Assessment of carnosine functional properties during in vitro digestion of meat differing in composition and cooking conditions

Yiyao Li

Department of Agricultural and Environmental Sciences

McGill University, Montreal

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Abstract

Meat can provide important nutrients, such as minerals and vitamins, but its consumption has been associated with increased risk of developing chronic diseases such as colorectal cancer and type 2 diabetes mellitus. At the molecular level, the formation of lipid and protein oxidation products including that of advanced glycation end products (AGEs) and other adverse compounds in meat and also potentially in the gastrointestinal tract which represents a pro-oxidative environment may contribute to this risk. The gastrointestinal tract also represents a pro-oxidative environment. Carnosine (β-alanyl-L-histidine), a dipeptide in meat with multiple properties including anti-oxidant capacity, carbonyl scavenging, and inhibition of AGEs formation, has potential to counteract the assumptive disadvantages of meat consumption. In order to determine the health beneficial properties of dietary carnosine during digestion, this study has taken into account the main factors with pro-oxidant potential susceptible to contribute to the formation of such adverse products. Therefore, meat samples prepared with two levels of carnosine, two levels of fat and cooked under two cooking intensities in a factorial arrangement were submitted to an in vitro digestion system mimicking the salivary, gastric and duodenal phases. Carnosine level and markers of lipid (hexanal, 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA)) and protein (protein carbonyls and thiols) oxidation along with the formation of N(epsilon)-(carboxymethyl)lysine (CML) as an AGEs marker were determined in each phase.

Markers of lipid and protein oxidation including AGEs formation generally increased during digestion. Enhanced fat level and cooking intensity, and in some case their interaction, also generally increased ($P<0.05$) lipid and protein oxidation markers including AGEs to different extents depending on the digestive phase. In counterpart, a decrease in carnosine level was observed throughout the different phases of digestion, but carnosine remained higher in the carnosine enhanced groups ($P<0.0001$). Results indicated that oxidized lipid and protein along with CML formation were significantly reduced ($P<0.05$) in most groups containing higher carnosine level in meat. These results show the potential of carnosine to counteract the formation of adverse compounds that have been associated with disadvantages of meat consumption and point toward the

importance of diet composition and preparation in order to reduce the formation of adverse compounds, increasing this way the bioavailability of carnosine for potential additional benefits upon absorption.

Sommaire

La viande, une excellente source de protéines fournit aussi d'importants nutriments tels les minéraux et les vitamines mais sa consommation est associée à un risque accru de développement de maladies chroniques telles, le cancer colorectal et le diabète mellitus. La formation dans la viande mais possiblement aussi dans le système digestif de composés d'oxydation des lipides et protéines incluant aussi celle des produits de glycation avancée (PGA) pourrait contribuer à ce risque accru. La carnosine (β-alanyl-L-histidine), un dipeptide intrinsèque du muscle possédant de multiples propriétés incluant un pouvoir antioxydant, la capacité de trapper les carbonyls et d'inhiber la formation de PGA pourrait contrer les désavantages présumés associés à la consommation de la viande. Afin de déterminer les avantages santé de la carnosine alimentaire durant la digestion, nous avons évalué les principaux facteurs pro-oxydants de la viande fraîche susceptibles de contribuer à la formation de ces produits délétères pour la santé. Des échantillons de viande de porc contenant deux niveaux de carnosine (intrinsèque et ajouté), deux niveaux de gras (intrinsèque et ajouté) et soumis à deux intensité de cuisson (faible et haute) suivant un arrangement factoriel, furent soumis à une digestion in vitro imitant les conditions buccales, stomacales et duodenales. Le niveau de carnosine et les marqueurs d'oxydation des lipides (hexanal, 4-hydroxynonenal (4-HNE), and MDA) et des protéines (proteine-carbonyl, thiols) incluant la formation de N(epsilon)-(carboxymethyl) lysine (CML) comme marqueur de PGA furent déterminé dans chaque phase digestive.

La formation des différents marqueurs d'oxydation des lipides et des protéines, incluant le CML a généralement augmenté au cours de la digestion. L'accroissement du taux de gras et de l'intensité de cuisson et dans certains cas de leur interaction a, de façon générale, augmenté ($P<0.05$) la formation des marqueurs d'oxydation des lipides et des protéines, incluant le CML mais de façon variable suivant chacune des phases. Une diminution des niveaux de carnosine fut par ailleurs observé d'une phase à l'autre en cours de digestion mais ces niveaux sont demeurés plus élevés dans le groupe enrichie en carnosine ($P<0.0001$). L'oxydation des lipides et des protéines ainsi que la formation de CML furent significativement réduits ($P<0.05$) en cours de digestion dans la plupart des

groupes contenant une plus haute teneur en carnosine. Ces résultats démontrent le potentiel de la carnosine alimentaire à contrer durant la digestion la formation de produits délétères à la santé associés à la consommation de la viande et suggère aussi l'importance que la composition et la préparation de la diète pourraient avoir pour la réduction de ces composés et la préservation de la biodisponibilité de la carnosine.

1. Introduction

Meat is an essential part of a healthy diet, providing high-quality proteins, minerals, vitamins and all eight essential amino acids [1]. However, high meat consumption has been associated with an increased risk of developing a number of chronic diseases, such as colorectal cancer, type 2 diabetes mellitus, and cardiovascular disease [2-4]. Although the mechanisms are not fully elucidated, the oxidation of some meat compounds is commonly believed to contribute to raising this risk [5-6]. Indeed, fat oxidation-derived aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) that can be absorbed from meat [7], are related to inflammation, ultimately leading to chronic diseases and are also capable of forming adduct with DNA [8-10]. Protein carbonyls, a typical result of dietary protein oxidation [11] are responsible for multiple protein damages and are involved in chronic diseases such as Alzheimer's disease, cataractogenesis and atherosclerosis [12]. There are other biomarkers of protein oxidation also found in meat and commonly used to study protein oxidation [13]. The thiol group which decrease upon oxidative formation of disulfide bridges [14] is related to functional impairment and protein misfolding, also potentially leading to protein aggregation which may raise the risk of diseases such as neurodegenerative disorders and ocular diseases [15-17]. Advanced glycation end products (AGEs) that arise from food preparation [18] are known to be absorbed from the diet [19] and add to the AGEs formed in vivo. Altogether, they can contribute to impair the secretion function of pancreas; the response of insulin and glucose uptake ability of cells, finally resulting in higher risk of type 2 diabetes mellitus [20-21].

Carnosine (β-alanyl-L-histidine), a dipeptide in meat with multiple functions has the potential to counteract the possible disadvantages of meat consumption due to its different properties which may interfere with formations of deleterious compounds mentioned above. Carnosine that was first reported by Gulevich in 1900 [22] has two common naturally occurring methylated derivatives: anserine and ophidine. Carnosine and its derivatives are found in skeletal muscles with a wide distribution among animal species including livestock, poultries and fishes, with anserine and ophidine being more prevalent than carnosine in poultry, some fishes and marine mammals [23-24]. Reviews on carnosine show that it possesses antioxidant capacity including metal ions chelation, and both carnosine and anserine have quite similar ability and efficiency in reactive oxygen species (ROS) scavenging, which can significantly contribute to reducing

oxidative stress [17]. In addition, carnosine also possesses carbonyls scavenging capacity, and the ability to inhibit the AGEs formation [25-27]. Other properties of carnosine, including accelerating wound healing [17], acting as a neurotransmitter and neuroprotective molecule [28], and enhancing exercise capacity and performance of muscle [29], can provide additional in vivo benefits.

Most studies on the physiological effects of carnosine in human and animals have been based on carnosine supplement [17]. No study, however, has been carried out on the functional aspects of dietary carnosine from meat during the course of digestion. Indeed, during food transit throughout the digestive system, carnosine from meat and compounds of the diet may participate in multiple reactions, considering the specificity of the digestion environment, involving pH variation and enzymes activities. There is scientific evidence that in fresh meat, fat content [30-31], cooking process [32] and digestion are the major factors that may increase oxidative stress [33]. The purpose of this study is, therefore, to evaluate the functional properties of two levels of carnosine during digestion of meat containing two different fat contents and cooked under two different intensities.

For this purpose, subcutaneous fat and carnosine were added to fresh pork *longissimus* muscle, and the mixed samples were cooked under different conditions before being submitted to an in vitro digestion system mimicking the condition in the mouth, stomach, and duodenum as used by Van Hecke et al. [31]. Samples of digests were taken from each phase and analyzed for different markers. Since polyunsaturated fatty acids of the n-6 series (n-6 PUFAs) are predominant in pork [34], their oxidation products were measured as lipid oxidation makers which include hexanal, a product of radical-mediated oxidation on several n-6 PUFAs, such as linoleic and arachidonic acid [35], and another two major toxic products of n-6 PUFAs peroxidation 4-HNE and MDA [5]. Protein carbonyls, the product of protein oxidation that can occur via multiple pathways [36] was determined, considering carnosine's carbonyl scavenging capacity and ability to directly react with protein carbonyls [25]. N(epsilon)-(carboxymethyl)lysine (CML) level in samples was determined as a biomarker of AGEs formation [37]. Finally, the levels of carnosine and anserine in both raw meat and digested samples were also determined as an assessment of their bioavailability. These determinations can further reveal the functional advantages of carnosine from meat in the gastrointestinal environment and might even change the paradigm of meat consumption.

2. Review of Current Studies

2.1 Benefits and risks of meat consumption

2.1.1 Nutrients in meat

To maintain a good health condition, a properly balanced diet made of vegetables, meat, cereal, fruits, and nuts is usually required [38], providing proteins, essential amino acids, lipids, essential fatty acids, vitamins, minerals, and adequate calories. Although each group is recognized for some particular nutritional advantages, meat has long suffered from a negative image. In fact, meat is an important part of a healthy diet considering it is a rich source of important minerals (zinc, large amounts of iron), and contains all eight essential amino acids, high-quality protein, fatty acids and B-vitamins (niacin and vitamin B12) [39].

Meat is a rich source of dietary iron, and most of this heme iron has a higher absorption rate (25%) than non-heme iron (5% to 15%) in the human body [5]. Iron is important and essential, as a vital structure of hemoglobin and myoglobin, for carrying oxygen from the lungs to tissues [5]. Zinc for its part is important for the health of the nervous system, including the development of the brain, and also for the metabolism of nucleic acid [40]. Its deficiency is associated with severe brain disorder such as Wilson's disease [40]. Zinc from meat also has a greater bioavailability [41]. Meat is also a major source of protein with on average 20–24 g protein per 100 g which is indispensable for maintenance and repair of the body as well as for the growth of children [42]. Because of its large amount of high-quality protein, lean red meat represents, in addition, a preferable choice for energy-controlled diet (less carbohydrate and more protein) [1]. With respect to fatty acids, meat contains essential fatty acids, including n-6 PUFAs (such as linoleic acid), possess beneficial roles in spite of their association with the production of inflammatory compounds (such as prostaglandins and leukotrienes) [43]. Indeed, essential fatty acids play vital roles in the prevention and treatment of osteoporosis by enhancing calcium absorption and bone collagen synthesis leading to improved bone strength [44]. Muscle foods are also the main source of Vitamin B12 with relatively high concentration and bioavailability [45] reported in beef, mutton, and pork [46]. Including meat in a daily diet represents a longstanding method to ingest enough amount of vitamin B12 and prevents Vitamin B12 deficient conditions such as neural tube defects in infants and permanent neurological damage in adults [47].

2.1.2. Purported health hazards of meat consumption

1. Colorectal cancer

Despite its major health advantages, many epidemiological studies have associated higher meat consumption and more particularly processed meat with several diseases, such as colorectal cancer (CRC). A meta-analysis has indicated that one serving of processed meat can increase this cancer risk by 8% [48]. Studies in medical, biological, nutritional and other related areas have provided some evidence linking some meat components and their reactions during digestion to a higher risk of developing CRC and have presented some hypotheses as well on the potential mechanisms involved since the exact causes are still not clear.

The most widely accepted hypothesis suggests that heme iron in meat may be the crucial element, with fat content being another indispensable factor involved in CRC. Based on reviews by Wolk et al. and Santarelli et al. [5, 49], heme iron can catalyze the endogenous production of free radicals which can damage polyunsaturated fatty acids and lead to the formation of cytotoxic and genotoxic compounds, such as malondialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE), and other oxidation products. These products of lipid oxidation can bind to DNA and alter its function. MDA has been reported to be able to target and alter genes [5] such as the p53, which codes for a tumor suppression protein, and also the oncogene KRAS gene [50]. 4-HNE can cause the mutation of the APC gene that codes for a tumor suppression protein in colon cells, resulting in increased risk of developing CRC [5]. Other lipid oxidation products can promote TGF-β level, which is related to many cell functions such as cell growth, apoptosis and so on, subsequently causing uncontrolled proliferation in the colon [5]. These lipid oxidation products, especially the α, β-unsaturated aldehydes, can be formed during meat cooking and also in the gastrointestinal tract from where they can be absorbed and reach the systemic circulation [7], with subsequent potential impact in the entire body.

For the preceding reasons, the World Cancer Research Fund/American Institute for Cancer Research recommends 500g as the maximum amount of red meat intake per week [51] in order to reduce the health risk to a minimum.

2. Type 2 diabetes mellitus

High level of meat consumption has also been associated with a high incidence of type 2 diabetes mellitus (T2DM) [3]. It is well-accepted that increased amount of meat consumption may significantly raise both fasting glucose and insulin concentrations [52] leading to an increased risk of T2DM. Some scientists report that women consuming a large amount of red meat before pregnancy may have the higher risk of developing gestational diabetes compared to women who did not [53]. Explanation of the role of meat on T2DM is not clear, and several compounds may be involved. In particular, heme iron could turn poorly reactive free radicals into highly reactive ones which would impair pancreatic cells resulting in inhibited insulin secretion (glucose disposal) and enhanced glucose production by more glucagon (glucose output) [54]. It has also been reported that saturated fatty acids and cholesterol, from red meat, could also increase insulin resistance [55]. The most probable mechanism would be that saturated fatty acids and cholesterol may raise the levels of intracellular fatty acyl-CoA and diacylglyceride, subsequently leading to reduced insulin activation in muscle and insulin-stimulated glucose transport activity, contributing this way to an increased risk of developing T2DM [56].

Advanced glycation end products (AGEs) formed in meat could also contribute to higher risk of T2DM. AGEs usually result from the non-enzymatic glycation and oxidation (such as Maillard reaction during cooking) of protein or lipids after their exposure to reducing sugars [57]. In tissues, after the engagement of AGEs with the receptor for AGE (RAGE), the sustained translocation of NF- κB from the cytoplasm to the nucleus is induced, leading to a series of endogenous reactions which produce a surge of reactive oxygen species and cause inflammation even injuries in different cells and tissues [20]. The injuries may seriously impair the insulin secretion function of the pancreas as well as the response to insulin and the glucose uptake, finally resulting in diabetes [21]. Higher post-meal glucose levels, lower insulin levels, lower glucose uptake in adipose tissue and a higher percentage of diabetes incidences have been observed on high AGEs diet-fed mice while, in contrary, long-term low AGE diet was shown to reduce and even reverse the insulin resistance and chronic inflammation [58-59]. Low AGEs intake can also decrease levels of both AGEs-modified low-density lipoprotein and insulin resistance in human [21].

Altogether, heme ions, saturated fatty acids, cholesterol, and dietary AGEs from meat have been associated with increased risk of T2MD.

3. Cardiovascular disease

Some prospective studies, meta-analysis and systematic reviews in epidemiology have established an association between meat consumption and the risk of increased cardiovascular disease (CVD) [3, 60]. According to a recent meta-analysis study covering a large number of stroke cases, each additional serving of 100 to 120g red meat per day could lead to an 11% higher risk of total stroke [4]. Saturated fat and cholesterol in meat may play important roles as contributing factors. According to Wolmarans et al. (1991) [61], levels of total cholesterol, low-density lipoprotein cholesterol, and triglycerides in plasma can be increased by ingesting saturated fat from meat, eventually resulting in increased CVD risk.

At the molecular level, the oxidation of the low-density lipoprotein and also AGEs absorbed from meat could lead to the formation of covalent cross-links of collagen molecule in the blood vessel resulting in increase protein aggregation in arteries leading to increased stiffness and reduced the buffering capacity of the elastic arteries around the heart with consequent increase in pulse wave velocity [6]. These effects may lead to increase systolic and pulse pressure, subsequently inducing cardiovascular disease. In addition, heme iron from meat which can catalyze the formation of hydroxyl free radicals and causes higher oxidative stress could also contribute to raising the CVD incidence [62].

Other than the diseases cited above, the oxidation of lipid and protein; sodium, nitrate and nitrite, and advanced glycation end products in processed meat could also cause other different damages that may potentially lead to various health conditions [5]. The evidence of a positive association between red meat consumption and a higher risk of all-cause mortality has also been reported [60]. According to these studies, daily meat, especially processed meat intake may be considered a health hazard.

2.1.3. Limitations of studies associating meat consumption with increased health hazards

As a consequence of what precedes, not only the consumers but also many scientists believe that limiting meat consumption and avoiding processed meat are excellent approaches for health

maintenance. Nevertheless, the exact mechanisms behind diabetes, CVD, and many other chronic diseases, especially cancer, are still at the hypothesis stage. In addition, it must be taken into account that these preceding conditions can also be caused by other lifestyle factors such as smoking and dietary habits outside meat. Indeed, a prospective study carried out in European countries reported no significant association between high processed meat consumption and increased cancer risk in people who have never smoked, while the association was significant in both current and former smokers [63]. It is well known that the data collection of dietary intakes in epidemiological studies, reporting the association between high level of meat consumption and increased risk of diseases, can be highly depended on subjects' memories which can be vague or even affected by current health conditions subsequently leading to a bias. Some epidemiological studies which did not establish a significant association between increased CRC risk and high meat intake also exist [65-66]. A recent meta-analysis on meat consumption and CVD and ischemic heart disease mortality revealed a high heterogeneity among studies and indicated the need to interpret these association with caution [60].

Another aspect worth considering is that the doses of carcinogenic inducer used in some experiments are usually much higher (1000- to 100000-times) than the amount that meat can provide to human [67-68]. In addition, carcinogenic heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs), which are usually used as determinants of CRC risk, are found in large amount in chicken whose consumption shows no association with higher CRC risk [69]. Studies even report that increasing poultry consumption in the daily diet would decrease both total mortality and cancer mortality [70-71]. Furthermore, people ingest most of their PAHs from cereals, grains, and bread which are not reported to contribute to the rise of CRC risk [72]. This information suggests that other explanations or factors may need to be considered since current studies may not provide adequate evidence to firmly conclude that meat ingestion leads to more risks than benefits. One particularly important aspect that has been barely covered at the present time is the dietary role of carnosine, a potent health protective molecule that exists only in muscle food. Therefore, the hypothesis of the present thesis is that depending on its behavior during processing, cooking, and digestion of the meat; carnosine may significantly interfere with many chemical reactions involved in the development of the age-related and chronic diseases mentioned owing to its the numerous biochemical and physiological advantages as published these years.

2.2 Distribution of carnosine

Carnosine (β-alanyl-L-histidine, Figure 1) has been studied by scientists since it was first discovered in 1900. Carnosine is synthesized by carnosine synthase that is mainly present in skeletal and heart muscle and certain brain regions such as olfactory neurons [17]. Carnosine on the other hand is degraded by carnosinases: one being tissue carnosinase (CNDP1) which barely exists in skeletal muscle, and the other being the serum carnosinase (CNDP2) which is absent from nonprimate mammals (with the only exception of Syrian golden hamster) but highly present in human serum [17, 73]. Carnosine is widely distributed in animals with mammals containing a relatively high level (such as livestock) compared with fishes, birds, amphibians, and reptiles that generally contain less carnosine but more anserine and also sometime ophidine (balenine) in the case of marine mammals (reviewed by Boldyrev et al. (2013) [17]). Carnosine and its main methylated derivative anserine are found in different proportions and concentrations among the main meat animals as shown in Table 1. Although carnosine derivatives anserine and ophidine commonly present in the animal skeletal muscles [17], they are absent in that of human, with the exception of anserine that has been recently reported in the cortex of human kidney [74]. Within the muscle, a higher carnosine level is found in fast-twitch muscle fibers as compared to the slow-twitch ones [75]. In addition to muscles, other tissues and biological fluids also contain carnosine, such as human renal tissue [74] human brain [76] as well as human blood [77].

Table 1. Carnosine and anserine concentrations in meat from different animal species

Meat	Carnosine (mg/100 g wet muscle)	Anserine (mg/100 g wet muscle)
Pork loin	313.0±35.7	14.5±1.4
Pork ham	449.5±59.2	22.9±5.1
Beef top loin	372.5±32.2	59.7±4.2
Chicken pectoral	180.0±10.9	772.2±50.3
Turkey breast*	66.2±0.5	420.0±3.0
Salmon	0.53±0.06	589.4±54.6

(Data are expressed as means±SD; adapted from studies of Aristoy et al. [24] * and Gil-Agustí et al. [78])

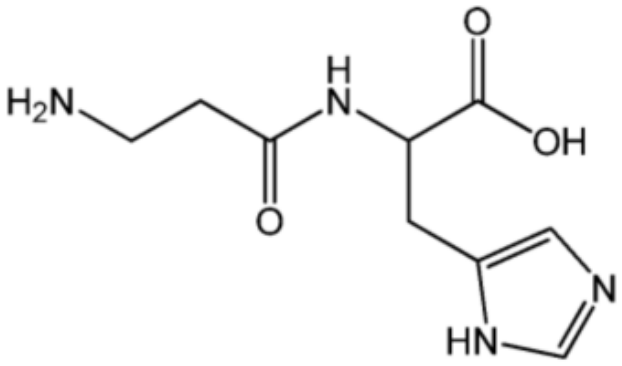

Figure 1. Carnosine structure

2.3 Properties of carnosine

Carnosine possesses many biochemical properties which altogether confer the molecule with numerous physiological roles.

2.3.1 PH-buffering

Due to a β-alanine residue, nitrogen atoms of imidazole ring and a carboxylic group, carnosine possesses three dissociation constant (pKa) 9.32, 6.72 and 2.76, respectively [79] that can act as an efficient muscle physiological buffer and react with acidic and alkaline substances and control the intracellular pH over a relatively wide range, especially in vertebrate muscle cells. Among the three groups, the imidazole ring, with its ability of protonating one of its nitrogen atom, contributes the most to the intracellular buffering capability by maintain the pH of the muscle cell close to its pKa value [80].

2.3.2 Anti-oxidation

Carnosine's antioxidant activity can be explained by different mechanisms, including chelating metal ion and reacting with ROS.

1. Metal ion chelation

Carnosine can form complexes with metal ions, such as Cu^{2+}, Zn^{2+}, Mn^{2+} and many other bivalent metal ions [81]. Due to their involvement in many metabolic processes and relevance to

ROS activation, the chelation of Cu^{2+} and Zn^{2+} are the two most widely studied ones among these bivalent metal ions.

1) Copper ion

Most of the copper ion (Cu^{2+}) in blood is bound to L-histidine for transport and part of Cu^{2+} binds to amino acids which are able to enhance its transport to tissues. As a common trace element in human, Cu^{2+} is a cofactor of various enzymes and proteins which are involved in electron transport, oxygen transport and metabolisms, such as cytochrome C oxidase and superoxide dismutase [82]. Except for the catalytic enzymes responsible for lipid and protein oxidation, Cu^{2+} can also contribute to oxidation by participating to the formation of reactive oxygen species (ROS), such as hydrogen peroxide, which may subsequently lead to apoptotic processes via several pathways [83-84]. By chelating Cu^{2+} in the body, carnosine can decrease Cu2+ activated oxidases and protect cells and tissues from Cu^{2+}-related oxidative damage. Moreover, in phosphate buffer 100mM (pH=7.0), copper-carnosine chelates have superoxide dismutase (SOD) similar activity against superoxide anion radicals from the xanthine-xanthine oxidase system [85].

2) Zinc ion

Zinc for its part, plays essential roles for the structure of the protein and is crucial for RNA polymerases and also acts as catalysts or co-catalysts for the proper folding of proteins [82]. Some Zn-proteins are also related to nucleic acid replication [82]. Similar to copper ion, zinc ion is also involved in the formation of ROS. Although Zn activated superoxide dismutase is known for reducing the level of hydrogen peroxide, it can also produce another radical, the hydroxyl radical, which is more reactive than hydrogen peroxide and may cause more damage [86].

The formation of Zn (II)-carnosine complexes can decrease the level of zinc ion, therefore reducing the related oxidative reactions. Different Zn (II)-carnosine complexes can exist under different conditions. Being similar to Cu (II)-carnosine complex, the dimer is the major form at neutral pH [87]. In an acid environment, the monomer of Zn (II)-carnosine complex is the primary form [87] and possesses protective ability in the stomach that has been approved as a drug for gastric ulcers treatment in some countries [88].

2. Scavenging reactive oxygen species

Reactive oxygen species (ROS) are free radicals (molecules with one or more unpaired electron(s)) containing oxygen that are mainly generated in the mitochondria as by-products of cellular metabolism [89]. Because of the unpaired electron (s), ROS are highly volatile and usually tend to react with ambient molecules to gain an electron and return to their stable ground. Thus, ROS have the strong oxidizing property which can be either beneficial or harmful. When the content of ROS does not exceed the need, ROS aid to resist infection by oxidizing chemicals, such as lipids, proteins, and even DNA, of microorganisms [90]. This ability of ROS, especially the superoxide ions, is also involved in the inhibition of the growth and spread of cancer cells [91]. At low concentration, ROS serve as regulatory mediators for the signal transduction processes for normal sperm function [92]. However, when ROS exceed the optimal level and cannot be compensated by available antioxidant capacity, ROS can modify proteins, fatty acids and other components of the cells and form toxic products [93]. As an example, ROS can react with PUFAs and form hydroperoxides whose breakdown will result in the formation of genotoxic and cytotoxic 4-HNE and MDA [94]. In addition to the direct damage to some molecule caused by oxidation, some other molecules such as inflammatory cytokines can be produced by the immune system in reaction to the oxidative damage that can lead to chronic inflammation and other more serious consequences [95].

Unlike many other chemicals that can scavenge ROS, the carnosine scavenging mechanisms may vary depending on the types of ROS produced and which one of the carnosine's functional groups takes part in anti-oxidative reactions [17]. Overall, however, many of these mechanisms are not related to reducing activity. Instead, it seems that the imidazole ring and the free amine group of carnosine can rapidly react with hypochlorite (HOCl) and form relatively stable chloramines through intramolecular chlorine transfer, restricting this way the HOCl-mediated oxidation [96]. The imidazole moiety of carnosine for its part is considered as the most important group for reacting with hydroxyl radicals ($\cdot OH^-$), to produce the more stable hydroxide (OH^-) [97]. The imidazole group in carnosine can also quench singlet oxygen several folds faster than other dipeptides and even slightly faster than the imidazole [98]. In addition, carnosine appears to have the similar ability as superoxide dismutase to directly react with superoxide radicals [99]. In

aqueous solutions, carnosine would form intramolecular charge-transfer complexes with superoxide ($O_2^{\cdot -}$) to reduce its reactivity [100].

2.3.3 Carbonyls scavenging

Carnosine is capable of quenching carbonyls produced from both lipid and protein, but the reactions between carnosine and lipid carbonyls are more clearly elucidated than reactions with protein carbonyl groups. For instance, the quenching mechanism of carnosine toward 4-HNE that forms a carnosine-HNE adduct, a cytotoxic lipid oxidation product, has been reported by Aldini et al. [101]. In the case of protein oxidation however, four different pathways leading to the formation of protein carbonyls (protein carbonylation) have been identified and are: a) metal-catalysed oxidation of arginine, proline, and lysine; b) radical-mediated oxidation; c) oxidation of lysine residues by glycation and glycoxidation products, such as ketoamines and deoxyosones; d) adduction of carbonyl species derived from polyunsaturated fatty acids peroxidation products, such as MDA and 4-HNE [36]. It is also well documented that these protein oxidation products accumulate in different tissues over time and can contribute through altered physiological function to many chronic diseases such as chronic renal failure, Parkinson's disease, Alzheimer's disease, CVD and more [102]. Carnosine can directly decrease protein carbonylation level and reduce its adverse consequences. Unlike a direct quenching ability as in the case of lipid carbonyl products, carnosine would reduce the concentrations of protein carbonyls by forming protein-carbonyl-carnosine-adducts and protect the protein from further crosslinking [25].

2.3.4 Inhibiting advanced glycation end products formation

Advanced glycation end products (AGEs) are formed by the non-enzymatic reaction between carbonyl groups of reducing sugars with the free amino groups of nucleic acids, protein, or lipids. AGEs formation can occur in the organism as a product of the metabolism and also in food through the Maillard during processing. Both exogenous AGEs ingested from foods and endogenous AGEs formed in the body can modify the structure of the proteins, inhibit cell communication and alter cellular and tissue metabolism and function [103].

AGEs are also capable of interfering with intracellular reactions by interacting with the receptor of AGE (RAGE) on the membrane, subsequently, transducing the cell signals (such as mitogen-activated protein kinases and NAD(P)H oxidase), leading to increased oxidative stress and

enhanced ROS formation [103]. Such an effect may damage important molecules, perturb the metabolism of the cells and even change the cellular functions inducing severe symptoms and chronic diseases. For instance, after the engagement with the receptor for AGE, the sustained translocation of NF-κB from the cytoplasm to the nucleus is induced, leading to a series of endogenous reactions which produce a surge of reactive oxygen species and cause inflammation even injuries in different cells and tissues [20]. The injuries may seriously impair the insulin secretion function of the pancreas as well as the response to insulin and the glucose uptake, finally resulting in type 2 diabetes mellitus [21]. Inducing the transcription factor NF-κB can also lead to the formation of prostaglandin E2 (PGE2) and the expression of oncogene Bcl-2 [104]. Levels of interleukin-1α, tumor necrosis factor-α, and many other pro-inflammatory cytokines or signal molecules have also been reported to significantly increase following the accumulation of AGEs [104].

For the preceding reasons, low AGEs diet is now advocated to avoid or decrease the adverse effects of AGEs and maintain good health [21]. Since the Maillard reaction is very common during food processing and cooking and is the major source of dietary AGEs, compounds that could reduce AGEs formation, such as phytochemicals phenolic compounds, could represent an important approach for health benefits [105]. Carnosine, which is relatively abundant in meat, has already been reported in several in vitro and in vivo studies as an inhibitor of AGEs formation [26-27]. Carnosine can block or reverse the glycation process [106]. This ability can occur through reversing the formation of the Schiff base resulting in the inhibition of the AGEs formation [107]. The nucleophilic attack of carnosine on the preformed aldosamine (the result of a spontaneous rearrangement of Schiff base) lead aldosamine to react with carnosine instead of taking part in the subsequent reactions of AGEs formation [107]. Carnosine is also capable of protecting some enzymes (such as aspartate aminotransferase) from inactivation caused by glycation, contributing in turn to reduce glycation [106].

2.4 In vivo functions of carnosine and diseases

As mentioned above, carnosine can provide protection to cells and organs and is even considered as a potential treatment for various diseases, especially those in which ischemic or oxidative stress are involved as reviewed by Boldyrev et al. (2013) [17]. However, the complexity of the

different in vivo environments where carnosine is present may influence its activity differently depending on the organs or tissues.

2.4.1 Skeletal muscles

As the first tissue where carnosine is found and as the main pool of carnosine in the organism the effects of carnosine in skeletal muscles have been quite documented, particularly with respect to its role in exercise physiology [108-109], although its entire functions and exact mode of actions are still being studied. Carnosine, nevertheless, would play a role as a pH buffer in skeletal muscles, particularly during the high intensity muscle contraction which would contribute to reduced skeletal muscle fatigue [110]. Indeed, under such conditions, the anaerobic glycolysis in the muscle is solicited to rapidly produce the extra energy needed, also producing lactic acid which is capable of immediately decreasing pH values to 6.5 and even lower, which has been associated to muscle fatigue [111].

In addition to its buffering capacity, carnosine can also regulate both the calcium release and calcium sensitivity of the muscle that also contribute to attenuated contractile muscle fatigue and a more efficient muscle contraction [109]. Incubating skeletal muscles of mice and also skinned muscle fiber of rats and frogs in the presence of carnosine enhanced the release of calcium ion for more efficient muscle contraction [110]. There is actually a clear positive concentration-dependent relation between muscle carnosine and calcium ion sensitivity. Indeed, at a certain concentration of calcium ion, the produced force is higher in the presence of carnosine in the sarcoplasm, in addition, muscle carnosine can also maintain a relatively higher force when less calcium ion is present such as during contraction-induced muscle fatigue [110].

In general, carnosine in skeletal muscle benefits both young and old people in exercise capacity and performance of muscle [29,112].

2.4.2 Brain

The brain is the center of the nervous system in all vertebrate and also in many invertebrates. The cerebral cortex of human being contains approximately 15–33 billion neurons, which subsequently connect to several thousand of other neurons and are responsible for the control of

information flow and implementation of algorithms [113]. Thus, the brain is the most complex and vital organ and maintaining its homeostasis is of utmost importance.

In spite that the research on functions of carnosine in the brain is still in its early stage, the antioxidant activity and ability to reduce AGEs formation make carnosine a potential efficient homeostatic and protective agent in the brain [17]. Studies on neurological disorder including brain cancer and Alzheimer's disease have shown the protective capacity of carnosine. Alzheimer's disease is mainly attributed to the deposition of β-amyloid plaque in brain tissue that damage neuronal cells [114]. AGEs are known as the accelerator of the aggregation of β-amyloid which in turn activates microglia and astrocyte cells of the brain which aggravate the condition through the production of radicals and cytokines [115-116]. Since AGEs formation can lead to protein aggregation and, accumulation of β-amyloid in the brain of Alzheimer's patient, it is likely that carnosine in the brain could simultaneously inhibit both the formation of AGEs and amyloid plaque as reviewed by Artioli et al. [117] Carnosine is shown to be capable of binding to existing β-amyloid and block its further aggregation by perturbing the hydrogen-bond network near residues and fibrillogenesis, reducing neuronal cells injury [118]. In addition, the carnosine anti-oxidant property could also decrease the oxidation of lipids and proteins, which result in more ROS and inflammation and induce cholinergic neurons to produce more β-amyloid which exacerbate Alzheimer's disease [119].

Carnosine also has the ability to act as an endogenous neuroprotective agent in the case of ischemic stroke which happens to more than 15 million people annually [120]. Stroke may happen when blood flow, especially blood flow in internal carotid arteries and vertebral arteries, is abnormally reduced. Ischemic stroke happens as a result of lack of blood flow, hence reduced the oxygen level in brain tissue. In such condition, carnosine can protect the brain by scavenging ROS which mediates much of the ischemia-induced brain damage [120]. According to Jin's group, carnosine can also increase the number of glial cells which protect neurons under stressful conditions such as lack of blood [121]. Carnosine's ability in crossing the blood-brain barrier may also improve its protection of the brain [121]. Carnosine is also capable of reducing infarct volume and damages in the brain by reducing mitochondrial impairment and deleterious formation of autophagosome, caused by stroke [122]. Moreover, carnosine can exert its neuroprotective and anti-apoptotic property through up-regulating mRNA expression after acute ischemic cerebral

stroke [123]. For these reasons, carnosine appears efficient in protecting against ischemic stroke both before and after the onset as well as offering protection of the brain after the disorder occurred.

Carnosine's metal ion chelating ability may also have some influence on neurotransmission since metal ions, such as copper and zinc, are neurotransmission modulators [124-125]. The high concentration of carnosine in olfactory neurons also suggests that carnosine has a putative role in sensory neurotransmission. Several studies provide proof that there is a co-localization of carnosine in synaptic terminals [126-127]. Recent research also indicated that carnosine could act as neurotransmitters in neuron-to-glia communication [128], with β-alanine, one of the two constituting amino acids, being also reported to possess neurotransmission properties [129].

2.4.3 Blood vessels and cardioprotection

The protection of carnosine is also significant for the cardiovascular system. Since most blood vessel lesions are the result of oxidation, the antioxidant activity of carnosine can protect blood vessels by reducing oxidative stress via chelating metal ions and scavenging radicals as indicated above.

Prevention of atherosclerosis is an important aspect of carnosine's blood vessel protective ability. Atherosclerosis is the thickening of the artery wall caused by oxidation of lipoproteins such as oxidized low-density lipoprotein (ox-LDL) and their consequential retention into the artery wall, leading to its inflammation which is accompanied by the accumulation of macrophages, T cells, proliferation of intimal-smooth-muscle cells, and finally creating a fibrofatty lesion which in turn traps more lipid near the lesion area, worsening the inflammation and immune cells accumulation [130]. Atherosclerosis not only causes the restriction in blood delivery but also is responsible for other diseases such as myocardial and cerebral infarction. In addition to carnosine anti-oxidative activity toward the formation of oxidants, such as ox-LDL, carnosine has also been reported to facilitate, in mice, the removal of aldehyde-modified proteins from existing atherosclerotic lesions, decreasing their deleterious effects and reducing this way the possibility of forming new lesions [131]. It is not clear how the presence of carnosinase in human plasma might influence this protective ability of carnosine. A study reports that carnosine supplementation caused no difference in either cardiovascular risk factors or plasma

inflammatory markers [132] suggesting other pathways might be involved for the mentioned efficacy of carnosine for cardiovascular conditions in human. Meanwhile, another recent study on human demonstrates that carnosine supplement is still effective in protecting plasma lipidome and preventing the incidence of CVD despite the existence of serum carnosinase [133].

In tissue level, AGEs also contribute to the problem by interacting with AGE receptor and modifying and trapping molecules such as LDL and structural proteins of the vessels through covalent cross-links considering the particular vulnerability of these vessels to this effect [134]. For instance, AGEs accumulated in vascular wall can form cross-links with type IV collagen in the basement membrane, inhibiting this way the collagen molecules from forming a normal network which therefore changes the structure of cell [134] and ultimately increases the vascular stiffness leading to increased risk of atherosclerosis-related diseases, such as macrovascular disease [135]. By reducing AGEs formation through carnosine anti-glycating property, reacting with the intermediate products of the Maillard reaction, scavenging ROS and decreasing inflammation, carnosine can contribute to different ways in maintaining the health of the blood vessels and heart.

2.5 Potential factors with influence on carnosine properties

The many properties mentioned above indicate the potential positive effects of carnosine in vivo. It is, therefore, possible that the carnosine present in meat as part of a daily meal may not only contribute to reducing the reported problems that have been associated with meat consumption [2-4] but also protect consumers from other health condition. Scientists have already suggested that the carnivorous diet could have health ameliorative properties [136-137]. So far, most studies on the in vivo benefits of carnosine were based on the use of supplements. The benefits of carnosine as a meat constituent, however, could be influenced by single or combined factors including the meat chemical composition and processing as well as meat digestion. [138].

Therefore, the single and combined effects of these factors need to be assessed to better understand the potential health benefits of carnosine associated with meat consumption. In corollary, any decrease in carnosine bioavailability during cooking and digestion, that may result from reacting and decreasing the formation and hence the bioavailability of lipid and protein oxidation by-products and AGEs, could also be regarded as another health beneficial effect of

carnosine in meat. However, no study has ever been conducted on the effect of carnosine in meat undergoing processing, cooking, and digestion altogether.

2.5.1. Processing and cooking

Fat, including added fat, is a common component of meat and processed meat products, contributing to mouthfeel and flavor. However, lipids, when oxidized, can have negative effects on meat physicochemical [139] and nutritional quality [140]. For instance, lipid oxidation can lead to the production of ROS and aldehydes as shown in Figure 2 [141]. These products may then react with other biomolecules and cause damages, such as MDA binding to DNA as reported earlier and (or) inducing protein crosslinking by interacting with lysine residues of different protein molecules [142]. In addition, lipids can also enhance oxidation related process such as AGEs formation by lipid peroxidation products participating in multiple pathways [143]. Considering that fat in meat can enhance oxidation related reactions [31, 144-145], it becomes possible that part of the carnosine in meat may be requested for its antioxidant and inhibition of AGEs formation properties, decreasing this way the amount of carnosine being bioavailable for postprandial protection of the organism.

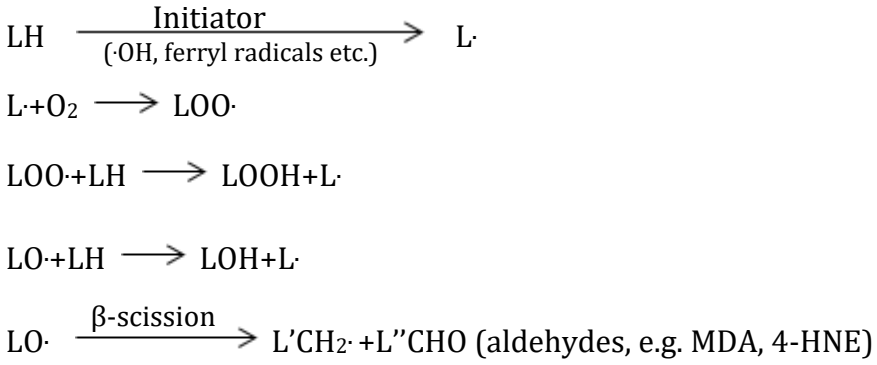

Figure 2. ROS and aldehydes produced from lipid oxidation [141]

With few exceptions, meat and meat products are mostly consumed cooked. However, there is only limited research focusing on the effects of cooking conditions on carnosine loss [146]. It has been documented that cooking meat at high temperature facilitates chemical reactions, including the oxidation of fat and protein that lead to AGE formation [147]. Indeed, studies on meat cooking methods indicate clearly that cooking conditions are highly responsible for the development of oxidation, imparting negative changes in quality [148-150]. At any given lipid level, cooking

temperature and time are the most important parameters leading to oxidation [148], with lipid oxidation increasing synergistically with increased cooking time and temperature. For instance, beef patties cooked at 20℃ higher temperature for 3 more minutes produced significantly more primary and secondary lipid oxidation products [149]. Cooking may, therefore, engage carnosine to counteract the formation of oxidative-related compounds, thereby reducing the availability of carnosine for other ulterior in vivo protective properties for the consumer.

Overall, fat level and cooking conditions are the two main factors leading to oxidation in the fresh meat of a given specie. No study, however, has taken into account the bioavailability of carnosine from the meat of different composition and cooking conditions upon digestion.

2.5.2. Digestion

Irrespective of processing and cooking conditions, the digestion process itself can directly influence properties of bioactive constituents in the diet. In order to be beneficial, bioactive compounds need first to be released from the food matrix and to become accessible to the digestive system. Such a process can be influenced by food composition, physicochemical properties, as well as reactions among different components [151]. With respect to carnosine, a study on its bioaccessibility during digestion of beef suggests that carnosine in food would not be fully available because part of it is absorbed by the food matrix which is dispersed in the digestion fluid [138]. Indeed, the human gastric fluid with its acidic pH represents an excellent medium for the amplification of lipid peroxidation and oxidation of other dietary constituents due to the higher activity of dietary endogenous catalyst (stability of ferryl species coming from metmyoglobin) at lower pH than that at alkaline conditions [152]. It may be hypothesized that such higher activity of catalyst in an acidic environment may decrease the level of bioavailable carnosine, owing to its potential reactions with radicals, AGEs and other adverse compounds formed during digestion. The acidic condition during digestion can also affect the bioavailability of carnosine considering the curvilinear relationship of pH on its solubility with a decrease observed from pH 2 to 5.3 followed by an increase up to pH 8 [138]. No research, however, has been carried out on how carnosine from meat differing in composition and preparation could react with products of oxidation and glycation during digestion, and to what extent its bioavailability could be affected.

In summary, since many studies have reported an association between meat consumption and chronic diseases, such as cancer, type 2 diabetes mellitus, and cardiovascular disease, it becomes of high interest to take into account the presence and role of dietary carnosine in the meat, which could modulate the existing information on health-related aspects of meat consumption. Indeed, carnosine's multiple functions, such as pH-buffering, anti-oxidative, and anti-glycation could potentially inhibit the mechanisms associated with the development of the aforementioned diseases associated with meat consumption, provide that the dipeptide would not be entirely involved in counteracting the oxidative conditions brought about by the diet and the digestion altogether. This project, therefore, was designed to evaluate the carnosine availability and potential ability to reduce lipid oxidation, protein oxidation, and inhibition of advanced glycation end products (AGEs) formation during in vitro digestion (salivary, gastric and duodenal phases) of meat processed with different fat contents and cooking conditions.

3. Methodology

All chemicals, enzymes, and solvents were from Sigma-Aldrich, except where indicated. Lean pork (entire fresh *longissimus* muscle) and subcutaneous fat were purchased from Olymel S.E.C. The entire protocols of digestion and determinations were repeated four times.

3.1 Meat sample preparation

Prior to the preparation of the meat treatments, 50g samples from each of the anterior, central and posterior portion of the longissimus muscle were collected and ground altogether (first with a 6mm plate, then with a 3.5 mm plate) for the determinations of the intrinsic intramuscular fat content according to AOAC (2000) (920.39) as well as that of carnosine and anserine [153]. Thereafter, the entire longissimus muscle was ground first with a 6mm plate, and the ground meat was divided into eight portions corresponding to the following 2 x 2 x 2 factorial arrangement of the treatments as shown in Table 2. Two carnosine levels, either 309.8mg/100g for (intrinsic) low carnosine treatment (LCar) or 600 mg carnosine /100 g meat for (added) high carnosine (HCar) group, were used with each of two fat levels being intrinsic or low fat (LF) vs 10 % or high fat (HF). Cooking treatments for all samples were either 65°C for 15 min for the low cooking intensity (LCI for) or 90°C for 30 min for the high cooking intensity (HCI). For HF groups, subcutaneous fat was added to the ground meat in order to reach 10% total fat that

corresponds to the maximum fat allowed in the extra lean ground meat category as per the Canadian regulation [154]. For the HCar group, the arbitrary 290.2 mg carnosine added per 100 g meat represents a broad estimate of the potential of pigs to deposit higher muscle carnosine level considering their commercial production on a vegetarian diet despite their omnivore nature altogether with the large proportion of high glycolytic muscles in commercial swine. The extent of carnosine increase observed in human studies upon supplementation with either beta-alanine or carnosine was also considered as a guide for selecting the added level. For each treatment, the ground meat and added fat and (or) carnosine was first thoroughly hand mixed for one minute (to reduce oxidation to a minimum) and the mixture was then ground again with a 3.5mm grid before being transferred in 250 ml Nalgene screw-cap containers with a fitted handmade rubber stopper in the lid for the cooking process. Containers were filled to their maximum and were cooked in a water bath under two different conditions used by Van Heck et al. (2014; 2015) [31-32]. After cooling cooked meat to room temperature (for around 30min), the entire content of the container including dripping was transferred to a food blender and fully ground for 2min. Every twenty grams of ground cooked meat was then vacuum packaged in the separate vacuum bag for each of the eight treatments and stored at -80° C until being used for digestion.

Table 2. Preparation of meat sample

Low Carnosine (LCar=309.8mg/100g *longissimus* muscle)*				High Carnosine (HCar=600.0mg/100g *longissimus* muscle) **			
Low Fat (LF=1.3%) *		High Fat (HF=10.0%) **		Low Fat (LF=1.3%) *		High Fat (HF=10.0%) **	
LCI	HCI	LCI	HCI	LCI	HCI	LCI	HCI

(* intrinsic levels of carnosine and fat in pork, ** targeted levels of carnosine and fat (increased) ;
Low cooking intensity (LCI): 65℃, 15min; High cooking intensity: 90℃, 30min)

3.2 In vitro digestion

In vitro digestion mimicking the mouth, stomach, and duodenum phases were conducted according to Van Hecke et al. (2014) [31] with some modifications. No colon digestion phase was carried out and the digests were not diluted to the final volume found in the duodenal phase, but the dilution caused by adding digestive fluids was taken into account in the following data analysis. For each treatment, three tubes of digest were run to follow the entire digestion process: salivary, gastric and duodenal phase. Briefly, digestive fluids shown in Table 3 were first prepared according to the study of Van Hecke et al. (2014) [31]. Approximately 4.5g of meat sample was placed into each of three 50ml centrifuge tube for each treatment. For the salivary

phase, 6ml of the saliva fluid was added in each tube and incubated in a 37°C water bath for 5 minutes. One of the tubes was then homogenized (10000rpm, 1min) and kept at -80°C until analyses. Then, 12mL of gastric juice was added in the two remaining tubes, making sure that pH would not exceed 3.0 with 5N HCl. The tubes were fixed to a rotating shaft to simulate 2-hour-stomach digestion at 50rpm and 37°C. After completion, one tube was again homogenized (10000rpm, 1min) and kept at -80°C for ulterior analyses. Thereafter, 20mL of duodenal fluid (duodenal juice, bile, and 1.008N $NaHCO_3$ (6:3:1, v/v/v)) was added in the remaining tube for an additional 2-hour-duodenal digestion and rotated at 10rpm at 37°C before being homogenized (10000rpm, 1min) and kept at -80°C for the analyses of the digests.

Table 3. Composition of digestion juices

Mouth		Stomach		Duodenum*				
Saliva		Gastric juice		Duodenal juice		Bile		NaH CO_3
pH range	6.8±0.2	pH range	1.3 ±0.02	pH range	8.1 ±0.2	pH range	8.2 ±0.2	
Inorganic		Inorganic		Inorganic		Inorganic		
0.90g	KCl	2.75g	Nacl	7.01g	NaCl	5.26g	NaCl	84.70g
0.20g	KSCN	0.27g	NaH_2PO_4	3.39g	$NaHCO_3$	5.79g	$NaHCO_3$	
0.90g	NaH_2PO_4	0.82g	KCl	0.08g	KH_2PO_4	0.38g	KCl	
0.57g	$NaSO_4$	0.40g	$CaCl_2{\cdot}2H_2O$	0.56g	KCl	0.15ml	37%HCl	
0.30g	NaCl	0.31g	NH_4Cl	0.05g	$MgCl_2$			
1.69g	$NaHCO_3$	6.50ml	37%HCl	0.18ml	37%HCl			
Organic and enzymes		Organic and enzymes		Organic and enzymes		Organic and enzymes		
0.20g	Urea	0.09g	Urea	0.10g	Urea	0.25g	Urea	
11.5mg	Uric acid	0.02g	Glucuronic acid	0.20g	$CaCl_2{\cdot}2H_2O$	0.22g	$CaCl_2{\cdot}2H_2O$	
6.90mg	$NaNO_2$	0.33g	Glucoseamine-HCl	1.00g	BSA	1.80g	BSA	
25.0mg	Mucin	0.65g	Glucose	9.00g	Pancreatin	30.0g	Bile	
2.50IU	Peroxidase	17.6mg	Ascorbic acid	1.50g	Lipase			
		11.2mg	$FeSO_4{\cdot}7H_2O$[1]					
		10 µl	H_2O_2(30%)					
		1.00g	BSA					
		2.50g	Pepsin					
		3.00g	Mucin					

* 20min in 37°C water bath before use

[1]frome Fisher Scientific International, Inc

3.3 Carnosine and anserine quantification by HPLC

For carnosine and anserine measurement, 1.5g of the digest was mixed with 2ml 0.01N HCl and centrifuged at 10000rpm at 4°C for 20min. The solution was filtered with glass wool, and 250µl

of the supernatant was mixed with 750µl of acetonitrile, held at 4° C for 20 min. The mixture was centrifuged at 10000rpm for 10 min at 4°C, and the supernatant was used for the HPLC analysis. HPLC instrument settings of Mateescu et al. (2012) [153] was followed with the use of an extra pre-column (Atlantis HILIC silica column (3.9 × 5 mm, 3 µm; Waters Corporation, Milford, MD)) with the following modifications of the flow rate: the solvent gradient was linear from 0% solvent B and 100% solvent A to 70% solvent B and 30% solvent A in 9.1min and then kept for 2.0min, followed by a return to 0% solvent B and 100% solvent A in 2.0min, and kept this for another 10.0min.

3.4 Lipid oxidation

Hexanal, MDA and 4-HNE determination with GC/MS

The measurement of hexanal, MDA, and 4-HNE in digests was developed based on the method of Tsikas et al. (2016) [155] using the SIM mode (electron impact ionization). For the first derivatization, 200µl of digest, 170 µl of PFB-HA (*O*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride) water solution (30mg/ml) and 10µl of HHE-d_3 (4-hydroxy Hexenal-d_3) ethanol solution (20ng/µl) were mixed with a vortex and incubated in the ultrasonic bath for 3 min (B1510R-MT , Branson Ultrasonic, USA). The extraction was then conducted once in 500 µl methanol and 1ml isooctane. Six drops of concentrated sulfuric acid were also added. The extract solution was mixed with a vortex for 1min and centrifuged at 2700rpm for 5min. 800µl of the supernatant was transferred into vials through anhydrous sodium sulfate and glass wool. The last step was the second derivatization in the oven (80° C, 1h), after 50µl of BSTFA (N,*O*-Bis(trimethylsilyl)trifluoroacetamide) being added in the completely evaporated vial. For quantification, the following ions were chosen for hexanal, HHE-d3 (internal standard), 4-HNE, and MDA, respectively: 239m/z (RT=10.57,10.60min); 187m/z, and 203m/z (RT=12.20,12.33min); 226m/z, 242m/z, 352m/z (RT=13.47,13.62min); and 250m/z (RT=13.61,13.75,13.78min).

3.5 Protein oxidation

3.5.1 Protein carbonyls determination

The determination of protein carbonyl content was adapted from Ventanas et al. (2006) [156]. Briefly, 200µl of each digest (0.1g meat/ml) was mixed with 20% trichloroacetic acid (TCA) and centrifuged (3000g, 5min). The pellet was kept and mixed with 2ml of 10mM (in 2N HCl) 2,4-dinitrophenylhydrazin. After centrifugation, the pellet was washed three times with 2ml of freshly prepared solvent (ethyl acetate and ethanol (1:1, v/v)) and dissolved completely with 2.5ml of 20 mM sodium phosphate buffer containing 6M guanidine hydrochloride (pH=6.5). Protein level was also determined as Ventanas et al. (2006) described [156]. The coefficient 21.0 nM^{-1} cm^{-1} was used for calculation and results were expressed as nmol/mg protein [156].

3.5.2 Protein thiols determination

Thiols were measured with the colorimetric method with 2, 2'-dithiobis (5-nitropyridine) (DTNP) [157] with some modifications. The protein level of the digest was determined with the biuret method [158] and adjusted to 5mg/ml with 200mM phosphate buffer (pH=7.4). An urea buffer (100Mm phosphate buffer (pH 8.0) containing 8M urea) was then used to adjust the protein level to 1mg/ml. The tubes were incubated for 1 hour at room temperature and the absorbance was read at 386nm. Readings of "Blank" and "DTNP" were subtracted and the coefficient $14mM^{-1}cm^{-1}$ was used for calculation according to Martinaud et al. (1997) [157]. Results were expressed as µmol/mg protein.

Table 4. Sample preparation

	200mM phosphate buffer	5mg/ml solution	Urea buffer	10mM DTNP (in ethanol)	Ethanol
Blank	-	400µl	1600 µl	-	20 µl
Test	-	400µl	1600 µl	20 µl	-
DTNP	400 µl	-	1600 µl	20 µl	-

3.6 N(epsilon)-(carboxymethyl)lysine level

OxiSelect™ CML competitive enzyme-linked immunosorbent assay (ELIZA) kit (Cell Biolabs, Inc) was used for the determination of N(epsilon)-(carboxymethyl)lysine as a marker of AGE formation according to the instruction provided. In brief, CML Conjugate was coated within 24h

before use. Samples and standards were then added to wells and incubated for 10 minutes on an orbital shaker at room temperature. The anti-CML antibody was then added and incubated for 1 hour, followed by the incubation and reaction of the substrate with horseradish peroxidase conjugated antibody. Absorbance was read at 450nm wavelength, and concentration was determined from the standard curve.

3.7 Statistical analysis

Statistical analyses of all data were carried out by the MIXED procedure of SAS version 9.4 (SAS, 2002-2012; SAS Institute Inc., Cary, NC, USA) according to a complete 2x2x2 factorial (2 carnosine levels, 2 fat levels and 2 cooking conditions) and using heterogeneous variances when appropriate. And results were expressed as mean (standard error). Since the entire protocol was repeated four times, the total number of observations was 32 (8 treatments with 4 repetitions) for each phase (salivary, gastric, and duodenum phases) respectively. $P<0.05$ was considered as significant with tendency defined as $0.05 < P < 0.10$. Interactions were further analyzed using partitioned analysis (slice option of the lsmeans statement). To have a better appreciation of the correlations among the determined compounds, a Principal Component Analysis was conducted and a graph of the first two principal components was used.

4. Results and discussion

4.1 Carnosine and anserine levels

Table 5. Carnosine and anserine levels

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
Carnsoine (%)	Mouth	79.69 (2.71)	79.18 (2.71)	86.27 (2.71)	71.40 (2.71)	80.33 (2.71)	88.46 (2.71)	89.61 (2.71)	78.27 (2.71)	**.0147**	.7862	**.0229**	.9697	.1251	**.0002**	.5109
	Stomach	56.34 (5.70)	68.20 (5.70)	72.02 (5.70)	66.35 (5.70)	59.22 (5.70)	63.86 (5.70)	71.01 (5.70)	62.94 (5.70)	.7183	.1390	.8562	.8662	.5566	.0732	.7674
	Duodenum	48.68 (5.99)	40.12 (5.99)	31.67 (5.99)	37.05 (5.99)	54.45 (5.99)	27.81 (5.99)	45.74 (5.99)	38.37 (5.99)	.6066	.2928	**.0381**	.2082	.0813	.0618	.7559
Anserine (%)	Mouth	97.41 (3.53)	82.95 (3.53)	90.98 (3.53)	79.08 (3.53)	94.17 (3.53)	101.86 (3.53)	100.88 (3.53)	95.13 (3.53)	**.0003**	.3118	**.0222**	.3135	**.0092**	.2873	.1226
	Stomach	92.99 (25.74)	89.05 (25.74)	103.59 (25.74)	94.08 (25.74)	98.26 (25.74)	95.83 (25.74)	101.79 (25.74)	111.67 (25.74)	.7055	.6349	.9594	.9348	.7764	.9270	.8080
	Duodenum	100.22 (10.32)	65.46 (10.32)	58.71 (10.32)	69.25 (10.32)	93.33 (10.32)	51.14 (10.32)	72.48 (10.32)	60.74 (10.32)	.5900	.1065	**.0132**	.3740	.3191	**.0159**	.6156

CAR: carnosine; F: fat; C: cooking intensities. Data are presented as mean (SEM).
%: the percent of their respective amount measured after cooking.

As reported in Table 2, the intrinsic level of carnosine in the fresh *Longissimus* muscle before treatments was 309.8 mg carnosine/100g. After cooking, irrespective of the intensity and fat level, the average concentration in the LCar group (without added carnosine) was 350.4±10.53 mg/100g meat (not shown in tabular form). Such an increase of carnosine level in the cooked batter suggests the potential occurrence of vapor losses during the cooking process. Indeed, the average difference in weight of the meat mixture before and after cooking was 4.5±1.8 % for the LCI samples vs 8.21± 2.46% for HCI ones, irrespective of the carnosine and fat treatments indicating greater losses occurring in samples cooked at higher intensity. Filling the Nalgene cooking containers to the brim with the meat preparation as per the described procedure has likely caused a sufficient increase in internal vapor pressure to allow some vapor to escape, bringing about an increased concentration of the content, including that of carnosine level. Given the hydrophilic nature of carnosine, vapor loss appears as the most likely explanation for an increase in the dipeptide level after cooking since any water leak would have carried along solubilized carnosine resulting instead in a reduced level. On this basis, potential loss of melted fat that could perhaps form at the surface of samples upon cooking cannot be ruled out and might contribute to concentrating the carnosine. In the preparation of the HCar group, carnosine was nearly doubled by adding external carnosine to the fresh meat in order to attain 600 mg /100 g of the meat mixture before cooking. After cooking, however, the average carnosine concentration in that group, irrespective of fat level and cooking intensity, was 438.55±37.45 mg/100g which represents about 73% of the targeted level (600 mg/100 g meat). Although the reason why carnosine level in the cooked batter of the enhanced group (HCar) is lower than the expected amount is not clear, it could be hypothesized that part of added carnosine might have been more easily involved in reactions with some unmonitored compounds or molecules in the meat either raw or cooked before digestion. In addition, the partial disruption of cellular and subcellular membranes caused by mincing [159] may account for this effect. Such a partial or incomplete disruption of the membranes could have potentially contributed to limiting the free carnosine movement either the instinct carnosine moving outward or the externally added carnosine moving inward. Based on the hydrophilic nature of carnosine, externally added carnosine might tend to dissolve and get lost with vapor, resulting in reduced amount of retained carnosine in the enhanced group. Overall, the carnosine content of the cooked samples intended for digestion

remained 20% higher in the HCar group compared to the LCar one (438.5 ±37.45/100g vs 350.4±10.53 mg/100 g).

The effects of digestion on carnosine and anserine expressed as percent of their respective amount measured after cooking as indicated above are shown in Table 5. A decrease in carnosine level occurred from the undigested cooked meat and after each of the salivary, gastric and duodenal digestion phase. A significant effect of the carnosine treatment in the salivary phase indicate also that in spite of the reported loss in carnosine with respect to the amount added, the carnosine level remained higher (P = 0.0147) in the high carnosine groups as expected.

As shown by Van Hecke et al. (2014; 2015) [31-32], increased fat content and the cooking intensity can promote oxidation of meat during digestion. These factors, therefore, may solicit more carnosine for in situ protection during digestion, reducing this way its bioavailability. Indeed, increased cooking intensity induced a significant reduction in both carnosine and anserine in each of the mouth (P=0.0229 for carnosine; P=0.0222 for anserine) and the duodenum (P=0.0381 for carnosine; P=0.0132 for anserine) phase. A sparing effect of carnosine on the anserine level in the saliva is also observable in the HCar groups where anserine remained higher (P = 0.0003), in spite that the level of anserine was not enhanced to maintain the relative ratio of carnosine and anserine in pork. Indeed, increased cooking intensity caused a larger anserine decrease in the low carnosine groups as the result of the interaction between carnosine level and cooking interaction in the saliva (P = 0.0092). Although fat content had no single effect on both carnosine and anserine throughout different digestion phases (P > 0.05), its interactive effects with cooking intensity (P=0.0002) showed lower available carnosine in the salivary digest from the HF-HCI sample compared to the other combination of treatments. In the case of anserine, a decreased level was detected in the LF- HCI duodenal digest as a result of the interaction between fat and carnosine levels (P=0.0159). These results, therefore, indicate that increased fat and cooking intensity have the ability to decrease both carnosine and anserine bioavailability.

Irrespective of the treatment, the level of available carnosine decreased progressively from the salivary to the gastric and duodenal phase as shown in Table 5. These results may be explained by the involvement of carnosine as an anti-oxidant in line with the pro-oxidative environment of the gastrointestinal tract [116] although the involvement of other properties of carnosine such as its pH buffering, carbonyl scavenging and metal ions chelation capacity cannot be precluded.

Unlike carnosine, a decrease in anserine was only observed between the stomach and duodenum phase while an apparent but small increase (6.53±52.23μg anserine/ml digest average values for the treatments) was measured between the salivary and gastric phases. Compared to the large decrease observed in carnosine between salivary and gastric phases (Δ = 295.73±235.35 μg carnosine/ml digest) the increase in anserine is minor and may have no other explanation than the experimental error and overall is unlikely to have any practical significance given anserine in pork did not represent more than 10% of the muscle carnosine content and this is reflected in the average amount of different digestion phases being 1081.44±374.12 μg carnosine/ml and 101.28±36.64 μg anserine/ml, respectively. Of particular interest, however, is the overall decrease of more than 50% that occurred in carnosine from the salivary to the duodenal phase while that of anserine occurred to a much lower extent. This suggests that carnosine activity during digestion might be more efficient than that of anserine considering the experimental conditions of the study, and/or due to its higher proportion, the probability of carnosine reacting with other components of the meat matrix during the mixing occurring during digestion was higher than that of anserine. In addition, it cannot be ruled out that according to a study with both serum and kidney carnosinase, in spite of the absence of carnosinase in the digestive fluid, carnosine methylation products, such as anserine, might be more resistant to enzymatic hydrolysis [160], hence anserine might have an increased resistance to digestion than carnosine. However, this aspect needs further investigation.

4.2 Lipid oxidation

4.2.1 Hexanal

Table 6. Hexanal concentrations

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
Hexanal	Mouth	117.69 (3.82)	90.64 (3.31)	140.67 (3.31)	219.47 (3.31)	82.11 (3.82)	106.35 (3.31)	134.88 (3.31)	252.33 (3.31)	.4683	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	.2076
(μg/ml digest)	Stomach	126.34 (7.19)	166.26 (7.19)	212.33 (7.19)	318.62 (8.30)	44.42 (7.19)	119.52 (7.19)	215.16 (7.19)	380.43 (7.19)	**.0052**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	.2633
	Duodenum	168.31 (7.24)	170.70 (7.24)	455.41 (24.32)	509.13 (24.32)	90.89 (7.24)	158.25 (7.24)	437.97 (24.32)	531.88 (24.32)	.1087	**<.0001**	**.0003**	.0729	**.0492**	.1379	.6299

CAR: carnosine; F: fat; C: cooking intensities.

Data are presented as mean (SEM).

Results of hexanal concentrations determined in the different digestion phases are presented in Table 6 and show the increase during digestion in most treatments, indicating the continuously increased lipid oxidation. Within each phase, additionally, hexanal formation was significantly increased by higher fat content ($P<0.0001$) and also by cooking intensity ($P<0.0001$), which have been documented for their pro-oxidative effect in meat [31-32, 163]. These results may explain in part the reported decrease in carnosine throughout digestion. In addition to single effect, fat content and cooking intensity also statistically interacted with carnosine level in each phase. The partitioned analysis (not presented) of the significant interactions obtained between carnosine level and fat content in the salivary ($P<0.0001$) and gastric ($P<0.0001$) along with a trend in the duodenal phase ($P=0.0729$), indicating that increasing the carnosine level in the meat was effective in decreasing hexanal formation in the low-fat treatment in each of the three phases. This result further demonstrates, from the health point of view, the benefit of either increasing muscle carnosine level or limiting the fat content of meat products. This reduction in lipid oxidation by carnosine is consistent with the results of Decker et al. [161] who showed decreased lipid oxidation with increase carnosine in cooked ground pork using thiobarbituric acid reactive substances (TBARS) measurements. In incubating hexanal in presence of carnosine in a phosphate buffer, Zhou et al. [162] provided further evidence of the potential of carnosine to directly decrease hexanal level. Interaction of carnosine with cooking intensity was also observed in each of salivary ($P<0. 0001$), gastric ($P<0. 0001$), and duodenal phase ($P=0.0492$). The partitioned analysis of this interaction indicates that lower hexanal was formed in the meat from the HCar-LCI group than in other combination of treatments. Interaction of fat content and cooking intensity was also observed in the salivary ($P<0.0001$) and gastric ($P<0.0001$) phase and show the synergistic effect of these two factors conducing to the highest hexanal formation in not only the high-fat samples cooked at high intensity in the salivary digest but also in both the LF-HCI and HF-HCI gastric digests as revealed by the partitioned analysis (not showed).

This study is reporting for the first time that carnosine can have a protective effect toward lipid oxidation occurring in the gastrointestinal tract. However, the carnosine level in the enhanced treatment was not sufficient to completely counteract the oxidation brought about by increased fat and cooking intensity particularly in the combined high-fat group cooked at a higher intensity. This limitation suggests a need for further increasing the carnosine level in some meat animals and/or also taking meat composition and processing into account for additional health

benefits. Although at the level reported in this study carnosine could not reduce hexanal formation in all treatments, the decrease in hexanal brought about by higher carnosine in LF groups and LCI groups reflects that with milder cooking and average intrinsic fat of pork (representing the level found in most commercial pigs), higher carnosine level in meat has the potential to contribute to the reduction of hexanal absorption through the intestine [164], following meat consumption, and also the reduction of potential adverse effects after hexanal being absorbed.

4.2.2 4-Hydroxynonenal (4-HNE)

Table 7. 4-HNE concentrations

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
4-HNE	Mouth	2.13 (0.06)	2.65 (0.03)	4.94 (0.02)	4.74 (0.15)	2.04 (0.09)	3.04 (0.36)	4.93 (0.12)	4.57 (0.19)	.7915	**<.0001**	.0511	.3148	.4905	**.0002**	.1893
(μg/ml digest)	Stomach	1.88 (0.30)	3.58 (0.30)	11.99 (1.01)	11.67 (1.01)	1.14 (0.03)	1.56 (0.03)	10.31 (0.35)	8.77 (0.35)	**<.0001**	**<.0001**	.8712	.2613	.1282	**.0189**	.9676
	Duodenum	1.20 (0.07)	1.19 (0.07)	8.03 (0.42)	5.70 (0.42)	0.07 (0.07)	1.28 (0.07)	8.26 (0.42)	6.72 (0.42)	.8032	**<.0001**	**.0042**	**.0119**	**.0255**	**<.0001**	.6000

CAR: carnosine; F: fat; C: cooking intensities.

Data are presented as mean (SEM).

Results for 4-HNE as an n-6 PUFAs peroxidation product are reported in Table 7. Contrary to hexanal, which showed an increase during the digestion process, the 4-HNE level generally increased from salivary to gastric phase (except in the LF-LCI combinations and HCar-LF-HCI group), followed by a decrease in the duodenal phase. A similar phenomenon has been reported and explained that the decrease in the duodenum could be attributed to the capacity of 4-HNE to react further with other compounds such as DNA and protein [30-31].

Similar to hexanal, however, increasing fat content increased the formation of 4-HNE in each of saliva ($P< 0.0001$), gastric ($P< 0.0001$) and duodenal ($P< 0.0001$) phase (Table 7). The effect of high cooking intensity throughout the three phases was more clearly defined through its interaction with the fat treatments ($P=0.002$, $P=0.0189$, $P<0.0001$, respectively for the salivary, gastric and duodenal phase) indicating its significant synergistic effect in enhancing 4-HNE formation in the low-fat samples in each phase. There is no clear explanation for no such significance in high-fat groups which is counterintuitive, given the known pro-oxidative effect of

fat. Only the hypothesis that there is a protective effect of increased fat content toward higher cooking intensity. However, there is no sufficient evidence on this and need further investigation. No single or interactive effect of carnosine was found in the salivary ($P > 0.05$) but in the gastric phase, increased carnosine significantly decreased 4-HNE concentrations ($P<0.001$). In the duodenum, an interactive effect of carnosine with fat ($P = 0.0119$) was obtained where, similar to hexanal, increased carnosine led to reduced 4-HNE in low fat groups, particularly in the HCar-LF-LCI one where 4-HNE got reduced below its detection limit. As the result of the carnosine by cooking interaction in duodenal phase ($P < 0.0255$), LCar-LCI and LCar-HCI groups were associated with the highest and lowest mean 4-HNE concentrations, respectively, compared with high carnosine groups. In spite of the pro-oxidative effect of high cooking intensity, such a result might be due to the high sensitivity of the method used for the determination of 4-HNE [155] because not only the difference was small (0.30 (0.14) μg /ml without calculating the dilution factor), but also the partitioned analysis of this interaction could not detect any significant effect of carnosine ($P>0.05$) in either LCI or HCI duodenal groups.

Nevertheless, the decrease in 4-HNE brought about by carnosine in the stomach and also in the duodenal digest from the low-fat groups agree with the results of Aldini et al. (2002) showing the efficiency of carnosine as a quencher of 4-HNE in phosphate buffer [165]. Therefore, the ability of carnosine in decreasing 4-HNE level during digestion represents another important health benefits for the consumer as it can contribute to reducing the absorption of genotoxic and cytotoxic 4-HNE [7].

4.2.3 Malondialdehyde (MDA)

Table 8. MDA concentrations

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
MDA	Mouth	81.98 (5.73)	87.09 (1.04)	113.83 (1.02)	110.05 (2.39)	65.19 (1.47)	75.10 (5.40)	107.41 (2.18)	107.70 (2.82)	**.0005**	**<.0001**	.2233	**.0403**	.3459	.0562	.9379
(μg/ml digest)	Stomach	96.65 (1.01)	137.74 (4.43)	237.05 (14.52)	254.15 (6.34)	50.65 (0.52)	103.59 (4.36)	237.28 (16.56)	246.29 (4.04)	**.0013**	**<.0001**	**<.0001**	**.0061**	.8774	**.0095**	.4163
	Duodenum	114.72 (7.40)	119.94 (7.40)	247.15 (7.40)	229.81 (7.40)	45.23 (7.40)	76.57 (7.40)	221.34 (7.40)	220.75 (7.40)	**<.0001**	**<.0001**	.3820	**.0010**	.0516	**.0156**	.6586

CAR: carnosine; F: fat; C: cooking intensities.

Data are presented as mean (SEM).

Table 8 shows the MDA levels according to the treatments during the digestion process. Compared to the salivary phase, MDA generally increased in the gastric phase to different extent depending on the treatment, followed by the decrease thereafter in the duodenum, similar to what was observed for 4-HNE. Also similar to 4-HNE, the decreased MDA levels between the stomach and duodenum phases may also be the result of MDA reacting with other molecules [16, 166] as discussed below.

As a single factor, high cooking intensity raised MDA concentration in the stomach ($P<0.0001$) while increased fat contributed to increasing MDA levels in all phases ($P<0.0001$). In addition, however, fat level also significantly interacted with cooking intensity in gastric ($P=0.0095$) and duodenal ($P=0.0156$) phases along with a trend in the salivary phase ($P = 0.0562$), all showing that increased cooking lead to significantly more MDA formation in the LF meat, while in the HF one the already significantly higher MDA was not affected by cooking intensity. These results are in accordance with those of other research which shows increased oxidation occurring during digestion procedure following increased cooking conditions and fat level on the formation of 4-HNE and MDA, as well as protein oxidation [32]. Increased carnosine, however, decreased MDA formation in salivary ($P=0.0005$), gastric ($P=0.0013$), and duodenal ($P < 0.0001$) phases and this effect was more effective in the low-fat groups as revealed by the interactions found in the mouth ($P = 0.0403$, decreasing from 84.54 (2.91) in LCar-LF to 70.14 (2.80) μg /ml in HCar-LF), stomach ($P = 0.0061$, decreasing from 117.19 (2.27) in LCar-LF to 77.12 (2.20) μg /ml in HCar-LF) and duodenum ($P = 0.0010$, decreasing from 117.33 (5.23) in LCar-LF to 60.90 (5.23) μg /ml in HCar-LF), as well as in the high-fat duodenal digest ($P=0.0363$, decreasing from 238.38 (5.23) in LCar-HF to 221.05 (5.23) μg /ml in HCar-HF). These reductions of MDA attributed to carnosine are consistent with several studies carried out in phosphate buffer, reporting carnosine decreasing MDA level as reviewed by Colzani et al. (2015) [167]. Considering that human can absorb MDA from ingested meat [168], reducing its formation in the gastrointestinal tract through consuming meat with higher carnosine content and hence reducing its absorption in the duodenum before it reaches the systemic circulation [7] could potentially contribute to reducing the potential formation of both DNA adduct [10] and LDL adduct with MDA in the body [166].

Altogether, the dynamic of hexanal, 4-HNE, and MDA as markers of lipid oxidation during digestion was generally indicative of the pro-oxidative environment occurring in the gastrointestinal tract. The oxidation occurring during digestion from mouth to duodenum has already been demonstrated by an in vitro study on lipid digestion [33]. Indeed, studies with a similar cooking procedure and digestion process [31-32] have shown that increasing fat content in pork from 1% to 5% resulted in increased level of hexanal, 4-HNE and MDA in both duodenal and colonic digest, and the use of higher cooking intensity also increased 4-HNE and MDA concentrations in digests. Carnosine has already been shown to reduce lipid oxidation in cooked meat such as decreased TBARS values in ground pork with 0.5%-1.5% (500mg-1500mg/100g raw meat) carnosine added [161] Reduced MDA-TBA (thiobarbituric acid) level was also reported in chicken patties with 1.5% carnoine (1500mg in each100g raw meat) added [169]. This study, however, is, to the best of my knowledge, the first report of the capacity of carnosine to reduce lipid oxidation during the digestion of meat. In the experiment, the addition of about 300mg carnosine/100g raw meat to obtain a targeted 600 mg carnosine/100 g meat only resulted in an additional 88.15±27.47mg carnosine/100g cooked meat in comparison with the intrinsic level measured in the control. This however implies that a much lower level of carnosine can still significantly decreased different lipid oxidation markers during digestion and even reduced 4-HNE level below the detection limit in the digest of the LF-LCI samples. Such an increase in pork could naturally be obtained by breeding, given the large variability that exists between individual pig [170].

Apart from hexanal which showed a constant increase during digestion, irrespective of the treatments, according to Table 7 and Table 8, the average concentrations of 4-HNE and MDA decreased respectively from 6.36 (0.82) to 4.06 (0.59) μg /ml digest and 170.42 (14.02) to 159.44 (13.47) μg /ml in the digest from the stomach and duodenum respectively, likely attributable to their capacity in attacking and forming stable adducts with protein during digestion [168]. According to studies [10, 171], MDA tends to react with basic amino acids, such as lysine [172], while 4-HNE can react with amino acyl chains: Cys, His, and Lys residues of the protein [173]. Indeed, the protein modification caused by 4-HNE and MDA has been reported after in vitro digestion of pork [32]. 4-HNE and MDA can also react with thiols (forming hemiacetal and providing the general structure $(RS)_2$-CH=CH-CHO [36], respectively) which may also contribute to their respective decline. Hexanal, however, whose reactions with primary amines are highly

reversible, is less likely to form a stable adduct, which may explain its stability and even increase during digestion. The research on interference between hexanal and different compounds including amino acids after incubation in phosphate buffer supports this explanation since no significant reduction in hexanal level was reported in the presence of different amino acids (except histidine) even at a 10-fold excess level [160].

4.3 Protein oxidation

4.3.1 Protein carbonyls

Table 9. Protein carbonyl levels

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
Carbonyls	Mouth	2.21 (0.18)	3.38 (0.18)	2.80 (0.18)	2.51 (0.18)	2.37 (0.18)	2.73 (0.18)	2.86 (0.18)	2.87 (0.18)	.8921	.5134	**.0240**	.0906	.3302	**.0018**	**.0441**
(nmol/mg	Stomach	2.93 (0.24)	3.11 (0.24)	4.42 (0.24)	3.83 (0.24)	2.54 (0.24)	2.98 (0.24)	3.29 (0.24)	3.84 (0.24)	**.0257**	**<.0001**	.4150	.3878	.0555	.3461	.2111
protein)	Duodenum	3.24 (0.40)	3.84 (0.30)	4.89 (0.76)	5.07 (0.06)	2.58 (0.19)	3.43 (0.20)	4.90 (0.51)	5.15 (0.13)	.3717	**<.0001**	.3032	.0937	.7682	.3525	.8681

CAR: carnosine; F: fat; C: cooking intensities.

Data are presented as mean (SEM).

Protein carbonyl levels expressed per mg of protein in the digest, are presented in Table 9. Similar to hexanal, an overall increase in protein carbonyls is observable from salivary to duodenal phase irrespective of the treatments which are consistent with the reported pro-oxidative environment that represents the gastrointestinal tract [152].

In the salivary phase, only cooking conditions showed a single effect (P=0.0240) but more importantly, interactions with fat (P=0.0018) and also the triple interaction of the treatments (P=0.0441) was obtained. The partitioning of the triple interaction indicated that the significance mainly came from the difference between the two cooking conditions in the low carnosine-low fat groups (P=0.0007) with more protein carbonyl being formed under the more intense cooking condition. Such an effect in low carnosine groups suggests a possibly physical even protective effect of added fat toward an increase in cooking intensity. There is little information supporting this hypothesis, except for some studies on mutagens in meat products reporting decreased mutagen levels in beef containing more fat (15% vs 5%; 30% vs 15%) and authors indicating

that the possible explanation could be the dilution of mutagenic precursors in meat by additional fat, without providing further evidence or explanation [174-175]. In the stomach, however, while cooking was the only factor that had no significant single impact (P>0.05), increasing fat content lead to higher protein carbonyl formation (P<0.0001). Increased carnosine, on the other hand, significantly decreased the formation of protein carbonyl (P=0.0257). The protecting effect of carnosine in the stomach was no longer present in the duodenum where the increased fat level was the only significant factor (P<0.0001) causing a large increase in protein carbonyls. Since the difference in carnosine level (between low-carnosine and high-carnosine groups) after gastric digestion was small (0.22±0.10 mg/ml digest on average, P>0.05), it is possible that carnosine was becoming limited with respect to counteracting the continuously increased protein carbonyls measured in high fat groups. Nevertheless, the decrease in protein carbonyls caused by increased carnosine level is a further evidence that carnosine can act as an antioxidant for the protection of not only lipids but also protein in a more complex environment such as the digestive system and also support the carnosine carbonyl scavenging property. These results, therefore, are in line with the antioxidant and carbonyl scavenging properties of carnosine [17, 176]. Scientists have shown that carnosine can form protein carbonyl-carnosine adducts, thereby preventing further related modifications [176]. It is also documented that increasing fat content and cooking intensity can both increased protein carbonyl formation. Indeed, researchers have shown that pork samples containing 20% fat had higher protein carbonyl than samples containing 1% fat [31]. At 100 °C, Traore et al. [177] have observed greater protein carbonyl level in pork samples cooked for 30min than in those cooked for 10min.

4.3.2 Thiols

Table 10. Thiols levels

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
Thiols	Mouth	44.87 (1.77)	38.92 (1.77)	30.90 (2.05)	25.46 (1.77)	46.51 (1.77)	47.04 (1.77)	34.77 (1.77)	32.38 (1.77)	**.0005**	**<.0001**	**.0164**	.8420	.0753	.6427	.5087
(μol/mg	Stomach	21.96 (2.42)	23.50 (2.42)	11.91 (2.42)	12.90 (2.42)	25.90 (2.42)	26.19 (2.42)	15.50 (2.42)	18.91 (2.42)	**.0261**	**<.0001**	.3709	.6671	.8659	.7093	.5960
protein)	Duodenum	6.38 (1.65)	11.78 (1.65)	8.18 (1.65)	6.60 (1.65)	5.10 (1.65)	8.31 (1.65)	4.00 (1.65)	7.97 (1.65)	.1194	.3140	**.0272**	.6827	.4787	.1956	.1110

CAR: carnosine; F: fat; C: cooking intensities.
Data are presented as mean (SEM).

Table 10 provides concentrations of thiols which gradually decreased throughout the digestion process from mouth to duodenum, irrespective of the treatments, indicating increased oxidation. The decrease in free thiol group can occur through the formation of disulfide bridges during heating and which have been reported to result in decreased protein digestibility [178].

In the mouth, increased fat level ($P<0.0001$), and cooking intensity ($P=0.0164$) resulted in significantly less free thiol remaining, in agreement with results reported above on lipid oxidation markers and protein carbonyls showing increased oxidation during digestion. Increased carnosine in meat, however, led to a significantly higher ($P=0.0005$) thiols level, in line with its anti-oxidant capacity [179]. In the gastric phase, the same pro-oxidant effect of increased fat ($P<0.0001$) and anti-oxidant effect of increased carnosine ($P=0.0261$) on respectively decreased and increased thiols were observed although cooking had no further effect ($P>0.05$). However, these effects of fat and carnosine observed in the salivary and gastric phases disappeared in the duodenum ($P>0.05$) but surprisingly, the statistical analysis revealed that increased cooking intensity led to increased thiols level ($P=0.0272$) except in the LCar- HF group. Given that oxidation was favored in this particular LCar-HF-HCI group, it is possible that interactions between thiols and other compounds may have occurred, in particular with lipid oxidation compounds since reactions of thiols have been reported with 4-HNE [34] and MDA [10,171] which could also contribute to explaining the reduced duodenal level of 4-HNE and MDA reported earlier.

In general, the respective increase in protein carbonyl and the decrease in thiol levels due to oxidation during digestion and amplified by both increase fat content and cooking intensity is consistent with other studies [31-32,177] and also with the results on lipid oxidation showing increased oxidation throughout the digestive process as reported earlier. The disappearance of the significant protective effect of carnosine toward protein oxidation (protein carbonyl and thiol) in the duodenum where the effect of carnosine remained significant in decreasing lipid oxidation suggests that the oxidation of protein was induced by that of lipid which likely contributed first in depleting carnosine to a level that became less effective for reducing protein oxidation. Although some proteins, such as myoglobin, also contribute to lipid oxidation in muscle food [180], it is documented that oxidation of lipid could generally lead to that of protein in meat [13, 181-182] although the reverse mechanism is also possible [181]. More importantly, the

reported increase in oxidation during digestion was accompanied by a gradual decrease in bioavailable carnosine that was concomitant with a reduction of most of the oxidation markers. Indeed, irrespective of treatments, only 64.99% carnosine (with respect to carnosine level after cooking) was left after digestion in the mouth and stomach for further reactions in the duodenum. What's more, there was 1230.92 (48.32) μg/ml digest (on average) left after gastric digestion in HCar groups while the average level left in LCar groups was 1010.58 (48.32) μg/ml digest. The difference between LCar and HCar was relatively minor (0.22 ± 0.10 mg/ml digest) and might be not enough to cause a difference in the duodenum.

4.4 N (epsilon)-(carboxymethyl)lysine level

Table 11. CML levels

		Low Carnosine (LCar)				High Carnosine (HCar)				P Values						
	Phase	Low Fat (LF)		High Fat (HF)		Low Fat (LF)		High Fat (HF)								
		Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	Low cooking intensity (LCI)	High cooking intensity (HCI)	CAR	F	C	CAR×F	CAR×C	F×C	CAR×F×C
CML	Mouth	17.08 (2.44)	19.04 (3.83)	25.82 (2.77)	68.13 (3.24)	13.83 (1.23)	17.66 (0.91)	12.48 (0.50)	15.30 (0.95)	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**
(ng/g meat)	Stomach	101.17 (10.02)	125.20 (24.22)	74.28 (2.84)	148.92 (16.19)	47.61 (11.09)	91.30 (5.50)	121.21 (12.48)	108.07 (28.08)	.0857	.0668	**.0090**	.0505	.1465	.8918	**.0265**
	Duodenum	635.79 (21.51)	832.01 (69.03)	1033.04 (77.25)	1529.71 (112.53)	596.92 (65.60)	741.41 (93.62)	854.11 (102.56)	924.52 (79.06)	**.0012**	**<.0001**	**.0013**	**.0123**	.0561	.3435	.1259

CAR: carnosine; F: fat; C: cooking intensities.

Data are presented as mean (SEM).

Levels of CML are shown in Table 11. Increase in CML, as a marker for AGEs formation, occurred and increased to a significant extent during digestion irrespective of treatments. This confirms the recent suggestion by Hipkiss (2018) that AGEs formation may occur in the digestive tract [183] following the report of dietary fructose, a very strong glycating carbohydrate, being a trigger of AGEs formation in the intestinal tract [184]. Increased cooking intensity led to increased CML levels in each of the salivary ($P<0.0001$), gastric ($P=0.0090$) and duodenal ($P=0.0013$) phase, while increased fat content lead to higher CML in the salivary ($P < 0.0001$) and duodenal ($P < 0.0001$) phases along with a tendency in the stomach ($P = 0.0668$) indicating their potential contribution in enhancing AGEs formation, in agreement with the results from Uribarri et al. (2005) that foods containing higher fat level and cooked at higher temperature are more prone to generate AGEs [19].

As a single factor, carnosine was effective in reducing CML formation in the mouth ($P < 0.0001$) and duodenum ($P = 0.0012$), with a trend also observed in the stomach ($P = 0.0857$). In salivary ($P < 0.0001$) and duodenal ($P = 0.0123$) phases, the higher level of CML was found in samples from the low carnosine group containing higher fat level as the result of the carnosine and fat interaction. In addition, high-intensity cooking tended to raise CML levels in salivary phase, with an increase to a larger extent in the low carnosine group ($P<0.0001$) as the result of the interaction between carnosine and cooking intensity. Moreover, a triple interaction of treatments was observed in the gastric phase where less CML was formed in the digest from HCar-LF-LCI groups ($P = 0.0265$), and another one was found in salivary phase ($P < 0.0001$) with the highest CML level determined in the LCar-HF-HCI groups, compared with other treatments, further emphasizing the efficacy of carnosine in reducing AGEs formation in the digestive environment. It cannot be ignored, however, the chemical environment, such as food matrix, may have influence on the result of ELIZA. For instance, fresh olive oil has 1000-fold more CML than milk with ELIZA technique [18] while olive oil is unlikely to contain a very high level of amino acid products [185]. Although food matrix may have impacts on CML determination, the antibody is selective to CML and the competitive ELIZA is used for a public database [18]. In general, carnosine still shows the ability of decreasing CML level in meat which is also in accordance with its reported property of inhibiting AGEs formation, as reviewed by Hipkiss (2009) [26]. This study is also reporting for the first time the ability of carnosine to inhibit AGEs formation in the gastrointestinal environment. Provided meat is not overcooked or contains too much fat, these results indicate that ingestion of carnosine from meat has the potential to reduce the health risk associated with dietary AGEs [19-20]. Indeed, it has been reported that 10%–30% of dietary AGEs is potentially absorbed, of which two-thirds are not excreted by urine and feces and therefore can be retained in tissues [19]. Consequently, the significant decrease in CML due to higher carnosine level particularly in the duodenum where their formation is the most prevalent emphasizes the beneficial effect of consuming meat containing higher level of carnosine.

4.5 Principal component analysis

Although this study focuses less on anserine, anserine is still included into principal component analysis. Because the sparing effect observed in carnosine and anserine quantification reveals that anserine is related to carnosine level changes; and anserine shares similar properties with

carnosine such as antioxidative ability, AGEs formation reduction, as well as similar in vivo functions such as putative neurotransmitter in brain and anti-fatigue ability in muscle [17, 107,186-188].

Results of the analysis are shown in Figure 3 and provide a good overview of the relationships between the different variables which accounted for over 75% of the total variance, with Principal Component 1 (the PC 1 axis), which appears to represent the oxid-reduction axis, alone explaining over 53% of the total variance. This also leaves the possibility that other unmeasured compounds might have reacted with carnosine or anserine and possibly also with thiol groups or other muscle components such as creatine which has been reported to have antioxidant property [189]. The positive association among the antioxidant compounds (carnosine and anserine, including also the thiol groups) in the upper left quadrant suggests their potential in sparing each other. The positive association between lipid oxidation products (hexanal, 4-HNE, and MDA) and protein oxidation products (protein carbonyl) in the upper right quadrant supports the interaction between lipid and protein oxidation as well as the potential oxidation of protein brought about by that of lipids as discussed before. More importantly, it is the opposition, hence the negative correlation between the antioxidant and oxidized compounds along the PC 1 axis that portrays the effective contribution of carnosine, anserine and thiols in reducing the formation of different oxidation markers shown in Figure 3 as presented throughout this document. The overall implication of the results in Figure 3, additional to the important role of dietary carnosine and other antioxidant compounds in reducing the formation and hence the potential absorption of health deleterious compounds, is the corollary showing at the same time how reducing the pro-oxidants in the diet can contribute to sparing carnosine leaving this way a higher bioavailable level for further health benefits for the consumers.

Of particular interest however, the negative correlation existing between CML and other oxidative markers, mainly lipid peroxidation products 4-HNE and MDA on PC 2 suggests that in meat, although the Maillard reaction cannot be ruled out from the reported markers, the formation of AGEs, especially CML, would derive more from lipid oxidation, although no direct link can be made with the markers measured. Nevertheless, the 24.4% of unexplained total variance by this analysis suggests that other molecules and reactions not measured in the study,

such as glycoxidation which is also related to CML formation [190], could also contribute and indicates a need for additional study.

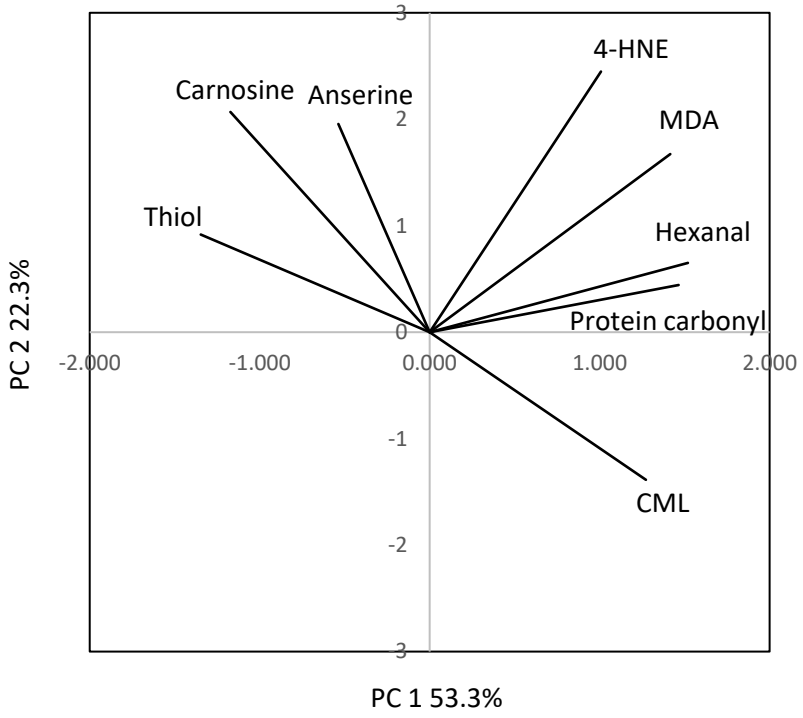

Figure 3. Principal component analysis

5. Conclusion and future prospects

Because of the presence of plasma carnosinase in humans, it remains unclear where and more importantly how can dietary carnosine be effective in terms of global health benefits and more particularly in tissues devoid of carnosine synthase considering the recent studies report the advantages of carnosine supplementation. For instance, carnosine supplementation shows protection against diabetes development in non-diabetic obese individuals [131] and in attenuating fasting glucose, triglycerides, AGEs and TNF-α levels in patients with type 2 diabetes [191]. In addition to glycotoxins formed in food at high temperature, recent research [183] raised the possibility that glycation might also occur in the digestive tract. Results of the study presented here possibly represent the first ever demonstration of the efficacy of carnosine in the gastrointestinal tract in reducing not only AGEs formation but also lipid oxidation products (hexanal, 4-HNE and MDA) and protein oxidation products (protein carbonyls) involved in the aetiology of diseases occurring in different organs or tissues in the body. It is important also to

point out that these beneficial effects were obtained by an increase of only about 25% carnosine in the cooked meat. Therefore, the carnosine functional effects showed in the gastrointestinal tract in vitro represent additional health benefits of dietary carnosine. Such activity of carnosine in the gastrointestinal environment is possibly a complementary but nonetheless potentially important contribution to its global health benefits through increased bioavailability and /or reduction of other deleterious compounds. Indeed, the concomitant decrease in bioavailable carnosine and AGEs along with both lipid and protein oxidation products points on the importance of a balanced omnivore diet that could potentially interfere with toxic compounds sparing and therefore increasing the available carnosine so that it can be absorbed in the intestine and protect cells and organs such as the intestinal epithelial cells [192-193] and blood vessel as reviewed above.

Moreover, the study presented here only included meat, which in general is usually consumed as part of a complete meal involving components that could interfere with or favor the roles of carnosine. Therefore, assessing through in vitro digestion, the functional roles of carnosine-enhanced meat as part of a complete meal system varying in composition and preparation would provide a better understanding of the potential of dietary carnosine in particular and the importance of meat in the diet in general. Such studies would be helpful in understanding the discrepancy between the epidemiological studies associating meat and processed meat consumption with the risk of developing chronic diseases and the impressive health benefits of carnosine that can only be found in muscle food.

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7. References

[1] McAfee, A. J., McSorley, E. M., Cuskelly, G. J., Moss, B. W., Wallace, J. M., Bonham, M. P., & Fearon, A. M. (2010). Red meat consumption: an overview of the risks and benefits. *Meat Science, 84, 1, 1-13.*

[2] Larsson, S. C., & Wolk, A. (2006). Meat consumption and risk of colorectal cancer: A meta-analysis of prospective studies. *International Journal of Cancer, 119, 11, 2657-2664.*

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

[4] Kaluza, J., Wolk, A., & Larsson, S. C. (2012). Red meat consumption and risk of stroke: a meta-analysis of prospective studies. *Stroke, 43, 10, 2556-2560.*

[5] Wolk, A. (2017). Potential health hazards of eating red meat. *Journal of Internal Medicine, 281, 2, 106-122*

[6] Greenwald, S. E. (2007). Ageing of the conduit arteries. *The Journal of Pathology, 211, 2, 157-172.*

[7] Grootvekl, M., Atherton, M. D., Sheerin, A. N., Hawkes, J., Blake, D. R., Richens, T. E., et al. (1998). In vivo absorption, metabolism, and urinary excretion of a,b-unsaturated aldehydes in experimental animals. Relevance to the development of cardiovascular diseases by the dietary ingestion of thermally stressed polyunsaturate-rich culinary oils. *The Journal of Clinical Investigation, 101, 6, 1210.*

[8]Bastide, N. M., Pierre, F. H., & Corpet, D. E. (2011). Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. *Cancer Prevention Research (philadelphia, Pa.), 4, 2, 177-84.*

[9] Calder, P. C., Ahluwalia, N., Brouns, F., Buetler, T., Clement, K., Cunningham, K., Esposito, K.,et al. (2011). Dietary factors and low-grade inflammation in relation to overweight and obesity. *The British Journal of Nutrition, 106, 5-78.*

[10] Antonio A., Mario F. M., & Sandro A. (2014). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity, 2014.*

[11] Stadtman, E. R., & Berlett, B. S. (1997). Reactive oxygen-mediated protein oxidation in aging and disease. *Chemical Research in Toxicology, 10, 5, 485-94.*

[12] Berlett, B. S., & Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *The Journal of Biological Chemistry, 272, 33, 20313-6.*

[13] Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods: A review. *Molecular Nutrition & Food Research, 55, 1, 83-95.*

[14] Shacter, E. M. (2000). Quantification and Significance of Protein Oxidation in Biological Samples. *Drug Metabolism Reviews, 32, 307-326*.

[15] Davies, K. J. A. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie, 83, 3, 301-310.*

[16] Grune, T., Jung, T., Merker, K., & Davies, K. J. A. (2004). Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and ‘aggresomes’ during oxidative stress, aging, and disease. *International Journal of Biochemistry and Cell Biology, 36, 12, 2519-2530.*

[17] Boldyrev, A. A., Aldini, G., & Derave, W. (2013). Physiology and pathophysiology of carnosine. *Physiological Reviews, 93, 4, 1803-45.*

[18] Uribarri, J., Woodruff, S., Goodman, S., Cai, W., Chen, X., Pyzik, R., Yong, A. et al. (2010). Advanced glycation end products in foods and a practical guide to their reduction in the diet. *Journal of the American Dietetic Association, 110, 6, 911-16.*

[19]] Uribarri, J., Cai, W., Sandu, O., Peppa, M., Goldberg, T., & Vlassara, H. (2005). Diet-Derived Advanced Glycation End Products Are Major Contributors to the Body's AGE Pool and Induce Inflammation in Healthy Subjects. *Annals of the New York Academy of Sciences, 1043, 1, 461-466.*

[20] Yamagishi, S., Ueda, S., & Okuda, S. (2007). Food-Derived Advanced Glycation end Products (AGEs): A Novel Therapeutic Target for Various Disorders. *Current Pharmaceutical Design, 13, 27, 2832-2836.*

[21] Kellow, N. J., & Savige, G. S. (2013). Dietary advanced glycation end-product restriction for the attenuation of insulin resistance, oxidative stress and endothelial dysfunction: a systematic review. *European Journal of Clinical Nutrition, 67, 3, 239-248.*

[22] Gulewitch, WS, Amiradzibi, S. (1900) Über das carnosine, eine neue organische Base des Fleischextraktes. *Ber Deutsch Chem Ges B33: S1902-1903.*

[23] Aristoy, M.C., & Toldrá, F. (1998). Concentration of free amino acids and dipeptides in porcine skeletal muscles with different oxidative patterns. *Meat Science, 50, 3, 327-332.*

[24] Aristoy, M. C., & Toldrá, F. (2004). Histidine dipeptides HPLC-based test for the detection of mammalian origin proteins in feeds for ruminants. *Meat Science, 67, 2, 211-217.*

[25]Hipkiss, A. R. (2005). Glycation, ageing and carnosine: Are carnivorous diets beneficial? *Mechanisms of Ageing and Development, 126, 10, 1034-1039.*

[26] Hipkiss, A. R. (2009). Chapter 3 Carnosine and Its Possible Roles in Nutrition and Health. *Advances in Food and Nutrition Research. 57, 87-154.*

[27] Finkel, S. E., Pepper, E. D., Farrell, M. J., & Nord, G. (2010). Antiglycation effects of carnosine and other compounds on the long-term survival of *Escherichia coli*. *Applied and Environmental Microbiology, 76, 24, 7925-7930.*

[28] Bellia, F., Vecchio, G., Cuzzocrea, S., Calabrese, V., & Rizzarelli, E. (2011). Neuroprotective features of carnosine in oxidative driven diseases. *Molecular Aspects of Medicine, 32, 4-6.*

[29] Sale, C., Saunders, B., & Harris, R. C. (2010). Effect of beta-alanine supplementation on muscle carnosine concentrations and exercise performance. Amino Acids : the Forum for Amino Acid, *Peptide and Protein Research, 39, 2, 321-333.*

[30] Steppeler, C., Haugen, J.-E., Rødbotten, R., & Kirkhus, B. (2016). Formation of Malondialdehyde, 4-Hydroxynonenal, and 4-Hydroxyhexenal duringin VitroDigestion of Cooked Beef, Pork, Chicken, and Salmon. *Journal of Agricultural and Food Chemistry, 64, 2, 487-496.*

[31] Van, Hecke. T., Vossen, E., Vanden, B. J., Raes, K., Vanhaecke, L., De, S. S., & Coulombe, R. A. (2014). Fat Content and Nitrite-Curing Influence the Formation of Oxidation Products and NOC-Specific DNA Adducts during In Vitro Digestion of Meat. *Plos One, 9, 6.*

[32] Van, Hecke. T., Vossen, E., Hemeryck, L. Y., Vanden, B. J., Vanhaecke, L., & De, S. S. (2015). Increased oxidative and nitrosative reactions during digestion could contribute to the association between well-done red meat consumption and colorectal cancer. *Food Chemistry, 187, 29-36.*

[33] Hur, S. J., Lim, B. O., Park, G. B., & Joo, S. T. (2009). Effects of Various Fiber Additions on Lipid Digestion during In Vitro Digestion of Beef Patties. *Journal of Food Science, 74, 9.*

[34] Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology and Medicine, 11, 1, 81-128.*

[35] Pryor, W. A., & Porter, N. A. (1990). Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radical Biology and Medicine, 8, 6, 541-543.*

[36] Dalle-Donne, I., Colombo, R., Milzani, A., Aldini, G., Carini, M., & Rossi, R. (2006). Protein carbonylation, cellular dysfunction, and disease progression. *Journal of Cellular and Molecular Medicine, 10, 2, 389-406.*

[37] Koito, W., Araki, T., Horiuchi, S., & Nagai, R. (2004). Conventional antibody against Nepsilon-(carboxymethyl)lysine (CML) shows cross-reaction to Nepsilon-(carboxyethyl)lysine (CEL): immunochemical quantification of CML with a specific antibody. *Journal of Biochemistry, 136, 6, 831-7.*

[38] USDA. (2013). USDA MyPlate & Food Pyramid. Retrieved from: https://web.archive.org/web/20131216085631/http:/fnic.nal.usda.gov/dietary-guidance/myplatefood-pyramid-resources/usda-myplate-food-pyramid-resources.

[39] Pereira, P. M. C. C., & Vicente, A. (2013). Meat nutritional composition and nutritive role in the human diet. *Meat Science, 93, 3, 586-592.*

[40] Pfeiffer, C.C., & Braverman, E.R. (1982). Zinc, the brain and behavior. *Biol Psychiatry, 17(4):513-32.*

[41] Hortin, A., Bechtel, P., & Baker, D. (1991). Efficacy of Pork Loin as a Source of Zinc and Effect of Added Cysteine on Zinc Bioavailability. *Journal of Food Science, 56, 6, 1505-1507.*

[42] Wyness, L. (2016). The role of red meat in the diet: nutrition and health benefits. *Proceedings of the Nutrition Society, 75, 3, 227-232.*

[43] Schmitz, G., & Ecker, J. (2008). The opposing effects of n−3 and n−6 fatty acids. *Progress in Lipid Research, 47, 2, 147-155.*

[44] Kruger, M. C., & Horrobin, D. F. (1997). Calcium metabolism, osteoporsis and essential fatty acids: A review. *Progress in Lipid Research, 36, 2, 131-151.*

[45] Watanabe, F. (2007). Vitamin B12 sources and bioavailability. *Experimental Biology and Medicine (maywood, N.j.), 232, 10, 1266-74.*

[46] Watanabe, F., & Bito, T. (2018). Vitamin B12 sources and microbial interaction. *Experimental Biology and Medicine (maywood, N.j.), 243, 2, 148-158.*

[47] Ashok, Nandhini G. (2014). Vitamin b12 in health: A review. *Journal of pharmaceutical sciences and research, 6, 3130-3131*

[48] Wang, X., Lin, X., Ouyang, Y. Y., Liu, J., Zhao, G., Pan, A., & Hu, F. B. (2016). Red and processed meat consumption and mortality: dose–response meta-analysis of prospective cohort studies. *Public Health Nutrition, 19, 5, 893-905.*

[49] Santarelli, R. L., Pierre, F., & Corpet, D. E. (2008). Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. *Nutrition and Cancer, 60, 2, 131-44*

[50] Thomas, J.S. & Chanjuan Shi (2016). Chapter 24 Molecular Testing in Colorectal Cancer. *Diagnostic Molecular Pathology. London: Elsevier Academic Press.*

[51] World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective; American Institute for Cancer Research: Washington, DC, USA, 2007; 517, www.dietandcancerreport.org/?p=ER (accessed Dec 24, 2013).

[52]Fretts, A. M., Follis, J., Nettleton, J. & Lemaitre, R., et al. (2015). Consumption of meat is associated with higher fasting glucose and insulin concentrations regardless of glucose and insulin genetic risk scores: A meta-analysis of 50,345 Caucasians. *The American Journal of Clinical Nutrition, 102, 5, 1266-1278.*

[53] Schoenaker, D., Mishra, G. D., Callaway, L. K., Callaway, L. K., & Soedamah-Muthu, S. S. (2016). The role of energy, nutrients, foods, and dietary patterns in the development of gestational diabetes mellitus: a systematic review of observational studies. *Diabetes Care, 39, 1, 16–23.*

[54] Wilson, J. G., Lindquist, J. H., Grambow, S. C., Crook, E. D., & Maher, J. F. (2003). Potential role of increased iron stores in diabetes. *The American Journal of the Medical Sciences, 325, 6, 332-339.*

[55] Feskens, E. J., Virtanen, S. M., Räsänen, L. et al. (1995). Dietary factors determining diabetes and impaired glucose tolerance. A 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study. *Diabetes Care, 18, 8, 1104-1112.*

[56] Kim, Y., Keogh, J., & Clifton, P. (2015). A review of potential metabolic etiologies of the observed association between red meat consumption and development of type 2 diabetes mellitus. *Metabolism, 64, 7, 768-779.*

[57] Schmidt, A. M., Hori, O., Brett, J., & Shi, D. Y. (1994). Cellular Receptors for Advanced Glycation End Products: Implications for Induction of Oxidant Stress and Cellular Dysfunction in the Pathogenesis of Vascular Lesions. *Arteriosclerosis and Thrombosis, 14, 10, 1521.*

[58] Hofmann, S. M., Dong, H. J., Li, Z et al. (2002). Improved insulin sensitivity is associated with restricted intake of dietary glycoxidation products in the db/db mouse. *Diabetes, 51, 7, 2082-2089.*

[59] Sandu, O., Song, K., Cai, W., Zheng, F., Uribarri, J., & Vlassara, H. (2005). Insulin resistance and type 2 diabetes in high-fat-fed mice are linked to high glycotoxin intake. *Diabetes, 54, 8, 2314-2319.*

[60] Abete, I., Romaguera, D., Vieira, A. R.et al. (2014). Association between total, processed, red and white meat consumption and all-cause, CVD and IHD mortality: A meta-analysis of cohort studies. *British Journal of Nutrition, 112, 5, 762-775.*

[61] Wolmarans, P., Benadé, A. J., Kotze, T. J., Daubitzer, A. K., Marais, M. P., & Laubscher, R. (1991). Plasma lipoprotein response to substituting fish for red meat in the diet. *The American Journal of Clinical Nutrition, 53, 5, 1171-1176.*

[62] Corpet, D. E. (2011). Red meat and colon cancer: Should we become vegetarians, or can we make meat safer? *Meat Science, 89, 3, 310-316.*

[63] Rohrmann, S., Overvad, K., Bueno-de-Mesquita, H. B. et al. (2013). Meat consumption and mortality- results from the European Prospective Investigation into Cancer and Nutrition. *Bmc Medicine, 11.*

[64] Chan, D. S., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. *Plos One, 6, 6.*

[65] Tabatabaei, S. M., Fritschi, L., Knuiman, M. W., Boyle, T., Iacopetta, B. J., Platell, C., & Heyworth, J. S. (2011). Meat consumption and cooking practices and the risk of colorectal cancer. *European Journal of Clinical Nutrition, 65, 6, 668-675.*

[66] Ollberding, N. J., Wilkens, L. R., Henderson, B. E., Kolonel, L. N., & Le, M. L. (2012). Meat consumption, heterocyclic amines and colorectal cancer risk: the Multiethnic Cohort Study. *International Journal of Cancer, 131, 7, 1125-1133.*

[67] Schwabn, C E, Huber, W W, Parzefall, W, Hietsch, GKassie, F. (2000). Search for compoiunds that inhibit the genotoxic anc carcinogenic effects of heterocyclic aromatic amines. *Crit Rev Toxicol, 30: 1–69.*
[68] Stavric, B. (1994). Biological significance of trace levels of mutagenic heterocyclic aromatic amines in human diet: a critical review. *Food and Chemical Toxicology, 32: 977–994.*
[69] Santarelli, R. L., Pierre, F., & Corpet, D. E. (2008). Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. *Nutrition and Cancer, 60, 2, 131-44.*

[70] Sinha, R., Cross, A. J., Graubard, B. I., Leitzmann, M. F., & Schatzkin, A. (2009). Meat intake and mortality: a prospective study of over half a million people. *Archives of Internal Medicine, 169, 6, 562-571.*

[71] Lee, J. E., McLerran, D. F., & Rolland, B. et al. (2013). Meat intake and cause-specific mortality: a pooled analysis of Asian prospective cohort studies. *The American Journal of Clinical Nutrition, 98, 4, 1032-1041.*

[72] Kazerouni, N., Sinha, R., Hsu, Che-Han, Greenberg, A., & Rothman, N. (2002). Analysis of 200 food items for benzo[a]pyrene and estimation of its intake ίη an epidemiologic study. *Food and Chemical Toxicology, 40, 1, 133.*

[73] Jackson, M. C., Kucera, C. M., & Lenney, J. F. (1991). Purification and properties of human serum carnosinase. *Clinica Chimica Acta, 196, 2, 193-205*

[74] Peters, V., Klessens, C. Q., Baelde, H. J., & Singler, B. et al. (2015). Intrinsic carnosine metabolism in the human kidney. *Amino Acids, 47, 12, 2541-2550.*

[75] Suyama Y., Suzuki T., Maruyama M., & Saito K. (1970). Determination of carnosine, anserine and beta-alanine in the muscle of animals. *Bull. Jpn. Soc. Sci.36, 10, 1048–1053*

[76] Jackson, M. C. & Lenney, J. F. (1996). The distribution of earnosine and related dipeptides in rat

and human tissues. *Inflamm Res, 45, 132-135.*

[77] Chaleckis, R., Murakami, I., Takada, J. et al. (2016). Individual variability in human blood metabolites

identifies age-related differences. *Proc Natl Acad Sci U S A. 113 (16), 4252-4259.*

[78] Gil-Agustí, M., Esteve-Romero, J., Carda-Broch, S., & Ghent Belgium (2008). Anserine and carnosine determination in meat samples by pure micellar liquid chromatography. *Journal of Chromatography A, 1189, 444-450.*

[79] Vistoli, G., Straniero, V., & Pedretti, A. et al. (2012). Predicting the physicochemical profile of diastereoisomeric histidine-containing dipeptides by property space analysis. *Chirality, 24, 7, 566-576.*

[80] Perrone, D., Monteiro, M., & Castelo-Branco, V. N. (2015) Chapter 3 The Chemistry of Imidazole Dipeptides. *Imidazole dipeptides: chemistry, analysis, function and effects. London: Royal Society of Chemistry.*

[81] Brown, C. E., & Antholine, W. E. (1979). Chelation chemistry of carnosine. Evidence that mixed complexes may occur in vivo. *The Journal of Physical Chemistry, 83, 26, 3314-3319.*

[82] Rainsford, K. D., Milanino, R., Sorenson, J. R. J., & Velo, G. P. (1998). Copper and Zinc in Inflammatory and Degenerative D1iseases. *Dordrecht: Springer Netherlands.*

[83] Martindale, J. L., & Holbrook, N. J. (2002). Cellular response to oxidative stress: Signaling for suicide and survival. *Journal of Cellular Physiology, 192, 1, 1-15.*

[84] Wang, X., McCullough, K. D., Franke, T. F., & Holbrook, N. J. (2000). Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. *The Journal of Biological Chemistry, 275, 19, 14624-14631.*

[85] Chan, K. M., & Decker, E. A. (1994). Endogenous skeletal muscle antioxidants. *Critical Reviews in Food Science and Nutrition, 34, 4, 403-426.*

[86] Yim, M. B., Chock, P. B., & Stadtman, E. R. (1990). Copper, zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. *Proceedings of the National Academy of Sciences of the United States of America, 87, 13, 5006-5010.*

[87] Torreggiani, A., Bonora, S., & Fini, G. (2000). Raman and IR spectroscopic investigation of zinc(II)-carnosine complexes. *Biopolymers, 57, 6, 352-364.*

[88] Takei, M. (2012). The Development of Polaprezinc Research. *Yakugaku Zasshi, 132, 3, 271-277.*

[89] Halliwell, B., & Gutteridge, J. M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymology, 186, 1-85.*

[90] Slauch J. M. (2011) How does the oxidative burst of macrophages kill bacteria? Still an open question. *Mol Microbiol. 80,3 580–583.*

[91] Huang, P., Feng, L., Oldham, E. A., Keating, M. J., & Plunkett, W. (2000). Superoxide dismutase as a target for the selective killing of cancer cells. *Nature, 407, 6802, 390-395.*

[92] Griveau, J.F., & Le Lannou, D.L.E. (1997). Reactive oxygen species and human spermatozoa: physiology and pathology. *International Journal of Andrology, 20, 2, 61-69.*

[93] Bongekile S., Ibukun P. O., & Stefan S. Du P. (2016). Chapter 1 Introduction to Reactive Oxygen Species: Emphasizing Their Importance in the Male Reproductive System. *Reactive oxygen species in biology and human health. Boca Raton: CRC Press.*

[94] Marnett, L. J. (2000). Oxyradicals and DNA damage. *Carcinogenesis, 21, 3, 361-70.*

[95] Ahmed N., Ahmed M. E., Mohammed M. A., & Sheikh F. A. (2016). Chapter 14 Pulmonary Oxidant–Antioxidant Dysregulation in Asthma. *Reactive oxygen species in biology and human health. Boca Raton: CRC Press.*

[96]Pattison, D. I., & Davies, M. J. (2006). Evidence for rapid inter- and intramolecular chlorine transfer reactions of histamine and carnosine chloramines: implications for the prevention of hypochlorous-acid-mediated damage. *Biochemistry, 45, 26, 8152-8162.*

[97] Torreggiani, M. T. A. (2009). A pulse radiolysis study of carnosine in aqueous solution. *International Journal of Radiation Biology, 74, 3, 333-340.*

[98] Hartman, P. E., Hartman, Z., & Ault, K. T. (1990). Scavenging of singlet molecular oxygen by imidazole compounds: high and sustained activities of carboxy terminal histidine dipeptides and exceptional activity of imidazole-4-acetic acid. *Photochemistry and Photobiology, 51, 1, 59-66.*

[99] Klebanov G.I., Teselkin Y., Babenkova I.V. et al. Vladimirov Y. (1997). Evidence for a direct interaction of superoxide anion radical with carnosine. *Biochemistry and Molecular Biology International, 43, 1, 99-106.*

[100] Pavlov, A. R., Revina, A. A., Dupin, A. M., & Boldyrev, A. A. (1993). The mechanism of interaction of carnosine with superoxide radicals in water solutions. *Biochimica Et Biophysica Acta, 1157, 3, 304.*

[101] Aldini, G., Facino, R. M., Beretta, G., & Carini, M. (2005). Carnosine and related dipeptides as quenchers of reactive carbonyl species: From structural studies to therapeutic perspectives. *Biofactors, 24, 77-87.*

[102] Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R., & Milzani, A. (2003). Protein carbonylation in human diseases. *Trends in Molecular Medicine, 9, 4, 169-76.*

[103] Chapman-Novakofski, K., & Luevano-Contreras, C. (2010). Dietary Advanced Glycation End Products and Aging. *Nutrients, 2, 12, 1247-1265*

[104] Goldin, A., Beckman, J. A., Schmidt, A. M., & Creager, M. A. (2006). Advanced Glycation End Products: Sparking the Development of Diabetic Vascular Injury. *Circulation, 114, 6, 597-605.*

[105] Shigetomi, K., Okada, N., Kumaki, T., Mitsuhashi, S., & Ubukata, M. (2018). Inhibitory activities of tree bark extracts against AGEs formation and their correlation with phenolic components. Wood Science and Technology: *Journal of the International Academy of Wood Science, 52, 3, 873-886.*

[106] Seidler, N., Shokry, S., & Nauth, J. (2001) Properties of a glycation product derived from carnosine. *J Biochem Mol Biol Biophys 5, 2, 153–162.*

[107] Szwergold, B. S. (2005). Carnosine and anserine act as effective transglycating agents in decomposition of aldose-derived Schiff bases. *Biochemical and Biophysical Research Communications, 336, 1, 36-41.*

[108] Begum, G., Cunliffe, A., & Leveritt, M. (2005). Physiological role of carnosine in contracting muscle. *International Journal of Sport Nutrition and Exercise Metabolism, 15, 5, 493-514.*

[109]. Everaert I & Derave W. (2015). Chapter 15 Human skeletal muscle carnosine and its function: a focus on homeostasis, muscle contractility and pH. *Imidazole dipeptides: Chemistry, analysis, function and effects.*

[110] Dutka, T. L., & Lamb, G. D. (2004). Effect of carnosine on excitation-contraction coupling in mechanically-skinned rat skeletal muscle. *Journal of Muscle Research and Cell Motility, 25, 3, 203-213.*

[111] Saunders, B., Artioli, G. G., Craig, S. & Gualano, B. (2015) Chapter 14 β-Alanine, muscle carnosine and exercise. *Imidazole dipeptides: Chemistry, analysis, function and effects*

[112] Serena, F., Hamilton, R., Marina, Y. S., Ana, P. H., et al. (2011). Beta-alanine (Carnosyn™) supplementation in elderly subjects (60-80 years): effects on muscle carnosine content and physical capacity. *Amino Acids, 43, 1, 49-56.*

[113] Churchland, P. S., Koch, C., & Sejnowski, T. J. (1990). What is computational neuroscience? *In E. L. Schwartz (Ed.), Computational neuroscience. Cambridge, MA: MIT Press 46–55.*

[114] Hashimoto, M., Rockenstein, E., Crews, L., & Masliah, E. (2003). Role of Protein Aggregation in Mitochondrial Dysfunction and Neurodegeneration in Alzheimer's and Parkinson's Diseases. *Neuromolecular Medicine, 4, 21-36.*

[115] Brownlee, M. D. M. (1995). Advanced Protein Glycosylation in Diabetes and Aging. *Annual Review of Medicine, 46, 223-234.*

[116] Wang, W. Y., Tan, M. S., Yu, J. T., & Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Annals of Translational Medicine, 3, 10.*

[117] Artioli, G. G., Sale, C., & Jones, R. L. (2018). Carnosine in health and disease. *European Journal of Sport Science, 4, 1-10.*

[118] Aloisi, A., Barca, A., Romano, A., Guerrieri, S., Storelli, C., Rinaldi, R., Verri, T., et al. (2013). Anti-Aggregating Effect of the Naturally Occurring Dipeptide Carnosine on Aβ1-42 Fibril Formation. *Plos One, 8, 7.*

[119] Boldyrev, A., Johnson, P., & Hipkiss, A. R. (2007). Could Carnosine or Related Structures Suppress Alzheimer's Disease? *Journal of Alzheimer's Disease, 11, 2, 229-240.*

[120] Davis, C. K., Rajanikant, G. K., Laud, P. J., Bahor, Z., & Majid, A. (2016). Systematic review and stratified meta-analysis of the efficacy of carnosine in animal models of ischemic stroke. *Journal of Cerebral Blood Flow and Metabolism, 36, 10, 1686-1694.*

[121] Jin CL, Yang LX, Wu XH, Li Q, Ding MP, Fan YY, Zhang WP, Luo JH, Chen Z.(2005) Effects of carnosine on amygdaloid-kindled seizures in Sprague-Dawley rats. *Neuroscience. 135, 939–947.*

[122] Baek, S. H., Noh, A. R., Kim, K.-A. et al. (2014). Modulation of Mitochondrial Function and Autophagy Mediates Carnosine Neuroprotection Against Ischemic Brain Damage. *Stroke, 45, 8, 2438-2443.*

[123] Wang, J.-P., Yang, Z.-T., Liu, C., He, Y.-H., & Zhao, S.-S. (2013). L-carnosine inhibits neuronal cell apoptosis through signal transducer and activator of transcription 3 signaling pathway after acute focal cerebral ischemia. *Brain Research, 1507, 125-133.*

[124] Boldyrev, A. (2001). Carnosine as a modulator of endogenous $Zn^{(2+)}$ effects. *Trends in Pharmacological Sciences, 22, 3, 112-113.*

[125] Trombley, P. Q., Horning, M. S., & Blakemore, L. J. (1998). Carnosine modulates zinc and copper effects on amino acid receptors and synaptic transmission. *Neuroreport, 9, 15, 3503-3507*.

[126] Sassoe-Pognetto, M., Cantino, D., & Panzanelli, P. et al. (1993). Presynaptic co-localization of carnosine and glutamate in olfactory neurones. *Neuroreport, 5, 1, 7-10*.

[127] Panzanelli, P., Cantino, D., & Sassòe-Pognetto, M. (1997). Co-localization of carnosine and glutamate in photoreceptors and bipolar cells of the frog retina. *Brain Research, 758, 1-2*.

[128] Baslow, M. H. (2010). A novel key-lock mechanism for inactivating amino acid neurotransmitters during transit across extracellular space. *Amino Acids : the Forum for Amino Acid, Peptide and Protein Research, 38, 1, 51-55.*

[129] Tiedje, K. E., Stevens, K., Barnes, S., & Weaver, D. F. (2010). Beta-alanine as a small molecule neurotransmitter. *Neurochemistry International, 57, 3, 177-88.*

[130] Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature, 362, 6423, 801-809.*

[131] Barski, O. A., Xie, Z., Baba, S. P. et al. (2013). Dietary carnosine prevents early atherosclerotic lesion formation in apolipoprotein E-null mice. *Arteriosclerosis, Thrombosis, and Vascular Biology, 33, 6, 1162-1170.*

[132] de, Courten. B., Jakubova, M., de, C. M. P., Kukurova, I. J., et al. (2016). Effects of carnosine supplementation on glucose metabolism: Pilot clinical trial. *Obesity (silver Spring, Md.), 24, 5, 1027-34.*

[133] Baye, E., Ukropec, J., de, C. M. P. J. & Vallova, S. et al. (2017). Effect of carnosine supplementation on the plasma lipidome in overweight and obese adults: a pilot randomised controlled trial. *Scientific Reports, 7, 1*

[134] Basta, G., Schmidt, A. M., & De, C. R. (2004). Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. *Cardiovascular Research, 63, 4, 582-592.*

[135] Karin, J.-D., & Mark, E. C. (2008). The Role of AGEs in Cardiovascular Disease. *Current Pharmaceutical Design, 14, 10, 979-986.*

[136] Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature, 414, 6865, 813-820.*

[137] Hipkiss, A. R. (2006). Would Carnosine or a Carnivorous Diet Help Suppress Aging and Associated Pathologies?. *Annals of the New York Academy of Sciences, 1067, 1, 369-374.*

[138] Marcolini, E., Babini, E., & Bordoni, A. et al. (2015). Bioaccessibility of the Bioactive Peptide Carnosine during in Vitro Digestion of Cured Beef Meat. *Journal of Agricultural and Food Chemistry, 63, 20, 4973-4978.*

[139] Love, J. D., & Pearson, A. M. (1971). Lipid oxidation in meat and meat products—A review. *Journal of the American Oil Chemists' Society, 48, 10, 547-549.*

[140] Kanner, J. (1994). Oxidative processes in meat and meat products: Quality implications. *Meat Science, 36, 169–189.*

[141] Morrissey, P. A., Sheehy, P. J. A., Galvin, K., Kerry, J. P., & Buckley, D. J. (1998). Lipid stability in meat and meat products. *Meat Science: Supplement 1, 49.*

[142]Stadtman, E. R. (2006). Protein oxidation and aging. *Free Radical Research, 40, 12, 1250-8.*

[143] Vistoli, G., De, M. D., Cipak, A., Zarkovic, N., Carini, M., & Aldini, G. (2013). Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. *Free Radical Research, 47, 3-27.*

[144] Sesink, A. L., Termont, D. S., Kleibeuker, J. H., & Van, D. M. R. (2000). Red meat and colon cancer: dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium. *Carcinogenesis, 21, 10, 1909-1915.*

[145] Lai, C., Dunn, D. M., Miller, M. F., & Pence, B. C. (1997). Non-promoting effects of iron from beef in the rat colon carcinogenesis model. *Cancer Letters, 112, 1, 87-91.*

[146] Peiretti, P. G., Medana, C., Visentin, S., Dal, B. F., & Meineri, G. (2012). Effect of cooking method on carnosine and its homologues, pentosidine and thiobarbituric acid-reactive substance contents in beef and turkey meat. *Food Chemistry, 132, 1, 80-85.*

[147] Promeyrat, A., Gatellier, P., Lebret, B., Kajak-Siemaszko, K., Aubry, L., & Santé-Lhoutellier, V. (2010). Evaluation of protein aggregation in cooked meat. *Food Chemistry, 121, 2, 412-417.*

[148] Domínguez, R., Gómez, M., Fonseca, S., & Lorenzo, J. M. (2014). Effect of different cooking methods on lipid oxidation and formation of volatile compounds in foal meat. *Meat Science, 97, 2, 223-230.*

[149] Rodriguez-Estrada, M. T., Penazzi, G., Caboni, M. F., Bertacco, G., & Lercker, G. (1997). Effect of different cooking methods on some lipid and protein components of hamburgers. *Meat Science, 45(3), 365–375.*

[150] Broncano, J. M., Petrón, M. J., Parra, V., & Timón, M. L. (2009). Effect of different cooking methods on lipid oxidation and formation of free cholesterol oxidation products (COPs) in Latissimus dorsi muscle of Iberian pigs. *Meat Science, 83, 3, 431-437.*

[151] Rein, M. J., Renouf, M., Cruz-Hernandez, C., Actis-Goretta, L., Thakkar, S. K., & da, S. P. M. (2013). Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. *British Journal of Clinical Pharmacology, 75, 3, 588-602.*

[152] Reeder, B. J., & Wilson, M. T. (2001). The effects of pH on the mechanism of hydrogen peroxide and lipid hydroperoxide consumption by myoglobin: a role for the protonated ferryl species. *Free Radical Biology and Medicine, 30, 11, 1311-1318.*

[153] Mateescu, R. G., Garmyn, A. J., & Vanoverbeke, D. L., et al. (2012). Genetic parameters for carnitine, creatine, creatinine, carnosine, and anserine concentration in longissimus muscle and their association with palatability traits in angus cattle. *Journal of Animal Science, 90, 12, 4248-4255.*

[154] CFIA. (2015). Guidance Document Repository: Ground Meat Standards. Retrieved from: http://www.inspection.gc.ca/food/retail-food/information-bulletins/ground-meat/eng/1331668408417/1331668480561

[155] Tsikas, D., Rothmann, S., & Schneider, J. Y., et al. (2016). Development, validation and biomedical applications of stable-isotope dilution GC-MS and GC-MS/MS techniques for circulating malondialdehyde (MDA) after pentafluorobenzyl bromide derivatization: MDA as a biomarker of oxidative stress and its relation to 15(S)-8-iso-prostaglandin F2α and nitric oxide (NO). *Journal of Chromatography B, 1019, 95-111.*

[156] Ventanas, S., Estevez, M., Tejeda, J. F., & Ruiz, J. (2006). Protein and lipid oxidation in Longissimus dorsi and dry cured loin from Iberian pigs as affected by crossbreeding and diet. *Meat Science, 72, 4, 647-655.*

[157] Martinaud, A., Mercier, Y., Marinova, P., Tassy, C., Gatellier, P., & Renerre, M. (1997). Comparison of Oxidative Processes on Myofibrillar Proteins from Beef during Maturation and by Different Model Oxidation Systems. *Journal of Agricultural and Food Chemistry, 45, 7, 2481-2487.*

[158] Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of biuret reaction. *J. Biol. Chem. 177, 751-766.*

[159] Honikel, K.O. (2004). Minced meats. *Encyclopedia of Meat Science (2nd Eds.), Oxford: Elsevier. 422-424.*

[160] Pegova, A., Abe, H., & Boldyrev, A. (2000). Hydrolysis of carnosine and related compounds by mammalian carnosinases. *Comp. Biochem. Physiol. B Biochem. Mol. Biol. 127, 4, 443-446.*

[161] Decker, E. A., & Crum, A. D. (1993). Antioxidant activity of carnosine in cooked ground pork. *Meat Science, 34, 2, 245-253.*

[162] Zhou, S., & Decker, E. A. (1999). Ability of amino acids, dipeptides, polyamines, and sulfhydryls to quench hexanal, a saturated aldehydic lipid oxidation product. *Journal of Agricultural and Food Chemistry, 47, 5, 1932-6.*

[163] Hernández, P., Navarro, J. L., & Toldrá, F. (1999). Lipids of pork meat as affected by various cooking techniques. *Food Science and Technology International, 5(6), 501–508.*

[164] Wilson, R., Lyall, K., Smyth, L., Fernie, C. E., & Riemersma, R. A. (2002). Dietary hydroxy fatty acids are absorbed in humans: implications for the measurement of 'oxidative stress' in vivo. *Free Radical Biology and Medicine, 32, 2, 162-168.*

[165] Aldini, G., Carini, M., Beretta, G., Bradamante, S., & Maffei Facino, R. (2002). Carnosine is a quencher of 4-hydroxy-nonenal: Through what mechanism of reaction? *Biochem Biophys Res Commun. 298: 699–706.*

[166] Berliner, J. A., & Heinecke, J. W. (1996). The role of oxidized lipoproteins in atherogenesis. *Free Radical Biology and Medicine, 20, 5, 707-727.*

[167] Colzani, M., Garzon, D., & Aldini, G. (2015). Chapter 8 Carnosine and Derivatives as Inhibitors of Protein Covalent Modifications Induced by Reactive Carbonyl Species. *Imidazole dipeptides: Chemistry, analysis, function and effects. 139 – 169.*

[168] Rosario, Z., & Francisco, H. (2005). Coordinate Contribution of Lipid Oxidation and Maillard Reaction to the Nonenzymatic Food Browning. *Critical Reviews in Food Science and Nutrition, 45, 1, 49-59.*

[169] O'Neill, L. M., Galvin, K., Morrissey, P. A., & Buckley, D. J. (1999). Effect of carnosine, salt and dietary vitamin E on the oxidative stability of chicken meat. *Meat Science, 52, 1, 89-94.*

[170] D'Astous-Pagé, J., Gariépy, C., Blouin, R., Cliche, S., Sullivan, B., Fortin, F., & Palin, M. F. (2017). Carnosine content in the porcine longissimus thoracis muscle and its association with meat quality attributes and carnosine-related gene expression. *Meat Science, 124, 84-94.*

[171] Ishii, T., Ito, S., & Kumazawa, S. et al. (2008). Site-specific modification of positively-charged surfaces on human serum albumin by malondialdehyde. *Biochemical and Biophysical Research Communications, 371, 1, 28-32.*

[172] Uchida, K. (2000). Role of reactive aldehyde in cardiovascular diseases. *Free Radical Biology and Medicine, 28, 12, 1685-1696.*

[173] Pizzimenti, S., Ciamporcero, E., & Daga, M. et al. (2013). Interaction of aldehydes derived from lipid peroxidation and membrane proteins. *Frontiers in Physiology, 4, 242.*

[174] Abdulkarim, B. G., & Smith, J. S. (1998). Heterocyclic Amines in Fresh and Processed Meat Products. *Journal of Agricultural and Food Chemistry, 46, 11, 4680-4687.*

[175] Knize, M. G., Andersen, B., Healy, S. K., Shen, N. H., Lewis, P. R., Bjeldanes, L. F., Hatch, F. T., & Felton, J. S. (1985). Effect of temperature, patty thickness, and fat content on the production of mutagens in fried ground beef. *Food Chem. Toxicol. 23, 1035-1040.*

[176] Hipkiss, A., Brownson, C., Bertani, M., Ruiz, E., & Ferro, A. (2002). Reaction of Carnosine with Aged Proteins: Another Protective Process? *Annals of the New York Academy of Sciences, 959, 1, 285-294.*

[177] Traore, S., Aubry, L., Gatellier, P., Przybylski, W., Jaworska, D., Kajak-Siemaszko, K., & Santé-Lhoutellier, V. (2012). Effect of heat treatment on protein oxidation in pig meat. *Meat Science, 91, 1, 14-21.*

[178] Opstvedt, J., Miller, R., Hardy, R. W., & Spinelli, J. (1984). Heat-induced changes in sulfhydryl groups and disulfide bonds in fish protein and their effect on protein and amino acid digestibility in rainbow trout (Salmo gairdneri). *Journal of Agricultural and Food Chemistry, 32, 4, 929-935.*

[179] Gumusyayla, S., Vural, G., Bektas, H., Neselioglu, S., Deniz, O., & Erel, O. (2016). A novel oxidative stress marker in migraine patients: dynamic thiol-disulphide homeostasis. *Neurological Sciences : Official Journal of the Italian Neurological Society, 37, 8, 1311-1317.*

[180] Baron, C. P. and Anderson, H. J. (2002). Myoglobin-induced lipid oxidation. A review. *J. Agric. Food Chem., 50: 3887–3897.*

[181] Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science, 89, 3, 259-279.*

[182] Xiong, Y. L. (2000). Chapter 8 Protein oxidation and implications for muscle food quality. *Antioxidants in Muscle Foods. Nutritional Strategies To Improve quality. Decker, E. A., Faustman, C., Lopez-Bote, C. J., Eds.; Wiley-Interscience: New York, 85-111.*

[183] Hipkiss, A. R. (2018). Glycotoxins: Dietary and Metabolic Origins; Possible Amelioration of Neurotoxicity by Carnosine, with Special Reference to Parkinson's Disease. Neurotoxicity Research : Neurodegeneration, Neuroregeneration, *Neurotrophic Action, and Neuroprotection, 34, 1, 164-172.*

[184] Guggliucci, A. (2017). Formation of fructose-mediated advanced glycation end products and their roles in metabolic and inflammatory diseases. *Adv Nutr. 8, 1, 54-62.*

[185] Henle, T. (2008). Chapter 2 Maillard reaction of proteins and advanced glycation end products (AGEs) in food. *Process-Induced Food Toxicants. John Wiley & Sons, Inc., Hoboken, NJ. 215-242.*

[186] Bauer, K., Hallermayer, K., Salnikow, J., Kleinkauf, H., & Hamprecht, B.(1982). Biosynthesis of carnosine and related peptides by glial cells in primary culture. *Biol. Chem., 257(7): 3593-7.*

[187] Everaert, I., Stegen, S., Vanheel, B., Taes, Y., & Derave, W.(2013). Effect of beta-alanine and carnosine supplementation on muscle contractility in mice. *Medicine & Science in Sports & Science, 45 (1), 43-51.*

[188] Goto, K., Maemura, H., Takamatsu, K., & Ishii, N. (2011). Hormonal responses to resistance exercise after ingestion of carnosine and anserine, *Journal of Strength & Conditioning Research, 25(2), 398-405.*

[189] Lawler, J. M., Barnes, W. S., Wu, G., Song, W., & Demaree, S. (2002). Direct antioxidant properties of creatine. *Biochem Biophys Res Commun., 290, 1, 47-52.*

[190] Fu, M. X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes J. W., &. Thorpe, S. R. (1996). The Advanced Glycation End Product, Ne -(Carboxymethyl)lysine, Is a Product of both Lipid Peroxidation and Glycoxidation Reactions. *The Journal of Biological Chemistry, 271, 17, 9982–9986.*

[191] Houjeghani, S., Kheirouri, S., Faraji, E., & Jafarabadi, M. A. (January 01, 2018). l-Carnosine supplementation attenuated fasting glucose, triglycerides, advanced glycation end products, and tumor necrosis factor-α levels in patients with type 2 diabetes: a double-blind placebo-controlled randomized clinical trial. *Nutrition Research, 49, 96-106.*

[192] Gardner, M. L., Illingworth, K. M., Kelleher, J., & Wood, D. (July 01, 1991). Intestinal absorption of the intact peptide carnosine in man, and comparison with intestinal permeability to lactulose. *The Journal of Physiology, 439, 1, 411-422.*

[193] Son, D. O., Satsu, H., Kiso, Y., & Shimizu, M. (January 01, 2004). Characterization of carnosine uptake and its physiological function in human intestinal epithelial Caco-2 cells. *Biofactors, 21, 395-398.*