This interesting range of samples was used to study the oxidative status of the pigs (Chapter II), to elucidate differences in the digestion metabolome (Chapter III) and to investigate differences in the microbiome and in colonic volatile organic compounds (Chapter IV).

In **Chapter II**, lipid oxidation products (malondialdehyde, 4-hydroxy-2-nonenal and hexanal) were higher in the chicken versus red & processed meat diets (1.7- to 8.3-fold) and subsequent *in vitro* (1.3- to 1.9-fold) and *in vivo* (1.4 to 3-fold) digests. These results were in contrast with the hypothesis, which was presumably related to the higher polyunsaturated fatty acid content in chicken meat and/or the added antioxidants in processed meat. Nevertheless, the dietary treatments (either as a result of meat type, background diet, or their interaction) only had a marginal or no effect on the systemic oxidative status as determined by plasma oxygen radical

absorbance capacity, malondialdehyde, glutathione and glutathione peroxidase activity in blood and organs, except for α-tocopherol, which was higher after the consumption of the chicken-Western diet.

In **Chapter III**, the alterations in the digestion metabolome of the small intestine and the colon content of the pigs were investigated by an untargeted UHPLC-HRMS-based polar metabolomics platform. Seventy-six unique metabolites (38 in small intestine, 32 in colon, and 6 in both intestinal compartments) responsible for the distinct gut metabolic profiles of pigs fed either chicken or red and processed meat were (tentatively) identified. Consumption of red and processed meat resulted in higher levels of short- and medium-chain acylcarnitines and 3-dehydroxycarnitine, irrespective of dietary context, whereas long-chain acylcarnitines and monoacylglycerols were specifically associated with the red&processed-Western diet. Several of these metabolites have been linked with pathophysiological effects and chronic diseases in literature, however, the causal link between the altered metabolites upon meat consumption and disease pathophysiology remains to be investigated.

In **Chapter IV**, the colon microbial community and volatile organic compounds were assessed (either quantified or based on their presence). Results show that *Lactobacilli* were characteristic for the chicken-prudent dietary pattern treatment and *Paraprevotella* for the red&processed-prudent dietary pattern treatment. Enterobacteriaceae and *Desulfovibrio* were characteristic for the chicken-Western dietary pattern treatment and *Butyrivibrio* for the red&processed-Western dietary pattern treatment. *Campylobacter* was characteristic for chicken, and *Clostridium XIVa* for red and processed meat consumption, irrespective of the dietary pattern. Ethyl pentanoate and 1-(methylthio)-propane were observed more frequently in pigs fed red and processed meat compared to chicken meat. The prevalence of 3-methylbutanal was >80% for pigs receiving a Western dietary pattern, whereas for pigs fed a prudent dietary pattern the prevalence was <35%. The concentration of butyrate was significantly higher when the prudent dietary pattern was given, compared to the Western dietary pattern, but no differences for other short chain fatty acids or protein fermentation products were observed.

In Chapter II, the question was raised if the lower concentrations of lipid oxidation products in the digests of red and processed meat compared to chicken meat could be related to the considerable proportion of luncheon meat products in these diets. Therefore, in the first experiment in **Chapter V**, the concentrations of lipid and protein oxidation products after *in vitro* gastrointestinal digestion of a wide range of ready-to-eat luncheon meat products (n = 24) was investigated. In a second experiment, oxidation products were evaluated in the digests of five different deep-fried meat products that were fried in either tallow or sunflower oil, which were used

either as a fresh or reused frying medium. Compared to unprocessed cooked pork mince, which was included as a reference in both experiments, levels of lipid oxidation products were low in the digests of most meat products. Only the digests of Parma ham had slightly higher or comparable levels as the reference pork. In contrast, the formation of protein carbonyl compounds was comparable or up to 6 times higher in the digests of the meat products compared to the reference pork. Although some differences in oxidation between the digests of the 5 types of deep-fried meat products and some small effects related to the type of frying oil and number of frying cycles were observed, these effects were limited compared to the differences between digests of the meat products and the reference pork.

In **Chapter VI,** the main findings of the different experimental chapters were integrated and discussed in the light of the current knowledge in literature. Regarding the oxidation products formed during meat digestion, it can be hypothesized that the presence of antioxidants in meat or its dietary context is probably the predominant factor in the final concentration of lipid oxidation products. Next, the combination and the synergistic effect of the heme-Fe content and the fatty acid profile are likely more important than the fatty acid profile or the heme-Fe content as such. On the other hand, the observation that protein oxidation products were frequently higher in the digests after processed meat digestion advocates more research on the effect of meat consumption on different protein oxidation products, and the resulting health impact. The general impact of the four dietary treatments on the gut metabolome and microbiome were discussed as well as the carnitine related metabolites upon red meat consumption, which was one of the most pronounced outcomes of the untargeted metabolomics analysis. In this chapter, also strengths and limitations of the present thesis and future perspectives of this research area were outlined.

SAMENVATTING

In de afgelopen decennia hebben verschillende epidemiologische onderzoeken gerapporteerd dat een hoge consumptie van rood en vooral verwerkt vlees het risico op het ontwikkelen van chronische ziekten zoals colorectale kanker, hart- en vaatziekten en diabetes verhoogt. Voor de consumptie van wit vlees daarentegen, zijn dergelijke associaties niet bekend. De meeste van deze onderzoeken maken enkel een onderscheid tussen rood en verwerkt vlees. Nochtans omvatten deze voedselgroepen een breed scala aan vlees, afkomstig van verschillende diersoorten en onderworpen aan verschillende verwerkingsprocessen, die verschillende gezondheidseffecten kunnen veroorzaken. Verschillende factoren die een rol kunnen spelen bij de associatie tussen een hoge consumptie van rood en bewerkt vlees en colorectale kanker werden reeds geformuleerd. De exacte mechanismen zijn echter nog niet volledig opgehelderd, aangezien veel van deze factoren niet specifiek zijn voor rood vlees of het hogere epidemiologische risico of verwerkt vlees in vergelijking met vers rood vlees niet kunnen verklaren. Bovendien is er waarschijnlijk niet één enkele factor verantwoordelijk voor elk van de associaties en is er waarschijnlijk sprake van een wederzijdse interactie tussen het gehele voedingspatroon, microbioom en (epi)genetische factoren. Meer inzicht in de onderliggende mechanismen is van groot belang om een oorzakelijke rol van vleesconsumptie in de etiologie van chronische ziekten te ondersteunen en strategieën te ontwikkelen om de samenstelling en/of verwerking van vlees te verbeteren.

Vlees wordt meestal geconsumeerd als onderdeel van een maaltijd waarin verschillende componenten met elkaar kunnen interageren. Eerdere studies hebben aangetoond dat de cumulatieve en interactie-effecten van meerdere componenten in het dieet bepaalde individuele effecten kunnen verzwakken of versterken. Bovendien worden consistentere resultaten verkregen wanneer wordt gefocust op voedingspatronen in plaats van op afzonderlijke nutriënten of voedingsmiddelen in epidemiologische studies. Daarom wordt aangeraden om voedingsonderzoek, en voornamelijk het onderzoek naar de complexe relatie tussen voeding en chronische ziekten, op verschillende niveaus uit te voeren, bij voorkeur volgens een ‘top down’ benadering. In deze 'top down' benadering wordt eerst de relatie tussen voedingspatronen en gezondheidseffecten onderzocht, gevolgd door de studie van kleinere eenheden zoals combinaties van voedingsmiddelen of geïsoleerde componenten om de oorzakelijke verbanden te onderzoeken en de achterliggende mechanismen van de waargenomen gezondheidseffecten beter te begrijpen. Voedingspatronen geassocieerd met een verminderd risico op verschillende chronische ziekten, hier benoemd als ‘gezonde’ voedingspatronen, hebben verschillende gemeenschappelijke elementen, zoals een hoge inname van fruit,

groenten, volkoren graanproducten en noten, en een lagere inname van suikers, vetten en rood en verwerkt vlees. Een Westers voedingspatroon daarentegen, vaak gekenmerkt door een hoge consumptie van vetten, suikers, geraffineerde granen, en rood en bewerkt vlees, werd vaker geassocieerd met hogere risico's voor het ontwikkelen van chronische ziekten zoals cardiovasculaire en metabole ziekten en colorectale kanker.

In deze thesis werd de impact van vleesconsumptie geëvalueerd in de context van een gezond en een Westers voedingspatroon, zowel volgens een doelgerichte ('targeted') en een niet-doelgerichte ('untargeted') benadering. De doelgerichte benadering (Hoofdstuk II en Hoofdstuk V) was voornamelijk gefocust op de rol van vleesconsumptie bij de vorming van cyto- en genotoxische oxidatieproducten tijdens vertering en postprandiale oxidatieve stress, wat een van de meest geavanceerde hypothesen is voor de associatie tussen rood vleesconsumptie en colorectale kanker. Bovendien is oxidatieve stress ook betrokken bij andere met rood vlees-geassocieerde chronische aandoeningen. Een niet-doelgericht metabolomics platform (Hoofdstuk III) en 16S rRNA-gen sequencing (Hoofdstuk IV) werden toegepast om veranderingen in het metabolisch profiel van de darm en het microbioom als gevolg van vleesconsumptie in een complexe voedingscontext te onderzoeken. Deze niet-doelgerichte benaderingen hebben de mogelijkheid om nieuwe inzichten te genereren en eerder voorgestelde mechanismen te bevestigen.

In **Hoofdstuk I** werden eerst het conceptuele kader en het doel van dit werk geformuleerd. In de algemene inleiding werd de geschiedenis van voedingspatronen en voedingsonderzoek kort beschreven. Vervolgens werden zowel het belang van vlees als voedingsbron als de epidemiologische associaties met chronische ziekten samengevat. Het mechanisme van oxidatie in vlees en tijdens vleesvertering, als een van de mogelijke onderliggende mechanismen die verantwoordelijk zijn voor deze associaties, werd toegelicht. Verder werden de basisprincipes van metabolomics en het gebruik van deze tool in voedingsonderzoek en in de ontdekking van vlees biomerkers beschreven. Tenslotte werden verschillende niveaus van voedingsonderzoek, waaronder de introductie van *in vitro* modellen en het gebruik van varkens als proefdieren in voedingsonderzoek, kort beschreven.

Een groot onderdeel van deze thesis **(Hoofdstuk II, III en IV)** is het resultaat van een uitgebreide varkensproef die werd uitgevoerd als verteringsmodel voor de mens. Een 2 × 2 experimenteel design met enerzijds kip of een mengsel van rood en verwerkt vlees in combinatie met een gezond of Westers achtergronddieet anderzijds, werd toegepast om de effecten van vleesconsumptie in een complexe context te onderzoeken. Na vier weken voederen met deze experimentele diëten werden de varkens (n = 8 per behandeling) geëuthanaseerd en werden stalen van bloed, orgaanweefsel en het gastrointestinaal stelsel verzameld. Deze stalen werden gebruikt om de oxidatieve status van de varkens te bestuderen (Hoofdstuk II), om

verschillen in het metabolisch profiel van de darminhoud op te helderen (Hoofdstuk III) en om verschillen in de microbiële samenstelling en verschillen in vluchtige organische componenten in de coloninhoud te onderzoeken (Hoofdstuk IV).

Hoofdstuk II – Vetoxidatieproducten (malondialdehyde, 4-hydroxy-2-nonenal en hexanal) waren 1,7 tot 8,3 keer hoger in de voeders die kip bevatten in vergelijking met de voeders die rood en bewerkte vlees bevatten. Eenzelfde trend werd ook teruggevonden in de digesten na *in vitro* (1,3- tot 1,9 keer hoger) en *in vivo* (1,4 tot 3 keer hoger) vertering. Deze resultaten stonden in contrast met de hypothese, en was vermoedelijk gerelateerd aan het hogere gehalte meervoudig onverzadigde vetzuren in kip en/of de toegevoegde antioxidanten in verwerkt vlees. Ondanks de verschillen in vetoxidatieproducten in de digesten hadden de verschillende diëten (hetzij als gevolg van het vleestype, het achtergronddieet of hun interactie) slechts een beperkt of geen effect op de systemische oxidatieve status. Deze werd onder meer bepaald door analyse van de antioxidant capaciteit van plasma, malondialdehyde en glutathion concentraties en de activiteit van glutathionperoxidase in bloed en organen. Enkel de concentratie α-tocoferol in de organen werd beïnvloed door de verschillende diëten en was hoger na de consumptie van kip in combinatie met het Westers achtergronddieet.

In **Hoofdstuk III** werden de veranderingen in het metabolisch profiel van de dunne darm en coloninhoud van de varkens onderzocht door een niet-doelgericht UHPLC-HRMS-gebaseerd polair metabolomics platform. Zesenzeventig unieke metabolieten (38 in de dunne darm, 32 in de dikke darm en 6 in beide darmcompartimenten), verantwoordelijk voor de verschillende metabolische profielen na consumptie van kip of rood en verwerkt vlees, werden (met een bepaalde zekerheid) geïdentificeerd. Consumptie van rood en verwerkt vlees resulteerde in hogere concentraties korte keten en middellange keten acylcarnitines en 3-dehydroxycarnitine, onafhankelijk van het achtergronddieet, terwijl lange keten acylcarnitines en monoacylglycerolen specifiek geassocieerd waren met de consumptie van rood en bewerkt vlees in combinatie met het Westers achtergronddieet. Verschillende van deze metabolieten werden in verband gebracht met pathofysiologische effecten en chronische ziekten in de literatuur, maar het oorzakelijk verband tussen deze metabolieten na vleesconsumptie en chronische ziekten moet nog worden onderzocht.

In **Hoofdstuk IV** werden de microbiële samenstelling en vluchtige organische stoffen in het colon onderzocht (gekwantificeerd of op basis van hun aanwezigheid). Resultaten tonen aan dat *Lactobacilli* kenmerkend waren voor de consumptie van kip in combinatie met het gezond achtergronddieet en *Paraprevotella* voor de consumptie van rood en verwerkt vlees in combinatie met het gezond achtergronddieet. Enterobacteriaceae en *Desulfovibrio* waren kenmerkend voor de consumptie van kip in combinatie met het Westers achtergronddieet en *Butyrivibrio*

voor de consumptie van rood en bewerkt vlees in combinatie met het Westers achtergronddieet. Campylobacter was kenmerkend voor consumptie van kip en *Clostridium XIVa* voor consumptie van rood en verwerkt vlees, ongeacht het achtergronddieet. Ethyl pentanoaat en 1-(methylthio)-propaan werden vaker waargenomen bij varkens die rood en verwerkt vlees kregen dan kippenvlees. De prevalentie van 3-methylbutanal was > 80% voor varkens met een Westers voedingspatroon, terwijl voor varkens met een gezond voedingspatroon de prevalentie <35% was. De concentratie boterzuur was significant hoger wanneer het gezonde voedingspatroon werd gegeven, vergeleken met het Westerse voedingspatroon, maar er werden geen verschillen waargenomen voor andere korteketenvetzuren of eiwitfermentatieproducten.

In Hoofdstuk II werd de vraag gesteld of de lagere concentraties vetoxidatieproducten in de digesten van rood en verwerkt vlees vergeleken met kip gerelateerd konden zijn met het aanzienlijke aandeel vleesproducten in deze diëten. Daarom werd in het eerste experiment in **Hoofdstuk V** de concentratie aan vet- en eiwitoxidatieproducten na *in vitro* vertering van een breed scala kant-en-klare vleesproducten (n = 24) onderzocht. In een tweede experiment werden oxidatieproducten geëvalueerd in de digesten van vijf verschillende gefrituurde vleesproducten die werden gebakken in vers of reeds gebruikt dierlijk vet of zonnebloemolie. De gehaltes aan vetoxidatieproducten in de digesten na vertering van de meeste vleesproducten waren laag, in vergelijking met de digesten van gekookt varkensgehakt, dat gebruikt werd als referentie in beide experimenten. Alleen de digesten van Parmaham hadden iets hogere of vergelijkbare gehaltes als het referentievarkensvlees. De concentraties aan eiwitoxidatieproducten daarentegen waren vergelijkbaar of tot 6 keer hoger in de digesten van de vleesproducten in vergelijking met het referentievarkensvlees. Hoewel enkele verschillen in oxidatie tussen de digesten van de 5 soorten gefrituurde vleesproducten en enkele beperkte effecten als gevolg van het type frituurolie en het aantal frituurcycli werden waargenomen, waren deze effecten beperkt in vergelijking met de verschillen tussen de digesten van de vleesproducten en het referentievarkensvlees.

In **Hoofdstuk VI** werden de belangrijkste bevindingen van de experimentele hoofdstukken geïntegreerd en gekaderd binnen de huidige kennis in de literatuur. Waarschijnlijk zijn de aanwezigheid van antioxidanten in vlees, of in de maaltijd, de belangrijkste factor in de finale concentratie aan vetoxidatieproducten na vertering. Vervolgens zijn de combinatie en het synergetische effect van het heem-Fe gehalte van vlees en het vetzuurprofiel waarschijnlijk belangrijker dan het heem-Fe gehalte of het vetzuurprofiel als zodanig. De vaststelling dat hogere concentraties aan eiwitoxidatieproducten werden gevormd na de vertering van verwerkt vlees motiveert om het effect van vleesconsumptie op verscheidene

eiwitoxidatieproducten en de daaruit voortvloeiende gezondheidseffecten beter te onderzoeken. De algemene impact van de vier experimentele diëten op het metabolisch profiel en de microbiële samenstelling van de darminhoud werd beschreven, evenals de carnitine-gerelateerde metabolieten op de consumptie van rood vlees, wat een van de meest uitgesproken resultaten was van het niet-doelgerichte metabolomics platform. In dit hoofdstuk werden ook de sterke punten en beperkingen van de thesis en enkele toekomstperspectieven in dit onderzoeksgebied beschreven.

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2010-2013 **BSc in Bioscience Engineering: Chemistry and Food Technology**
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Scientific Output

A1 Publications

Goethals, S., Van Hecke, T. Vossen, E., Vanhaecke, L., Van Camp, J., De Smet, S. (submitted to Food Research International). Commercial luncheon meat products and their *in vitro* gastrointestinal digests contain more protein- but less lipid- oxidation products compared to fresh pork.

Goethals, S., Rombouts C., Hemeryck L. Y., Van Meulebroek L., Van Hecke T., Vossen E., Van Camp J., De Smet S., Vanhaecke L. (2020). Untargeted metabolomics to reveal red versus white meat-associated gut metabolites in a prudent and Western dietary context. *Molecular Nutrition & Food Research,* doi: 10.1002/mnfr.202000070

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Oral and poster presentations

Goethals, S., Van Hecke, T., Vossen, E., Opdenacker, K., De Smet, S. 'Lipid and protein oxidation during *in vitro* digestion of deep-fried meat products' – BAMST Symposium 'Meat the Belgian Meat Researchers', Melle, Belgium, December 5, 2019 – oral presentation

Goethals, S., Rombouts, C., Hemeryck, L.H., Van Meulebroek, L., Van Hecke, T., Vossen, E., Van Camp, J., De Smet, S., Vanhaecke, L. 'Untargeted metabolomics to reveal red versus white meat-associated gut metabolites in a prudent and Western dietary context' – Metabolomics, June, 23-27, 2019, The Hague, The Netherlands – e-poster presentation

Goethals, S., Van Hecke, T., Vossen, E., Vanhaecke, L., Van Camp, J., De Smet, S. 'Lipid and protein oxidation during *in vitro* digestion of different types of luncheon meat' – 6th International Conference of Food Digestion, Granada, Spain, April, 2-4, 2019 – poster presentation

Goethals, S., Vossen, E., Van Hecke, T., Rombouts, C., Hemeryck L.Y., Van Camp, J., Vanhaecke, L., De Smet, S. 'Impact of red versus white meat consumption in a prudent or Western dietary pattern on the oxidative status in a pig model' – Masstrplan conference: Advances in the Study of Lipid and Protein Oxidation: From Methods to Targets, Ghent, Belgium, March, 13-15, 2019 – oral and poster presentation

Goethals, S., Rombouts, C., Hemeryck, L.H., Van Meulebroek, L., Van Hecke, T., Vossen, E., Van Camp, J., De Smet, S., Vanhaecke, L. 'Formation of metabolites during *in vitro* and *in vivo* digestion of red and processed meat' – BAMST Symposium 'Meat the Belgian Meat Researchers', Melle, Belgium, December, 6, 2018 – oral presentation

Goethals, S., Rombouts, C., Hemeryck, L.H., Vanderbeke, A., Van Hecke, T., Vossen, E., Van Camp, J., De Smet, S., Vanhaecke, L. 'Metabolites in digestion fluids of pigs fed human diets : red versus white meat in the context of a prudent and western diet' – Benelux Metabolomics Days, Rotterdam, The Netherlands, September, 19-20, 2018 – poster presentation

Goethals, S., Vossen, E., Van Hecke, T., De Smet, S. 'The effect of meat consumption on the liver antioxidant status in pigs is modulated by the food matrix'– BAMST Symposium 'Meat the Belgian Meat Researchers', Melle, Belgium, December, 8, 2016 – oral presentation

DANKWOORD

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