Health related properties of dietary carnosine during in vitro digestion of meal models and the effect of carnosine on nitrosamine formation

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Abstract

Meat is a good source of multiple nutrients, such as high-quality proteins and minerals. Furthermore, meat is also popular due to its desirable flavor. However, red meat, in particular, has developed a generally negative image in the eyes of the public because its consumption has been associated with increased risks of chronic diseases, which are mainly due to the potential of red meat to promote oxidation, inflammation, and generation of carcinogenic molecules, such as nitrosamines. The multifunctional carnosine, which naturally exists in commonly consumed meat (and meat products), such as pork and beef, may impart important health benefits to meat consumers. Carnosine (β-alanyl-L-histidine) has multiple properties, including antioxidant, anti-glycation, and anti-inflammatory abilities. Its therapeutic potential for diabetes, cardiovascular diseases, and other oxidative stress-related health problems is currently under study. However, limited information is available on the health effects of carnosine in its natural meat matrix and in a more complex meal matrix, that includes other food components or additives, such as nitrite salts, used during the processing of meat products. Therefore, studies are needed to examine the effect of carnosine on nitrosamine formation and to assess the performance and bioaccessibility during the digestion of different types of meals for the purpose of optimizing the formulations of meat-containing meals to minimize the potential negative impact on health.

The bioaccessibility and effects of carnosine were evaluated during in vitro digestion of a model of an unhealthy meal containing intrinsic (low) and enhanced (medium and high) carnosine levels in a mix of minced pork and bread, with or without supplemental level of

ascorbic acid (AA) and/or dietary level of fructose as anti- and pro-oxidants, respectively. In the presence of either AA or fructose, the pro-oxidative potential of carnosine could be observed during in vitro digestion of the meal at the medium carnosine level. However, high levels of free carnosine exerted a protective effect, reducing the formation of 4-hydroxynonenal in the gastric phase and glyoxal (GO) in both the gastric and duodenal phases.

In a model of a burger meal combo including intrinsic (low; LCar) and enriched (medium, MCar; high, HCar) carnosine in ground pork, with or without dietary levels of AA and/or fructose (Fru), the effect of carnosine on oxidation and the formation of di-carbonyls and advanced glycation end products (AGE) were also assessed during in vitro digestion. The antioxidant activity of carnosine in MCar and HCar treatments was demonstrated, irrespective of the digestion phase and the presence of pro- and/or antioxidants (Fru and/or AA). The highest antioxidant activity was observed in the MCar treatment, with no further benefits obtained in the HCar, indicating a ceiling-like effect of carnosine benefits in vitro.

During in vitro digestion of a meat-included Mediterranean meal, the antioxidant effect of carnosine was demonstrated on lipid and protein oxidation. However, an unexpected increase in malondialdehyde content in the MCar gastric digests was observed in the absence of significant changes in the ferric-reducing power. These observations may suggest that the ferric-reducing capacity of carnosine contributing to the Fenton reaction may not be the only or major mechanism of this observed pro-oxidant outcome.

The effect of different concentrations of carnosine on nitrosamine formation was assessed in an aqueous and a biphasic water-lipid systems to collect fundamental knowledge and information in the stomach-simulating environment. The capacity of carnosine to inhibit nitrosamine formation was confirmed in aqueous and lipid environments, suggesting the potential of dietary carnosine to lower the risks associated with the consumption of processed meat products.

Overall, experimental evidence was provided regarding the beneficial effect of dietary carnosine during digestion of meat-containing unhealthy or healthy meals, in the presence of other anti- or pro-oxidants, and even the dual effect of carnosine, fructose, and AA depending on their concentrations and interactions. The effect of carnosine on suppressing nitrosamine formation was also demonstrated.

Résumé

La viande est une bonne source de nombreux nutriments incluant des protéines de haute qualité et de minéraux. Elle est par ailleurs appréciée pour sa saveur désirable. Cependant, la viande rouge, en particulier, possède maintenant une image généralement négative aux yeux des consommateurs due à sa consommation qui a été associée à un risque accru de maladies chroniques attribuable à son potentiel à promouvoir l'oxydation, l'inflammation et la génération de molécules carcinogènes, telles les nitrosamines dans les produits transformés. La multifonctionnalité de la carnosine, naturellement présente dans la viande (et les produits de viande) communément consommés telles le porc et le

bœuf pourrait apporter de nombreux bénéfices pour la santé des consommateurs. Les nombreuses propriétés de la carnosine (β-alanyl-L-histidine) incluent son pouvoir antioxydant, anti-glycation et sa capacité anti-inflammatoire. Son potentiel thérapeutique dans les cas de diabètes, de maladies cardio-vasculaires ou d'autres problèmes de santé associés au stress oxydatif fait présentement l'objet de différentes études. L'information ayant trait aux effets santé potentiel de la carnosine alimentaire; celle présente dans sa matrice musculaire ou par extension comprise dans un repas incluant viande et autre aliments, ou encore additifs alimentaires tels les sels de nitrite utilisés en charcuterie, est très limitée, voire inexistante. Afin de mettre en évidence les avantages santé potentiels de la carnosine alimentaire, des études sont d'abord requises afin de déterminer, lors de la digestion, les effets du dipeptide et ses interactions potentielles avec d'autres composés alimentaires de système repas susceptibles d'influencer sa bioaccessibilité, ou encore son potentiel à réduire la formation de nitrosamines.

La bioaccessibilité et les effets de la carnosine furent d'abord étudiés durant la digestion in vitro d'un repas modèle pro-oxydant de type hamburger incluant des teneurs intrinsèques (faible) ou encore enrichies (intermédiaire et élevé) en carnosine dans un mélange de porc haché et de pain, avec ou sans acide ascorbique (AA) et/ou fructose à titre d'anti- ou pro-oxydant, respectivement. En présence d'AA ou de fructose, un effet pro-oxydant de la carnosine en concentration intermédiaire fut observé durant la digestion in-vitro du repas. Un niveau plus élevé de carnosine a cependant démontré l'effet protecteur de la molécule, réduisant la formation de 4-Hydroxynonénal dans la phase gastrique et de glyoxal dans chacune des phases gastriques et duodénales

Un second modèle hamburger ayant les teneurs en carnosine intrinsèques et enrichies dans la viande de porc hachée avec ou sans AA et/ou fructose à des concentrations alimentaires fut aussi utiliser pour déterminer l'effet de la carnosine sur l'oxydation et la formation de di-carbonyls et de produits de glycation avancés durant la digestion in vitro. Un effet constant de l'enrichissement en carnosine (MCar et HCar) fut démontré, indépendamment de la présence ou non des composés pro- ou antioxydants (Fru et AA) à des concentrations alimentaires. La plus forte activité antioxydante de la carnosine fut cependant démontrée au niveau MCar, sans gain additionnel au niveau HCar, indiquant un plafonnement de la concentration bénéfique en carnosine dans ce modèle.

Durant la digestion d'un repas modèle de type Méditerranéen en présence de viande, l'effet antioxydant de la carnosine fut démontré sur l'oxydation des lipides et des protéines. Cependant, une augmentation imprévue de la teneur en malondialdéhyde dans les digestats gastriques fut observé en absence de changement significatif du pouvoir de réduction ferrique. Une telle observation suggère que le pouvoir de réduction ferrique de la carnosine lors de la réaction de Fenton pourrait ne pas être le seul ou le mécanisme principal de cet effet prooxydant.

L'effet de différentes concentrations de carnosine sur la formation de nitrosamines fut évalué à l'aide d'un système aqueux et d'un système biphasique eau-lipide pour obtenir des informations fondamentales dans un environnement simulant un milieu gastrique. La capacité de la carnosine à inhiber la formation des nitrosamines en environnements

aqueux et lipidiques suggère le potentiel de la carnosine alimentaire à réduire les risques pour la santé associés à la consommation de produits de viande transformés.

Globalement, des évidences expérimentales ont été apportées pour supporter les effets bénéfiques de la carnosine alimentaire et sa teneur musculaire lors de la digestion de repas modèles différant quant à leur potentiel santé suivant la présence et la concentration d'autres composés ayant un pouvoir redox. Des effets pro- et/ou antioxydant de la carnosine, du fructose et de l'AA furent observés selon leur concentration respective et leurs interactions. La capacité de la carnosine à réduire, voire supprimer, la formation des nitrosamines a aussi été démontrée.

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Contribution to original knowledge

1. The first report of a functional role of carnosine as a meal component observed in the gastrointestinal tract (GIT).

2. The first evaluation of the effect of dietary carnosine during in vitro digestion of a typical Western meal with a popular ascorbic acid supplement:

2.1 The first report on the pro-oxidant synergistic effect between dietary carnosine and ascorbic acid at a supplement.

2.2 The first report on the interaction between dietary carnosine and fructose during in vitro digestion, showing a synergistic pro-oxidant effect.

2.3 The first report indicates that the anti- or pro-oxidative outcomes of the interaction between dietary components depend on the levels of carnosine in the meat and on the presence of other dietary components with anti- and/or pro-oxidant potentials.

3. The first assessment of the effect of carnosine as a meat constituent during in vitro digestion of a model of burger meal combo as a representative of a typical Western meal:

3.1 Irrespective of interactions among carnosine, fructose, and ascorbic acid, increased levels of carnosine efficiently reduced oxidation and glycation, and the formation of advanced glycation end-products.

3.2 The benefits of carnosine toward lipid oxidation reached a plateau at the intermediate levels of carnosine.

4. The first evaluation and the first report of the health-beneficial properties of carnosine in a meat matrix during in vitro digestion of a healthy meal model system, which includes considerable amounts of other dietary antioxidants.

5. The first-ever study emphasizes the importance of understanding the interplay among dietary components and provides new and fundamental information on the bioaccessibility of carnosine and its contribution to health.

6. The first report to demonstrate the role of carnosine in suppressing the formation of different types of nitrosamines in both aqueous and lipid environments.

Contribution of authors

The author (YiYao Li) was responsible for the design of experiments, experimental work, and preparation of all chapters. Dr. Varoujan Yaylayan, Dr. Claude Gariépy, and Dr. Marie-France Palin were involved in funding acquisition and support. Dr. Claude Gariépy and Dr. Marie-France Palin contributed to the concepts and design of the entire project. Dr. Varoujan Yaylayan, Dr. Claude Gariépy, Dr. Tania Ngapo, and Dr. Marie-France Palin critically edited the manuscripts prior to their submissions to different journals. Dr. Varoujan Yaylayan and Dr. Claude Gariépy mentored the work from Chapter 3 to Chapter 6 and guided the preparation and improvement of the dissertation. Simon Cliche assisted importantly with the experimental work involved in studies from Chapter 3 to Chapter 6. Dr. Hassan Sabik and Fleur Gagnon guided the development and modification of the Mass-Spectrometry-related methods included in all studies in Chapters 3-6.

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Chapter 1 Introduction

1.1 Introduction

Carnosine (β-alanyl-L-histidine, Figure 1), a dipeptide first identified in meat broth, is a multifunctional dipeptide naturally present exclusively in muscle and, therefore, found in muscle foods such as pork, beef, and poultry, and some fish (Boldyrev et al., 2013). Since its discovery, the remarkable properties of carnosine and its various benefits, such as scavenging radicals, reducing glycation and inflammation, and acting as a neuromodulator, have been continuously reported in studies using aqueous in vitro models, experimental animals, as well as human subjects (as reviewed by Boldyrev et al., 2013; Wu, 2020).

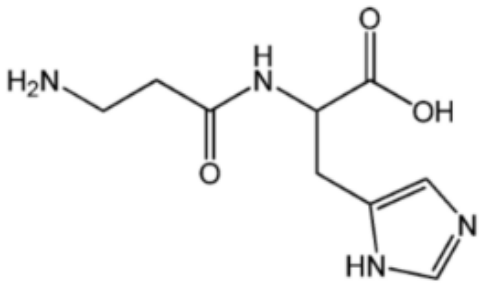

Figure 1. Carnosine structure

Although meat is the most important natural food source of carnosine, meat consumption does not enjoy a positive reputation. Numerous studies have contributed to this negative image. For instance, in a meta-analysis of published prospective cohort studies, Wang et al. (2016) found that one serving of processed red meat per day was associated with an 8% increase in the risk of cancer mortality. Other studies have shown that oxidation of meat-derived lipids and proteins leads to increased oxidative stress, impairs the function of vital molecules, such as LDL (low-density lipoprotein) and DNA (Lewin et al., 2006), interferes with cell signaling (Sottero et al., 2019) and damages cells (reviewed by Huang

& Ahn, 2019), subsequently increasing the risk of chronic diseases. In addition, further negative image of meat is exacerbated by reports of toxicity of some meat product ingredients, particularly nitrite salts. Apart from increased oxidative stress, nitrite salts ingested along with processed meat products, such as bacon and sausages, are reported to be related to the formation of nitrosamines (Toldrá, 2017), considered to be carcinogenic owing to their metabolic activation (by cytochrome P450 mediated hydroxylation) to produce an α-hydroxynitrosamine capable of undergoing a series of reactions that generates reactive nitrogen species able to modify DNA and imperil the health of consumers (Hotchkiss, 1988; Shephard et al., 1987).

Despite these studies and the associated poor health image, meat is still included in the daily diets of many people, not only because of its sensory properties, but also its contribution to the healthy diet being a source of multiple assimilable nutrients, for example, zinc, iron (heme iron), high-quality protein, fatty acids, and vitamins (as reviewed by Wyness 2016). In addition, the beneficial role of carnosine, as a meat constituent, during in vitro digestion of meat was also demonstrated, and even suggested potential health advantages of meat consumption as part of a meal (Li et al., 2021). Specifically, enhanced dietary carnosine in a meat matrix with varying fat content and subject to different cooking intensities reduced adverse reactions, such as lipid and protein oxidation and AGE formation during in vitro digestion. Meat, however, is rarely consumed by itself in a meal, and the presence of other dietary components and the possible interaction among them may lead to contradictory results; therefore, more information is required to understand and achieve optimal benefits of carnosine in a

meat-containing meal. For instance, it was reported that consumption of reducing sugars may contribute to the generation of free radicals (Vistoli et al., 2013), and countering these adverse reactions may necessitate the presence of more protective molecules such as carnosine. Alternatively, well-known antioxidants, such as ascorbic acid present naturally in vegetables or supplemented in beverages, might synergistically work with carnosine, decreasing its consumption as an antioxidant and thereby making more carnosine available to perform other in vivo beneficial functions, such as healing wound s and normalization of vascular permeability (Artioli et al., 2019).

The assessment of health-beneficial outcomes from dietary components, such as carnosine, should not rely only on the information obtained from studies based on simple matrices without considering the possible interactions with other food components, not to mention the digestion process itself, which may interfere by releasing hydrolysates vulnerable to oxidation or by favoring acidic environment promoting oxidation and nitrosamine formation (Kanner et al., 2001; Tricker, 1997). Studies must be conducted to explore, determine, and understand the properties of dietary carnosine during in vitro digestion of meal models closer in composition to that of daily meals, including both pro-oxidative (unhealthy) and less oxidative (healthy) meals.

In order to understand how dietary carnosine could perform or to what extent it may exert a beneficial effect during digestion of relatively pro-oxidative meals, models of a typical Western meal were used. Burger-based meal systems including a beverage-simulating fructose solution were chosen for this purpose for the following reasons. Firstly, such

burger meals contain relatively high levels of carbohydrates and lipids, two dietary components known to contribute to the increased risk for many chronic diseases (Bahadoran et al., 2015; World Health Organization, 2021), and which could provide a relatively high pro-oxidative dietary environment. Secondly, in addition to the presumptive association between fructose and chronic diseases (DeChristopher, 2017; Gugliucci, 2017), another reason for including the beverage-simulating fructose solution is the availability of soft drinks in fast-food outlets. Thirdly, the burger meal is popular worldwide, with ten billion burgers consumed each year in the United States alone (Waite, 2018). To varying extents, most consumers are aware of the unhealthiness of fast food, of which the burger meal with a fructose-containing beverage is included, and in a variety of ways, some consumers attempt to counteract or nullify nutritional issues. A prime example of such remediation is the use of widely available dietary supplements, including antioxidants. Indeed, ascorbic acid is the world's leading antioxidant supplement and is available in a range of concentrations (Wunsch, 2021), a relatively high level of ascorbic acid can be a representative of the most common antioxidant. Considering that soft drinks are often served in fast food outlets, using aqueous solutions of fructose at a dietary level and ascorbic acid at a supplemental level in the burger meal model allows to simulate a situation in which a burger meal is consumed with an antioxidant as a potential remediation. Some consumers may also choose beverages containing antioxidants, such as fruit juices, as the remediation. Considering that both fruit juices and soft drinks are available in the burger meal combo in fast food outlets, using aqueous solutions of dietary levels of ascorbic acid and/or fructose allows to

simulate potential anti- and/or pro-oxidants present in the diet when consuming a burger meal combo.

Altogether, these two dietary simulations provide for the fundamental studies of potential antagonistic or synergistic interactions among different bioactive molecules (such as carnosine, ascorbic acid, and fructose) and their overall effect.

In contrast to the relatively unhealthy meal represented by a burger, the Mediterranean diet (MD), which usually includes olive oil as the main source of lipids and abundant of plant foods, is a well-known reference for healthy meals (Salas-Salvadó & Papandreou, 2020). The MD contains multiple health-beneficial compounds possessing properties similar to those of carnosine and may show synergistic properties to mitigate the effect of oxidation during digestion of meat-containing meals (Azzini & Maiani, 2015; Gorelik et al., 2013; Kanner et al., 2017). For this reason, a meat-containing MD was chosen as a model to explore the role of dietary carnosine during digestion of a healthy meal.

Finally, referring back to the poor public image often accorded to meat consumption, nitrite salts are invariably at the forefront of this negativity, and in particular, the formation of carcinogenic nitrosamine from these salts. However, the nitrosamine-suppressing potential of carnosine is still debatable, with no direct quantitative data available on nitrosamine levels after carnosine treatments. The potential effect of carnosine on the production of nitrosamines was, therefore, undertaken using an aqueous system including different levels of carnosine, including secondary amines and sodium

nitrite. In addition, an aqueous-lipid biphasic system was also chosen to mimic the gastric environment and assess how carnosine could affect nitrosamine formation during digestion.

1.2 Hypothesis

- Carnosine, a health-beneficial dipeptide naturally found in muscle foods, could reduce oxidation and glycation during digestion of meat-containing meals depending on the presence, nature, and concentration of the bioactive dietary components present in the meal.
- Carnosine is able to suppress the formation of different species of nitrosamine in both aqueous and lipid environments.

1.3 Objectives

1.3.1 Obtain information on the effect of carnosine as a meat constituent during in vitro digestion of a typical Western meal model with ascorbic acid at a supplemental level. To achieve this objective, the following aims were defined:

- Develop a pro-oxidative burger meal model simulating a typical Western meal including a supplemental (high) level of ascorbic acid
- Evaluate the effect of carnosine on oxidation and glycation, including AGE formation during digestion of this model
- Establish single and potential interactive effects of bioactive molecules in the meal, including carnosine, on the bioaccessibility of carnosine

1.3.2 Determine the effect of carnosine as a meat constituent during in vitro digestion of a burger meal combo. Three aims were defined to achieve this objective:

- Develop a burger meal combo, including aqueous solutions with or without dietary levels of ascorbic acid and/or fructose for the simulation of beverages that are usually included in a burger meal combo
- Determine the effect of ascorbic acid and fructose in the current model and their potential interactions with carnosine
- Assess the bioaccessibility and effect of dietary carnosine in its pork matrix during in vitro digestion of the model burger meal combo with or without ascorbic acid and fructose as anti- and pro-oxidants from beverages

1.3.3 Study the effect of carnosine as a meat constituent during in vitro digestion of a healthy meal. The following aims were included:

- Develop a meat-containing Mediterranean meal as the model healthy meal
- Assess the bioaccessibility and the potential antioxidant and anti-glycation effect of carnosine during in vitro digestion of this healthy meal

1.3.4 Explore the direct effect of carnosine on nitrosamine formation. The aims of this objective included:

- Development of an aqueous model for the assessment of carnosine's effect on the formation of different nitrosamine species
- Development of an aqueous-lipid model to study the effect of carnosine on nitrosamine formation in a stomach-simulating environment

- Development of methods for the determination of nitrosamine in samples from both models
- Assessment of the effect of carnosine on the formation of different nitrosamine species in aqueous and lipid environments, respectively

2. Literature review

2.1 Introduction to meat

The flesh of animals, including muscle fibers, connective tissues and fat associated with muscle, are referred to as meat. The commonly available kinds of meat are skeletal muscles from birds and domesticated livestock, such as chickens, turkeys, cattle, and hogs (Savell, 2022). The general structure of the skeletal muscle is shown in Figure 2.1.

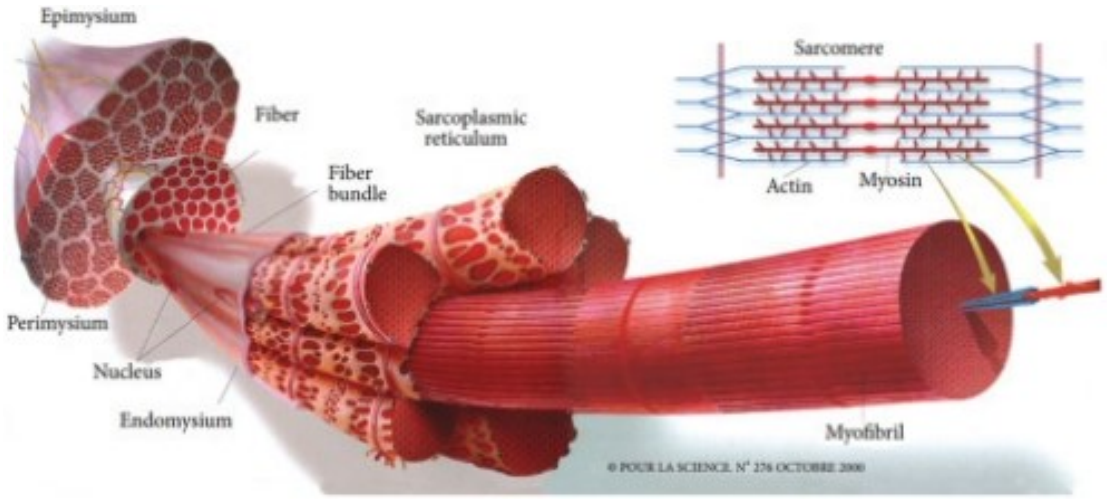

Figure 2.1. The general structure of skeletal muscle (Purslow, 2022)

Irrespective of the type of meat, water is the most abundant component in meat (about 75%). Numerous proteins are also contained in meat, for example, myoglobin in the sarcoplasm and myofibrillar proteins such as myosin (the major protein in thick filaments) and actin, tropomyosin, and troponin (in thin filaments) (Purslow, 2022),

making proteins the second most abundant component in meat (about 19%) (López-Bote & Calvo, 2022). Lipids are the third most abundant (about 2.5%), while carbohydrates are the fourth most abundant (about 1.2%), followed by nonprotein nitrogenous substances and micronutrients (López-Bote & Calvo, 2022).

2.2 Health benefits and attractive sensory qualities

Meat provides high-quality protein, essential amino acids, and essential fatty acids, in addition to contributing to meeting energy requirements. In addition, important micronutrients contained in meat further support its nutritional value, particularly considering these micronutrients could be lacking in vegan diets; for example, meat is a good dietary source of vitamin A, B vitamins, zinc, and heme-iron (which is the easily absorbed form of iron) (Ahmad et al., 2018). Furthermore, several red-meat-based healthy meals have been designed, and their benefits have been confirmed in studies with healthy men and women volunteers (Roussell et al., 2012).

Many meat constituents can also contribute to the formation of desirable sensory qualities. For instance, myoglobin, consisting of apoprotein and heme, is the key protein supplying oxygen to the muscle cells of living animals and is responsible for color development in meat. Briefly, myoglobin at the surface of meat is usually in its deoxymyoglobin (purplish red) state. When exposed to air, the heme-iron, which is in the Fe(II) oxidation state, is able to bind a molecule of oxygen, yielding oxymyoglobin (red), whereas slow oxidation of Fe(II) to Fe(III) will generate brown-colored metmyoglobin (Faustman et al., 2022). Lipids can contribute to flavor development through oxidation reactions. Among different types of lipids, phospholipids play a vital role in generating

desirable meat flavors because their high polyunsaturated fatty acid content makes them prone to oxidization, generating aldehydes, ketones, alcohols, and other volatile compounds. Lipid-involved Maillard reaction also provides essential contributors to the flavor of meat through its ability to generate carbonyl compounds. Similarly, proteins and carbohydrates can contribute to the flavor also through the Maillard reaction (particularly Strecker degradation), generating odor-active heterocyclic compounds and aldehydes, as reviewed by Mottram (1998) and Flores (2017). In addition, the degradation of specific molecules, such as thiamine, generates heterocyclic sulfur compounds, which contribute to kokumi and umami flavors. Ribonucleotides, through the Maillard reaction, also produce 5′-ribonucleotides as the umami flavor contributor.

Overall, meat as a nutritious and palatable food will remain popular for some time to come.

2.3 Health concerns raised by meat consumption

Many studies have reported that high meat consumption (particularly red meat, such as lamb, beef, and pork, as well as processed meat products) increases the risk of developing oxidation- and inflammation-related chronic diseases, such as cancer, type 2 diabetes mellitus, and cardiovascular diseases (reviewed by Richi et al., 2015; Wolk, 2017). Some meat components, such as lipids and added nitrites, can be associated with health concerns.

2.3.1 Lipids proteins and iron in meat

Meat (fresh and processed) can be a source of saturated lipids, *trans*-fatty acids, and cholesterol, whose intake is recommended to be controlled by the World Health Organization (2020). Ingestion of these meat constituents contributes to increased blood levels of low-density lipoproteins (LDLs), whose oxidation could lead to modifications in blood and vascular walls, triggering multiple signaling pathways, changes in atherogenic properties, and finally inducing atherosclerosis, cardiovascular disease among others, as reviewed by Poznyak et al., (2021) and Gehring (2022).

Proteins and lipids in meat can also contribute to the formation of AGE (Advanced Glycation End Products) through non-enzymatic glycation and oxidation (such as Maillard reactions). The generated AGEs can interact with other in vivo bioactive molecules and be responsible for AGE-related health concerns. For instance, the modifications of LDL (low-density lipoprotein) and apolipoprotein B (apo-B) by AGE and cross-links between AGE and type IV collagen are well-known reasons for the increased risk of atherosclerosis (reviewed by Vasdev et al., 2007). The engagement of AGEs with receptors for advanced glycation end products (RAGE) can lead to the activation of NF-κB, which binds to certain DNA sequences and activates transcription of inflammation-related genes, such as cytokine and adhesion molecule genes (Bierhaus et al., 2005). The induced oxidation and inflammation can lead to dysfunction in cells and even diseases, for example, damaging the pancreas and cells throughout the body, affecting insulin secretion and glucose uptake (Yamagishi et al., 2007; Kellow & Savige, 2013), subsequently increasing the risk of type 2 diabetes.

Iron content in meat can act as a catalyst for many reactions. For instance, heme-iron can catalyze lipid oxidation through various mechanisms depending on the iron oxidation state, such as the Fenton-like mechanism, heme—Fe(III)/heme—Fe(IV)=O mechanism, pseudoperoxidase mechanism, as reviewed by Carlsen et al. (2005). The increased oxidation by heme-iron has been observed not only in muscle foods, such as beef (Purohit et al., 2015), but also during meat digestion in both animals and humans (as reviewed by Van Hecke et al., 2017). Therefore, heme-iron provided by meat is associated with a high risk of chronic diseases, such as cardiovascular disease, because of its promotion of oxidation, increased severity of inflammation, and multiple related reactions in the body (Czerwonka & Tokarz, 2017).

2.3.2 Nitrite salts in processed meat products and nitrosamine

Apart from meat's inherent components, nitrite salts, the most common additives in processed meat, are also associated with health concerns. Processed meat products, such as bacon and sausages, are popular around the world, for example, being consumed on a daily basis by many North American consumers (Frank et al., 2021). Nitrite salts in processed meat products are irreplaceable for their beneficial roles in controlling oxidation, improving flavor, and acting as anti-bacterial agents and colorants (Sebranek, 2008). However, the ingested nitrites can generate nitrogen oxides and nitrosamines through a sequence of reactions presented in Figure 2.2. Briefly, in the acidic environment of the stomach, the nitrite can generate HNO_2 and then N_2O_3 and the

nitrosonium cation (in the presence of a transition metal ion), which can directly react with secondary amines and produce nitrosamines (Toldrá, 2017).

$$NO_2^- + H^+ \leftrightarrow HNO_2$$

$$2\ HNO_2 \leftrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \leftrightarrow NO + NO_2$$

$$HNO_2 + H^+ \rightarrow H_2NO_2^+ \leftrightarrow NO^+ + H_2O$$

$$H_2NO_2^+ + X^- \rightarrow XNO + H_2O$$

$$NO + M^{n+} \rightarrow NO^+ + M^{(n-1)+}$$

$$XNO + R_2NH \rightarrow R_2N\text{-}NO + HX$$

$$NO+/N_2O_3 + R_2NH \rightarrow R_2N\text{-}NO$$

Where X^- represents catalyzing ions such as SCN^-, I^-, Br^-, and Cl^-; and $M^{n+}/M^{(n-1)+}$ represent transition metal ions

Figure 2.2. Nitrosamine formation (Toldrá, 2017)

The nitrosamines are secondary amine derivatives possessing the structure shown in Figure 2.3, in which a nitroso group (−N=O) is bonded to the nitrogen of the deprotonated amine. With respect to meat products, N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR) are the typical nitrosamines (Andrée et al., 2010) along with N-nitrosodiethylamine (NDEA), N-nitrosomorpholine (NMOR), N-nitrosodipropylamine (NDPA), and N-nitrosodibutylamine (NDBA) that can also originate from dietary exposure (Li & Hecht, 2022), with the secondary amines usually formed in raw meat and meat products. For instance, putrescine, which is frequently found in fermented sausages, can contribute to NDMA formation with heating treatments (Drabik-Markiewicz et al., 2011); spermidine

and spermine, two secondary amines present at significant levels in raw meat, can contribute to the formation of NPYR and NPIP; and cadaverine, which is usually observed in meat products, can be converted to piperidine and then participate in the generation of NPIP (as reviewed by Sallan et al., 2023). Epidemiological studies have also suggested the carcinogenicity of high intake of nitrites, which causes increased nitrosamine levels, and nitrosamine is associated with concerns of carcinogenesis (Song et al., 2015; Pegg & Shahidi, 2000).

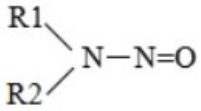

Figure 2.3. Nitrosamine

Irrespective of the origin of nitrosamines, many of them can be classified as probable or possible carcinogens (category 2A or 2B), for example, N-nitrosodiethylamine (NDEA) belongs to category 2A, and N-nitrosodimethylamine (NDMA) belongs to category 2B (World Health Organization, 2023). In the research studies that have been conducted with various experimental animals, approximately 90% of nitrosamines tested were deemed to be carcinogenic (Bryan & van Grinsven, 2013). Apart from some exceptions, there is a commonly shared mechanism behind the carcinogenic effect of nitrosamines. The α-carbon atom of the nitrosamine is first hydroxylated, producing an unstable structure (Hotchkiss, 1988). The latter takes part in reactions that generate reactive nitrogen compounds, such as diazoalkane, diazonium cation, and diazohydroxide, that can react with DNA, yielding pro-mutagenic DNA (Shephard et al., 1987). In addition to damaging the DNA, exposure to nitrosamine also promotes oxidation of low-density lipoprotein and other lipids, generating reactive oxygen species (ROS) (such as superoxide anion and

hydrogen peroxide), leading to increased oxidative stress (Liu et al., 2001). The enhanced oxidative stress can result in tissue and organ injuries (for example, damaged pancreatic cells and blood vessel lesions), impacting cell signaling pathways and increasing the risk of chronic diseases (Liu et al., 2001; Wilson et al., 2003; Mueller et al., 2005; Barski et al., 2013).

Overall, multiple clinical and epidemiological studies and research on potentially involved mechanisms have reported the relationship between meat consumption (especially red meat) and adverse health outcomes, forming the basis for the widespread negative image of meat consumption among the public. However, recent publications that evaluate the previous studies reporting unfavorable health consequences of meat consumption have pointed out the lack of reliable evidence regarding the effects of meat consumption on health and the existence of only weak associations between different chronic diseases and consumption of unprocessed red meat (Zhang et al., 2020; Lescinsky et al., 2022). Potential bias during epidemiological studies may provide a partial explanation for the existing contradictory data; for example, the process of assessing dietary intake, such as by relying on the memory of the volunteers, may not be accurate (Gehring, 2022). Furthermore, inadequate attention has been paid to some components of meat itself that possess health-beneficial properties and may even counteract undesirable consequences brought about by meat consumption. Carnosine, an inherent muscle histidine-containing dipeptide, is worthy of attention owing to its versatility.

2.4 Introduction to carnosine

Carnosine (β-alanyl-L-histidine, Figure 1) is a multifunctional dipeptide that was first discovered over a century ago in meat extract. While it can be found in several tissues, such as the kidney, plasma, and certain brain regions, the vast majority (⩾ 99% in many vertebrates) is in skeletal muscles (Boldyrev et al., 2013) and is therefore found exclusively in meat. Carnosine in different types of meat originates from the same general synthetic pathway (as included in Figure 2.4). In brief, dietary β-alanine and L-histidine can be transported into the muscle by the Tau-T taurine/β-alanine transporter (TauT), the proton-coupled amino acid transporter 1 (PAT1), and the peptide/histidine transporter 1 (PHT1). In addition, dietary carnosine can be absorbed by the oligopeptide transporter 1 (PEPT1) and provide β-alanine and L-histidine for carnosine synthesis as shown in Figure 2.4. Then, carnosine can be synthesized in the muscle in the presence of carnosine synthase (a type of ATP-grasp domain-containing protein 1), Mg^{2+}, and ATP (Kalyankar & Meister, 1959; Drozak et al., 2010).

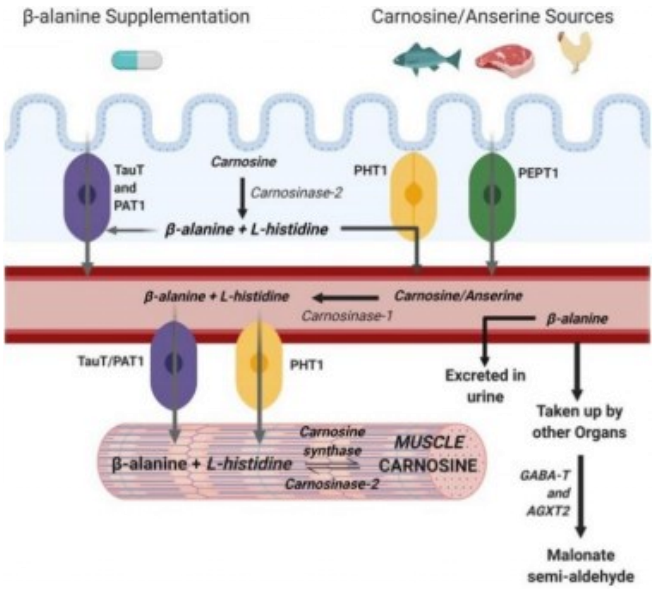

Figure 2.4. Metabolism of muscle carnosine (Perim et al., 2019)

The variability of carnosine content in muscle originating from different animals can be related to the species, breeds, gender, and nutrition of the animals (Peiretti & Meineri, 2015; Harris et al., 2012); for example, the gene expression of the transporters and enzymes involved in carnosine synthesis can be breed-related (D'Astous-Pagé et al., 2017), and the amounts of β-alanine and L-histidine available can be related to feeding (Kopec et al., 2020; Paniagua et al., 2023). In addition, the composition of muscle fibers can also depend on species, breed, gender, and age, and meat containing more glycolytic muscle fibers tends to possess a higher intrinsic level of carnosine (Mora et al., 2008; Harris et al., 2012). Therefore, commonly available types of meat from different parts of animals as well as different animals have different concentrations of carnosine, as illustrated by the information presented in Table 2.1.

Table 2.1. Carnosine concentrations in different types of meat[1]

Type of meat	Carnosine level (mg/100 g meat)
Pork loin	313.0±35.7
Pork trapezius	181.0±10.1
Beef top loin	372.5±32.2
Chicken pectoral	180.0±10.9

[1]Aristoy & Toldrá, et al. (1998; 2004)

Muscle food (meat), therefore, can be a good dietary source of this multifunctional dipeptide and might thereby ameliorate pathologies and optimize health conditions (Hipkiss, 2005; Wu, 2020) owing to carnosine's properties, such as metal ion chelation, antioxidant and anti-glycation activities, and the potential to suppress nitrosamine formation (Boldyrev et al., 2013; Artioli et al., 2019; Prakash et al., 2021; Nicoletti et al., 2007).

2.5 Properties of carnosine

2.5.1 pH buffering and metal ion chelation

In the carnosine molecule, the β-alanine residue, the nitrogen atoms of the imidazole ring, and the carboxylic group are capable of reacting with acidic and alkaline substances and controlling the pH of the environment over a relatively wide range (Boldyrev et al., 2013). Furthermore, considering that the pka value (7.01) of the imidazole ring is close to the intracellular pH of muscle, the ability of the imidazole ring to maintain the surrounding pH close to its pk value can contribute to the intracellular buffering property of carnosine (Perrone et al., 2015).

The pH of the environment is also related to the metal ion chelation capacity of carnosine. This metal-chelating ability is well described in terms of copper and zinc ions and can contribute to various functions of carnosine. Depending on the ratio of metal ions to carnosine and the pH value of the environment, the Nτ or Nπ atom of the imidazole ring, the nitrogen atoms from amino groups, and one carboxyl oxygen atom can all provide chelation sites (Boldyrev et al., 2013). Despite iron ion is always on the list when the metal ion chelating ability of carnosine is mentioned, the generation of carnosine-iron complexes is not well understood, although carnosine is reported to chelate ferrous ions (Canabady-Rochelle et al., 2015).

Transition-metal ions are reported to facilitate oxidation and contribute to the nitrosation of secondary amines (Carlsen et al., 2005; Toldrá, 2017; Challis et al., 1978). Carnosine's

chelating capacity may, therefore, contribute to its antioxidant function and its potential to control nitrosamine formation.

2.5.2 Antioxidant activity and inhibition of advanced glycation

a) Scavenging of reactive oxygen species (ROS)

The ability of carnosine to scavenge ROS contributes to its observed antioxidant activity. The mechanism of scavenging is dependent on the nature of the free radical. For instance, carnosine scavenges hydroxyl radicals by the interaction of the carbon atom (C (2) position) of its imidazole moiety with hydroxyl radicals, yielding more stable intermediates (Decker et al., 1992). Carnosine, on the other hand, can donate a hydrogen atom to the peroxyl radical, thereby terminating the free radical chain reaction (Kohen et al., 1988). A complex scheme can also be involved in the scavenging of the strong oxidant, hypochlorous acid/hypochlorite. Reaction of hypochlorous acid with carnosine initially results in chloramine formation at the Nτ atom on the imidazole ring or at the terminal amino group on the β-alanyl moiety but is followed by an intra- or intermolecular transfer of chlorine (depending on the pH) shown in Figure 2.5 and forms relatively stable structure (Carroll et al., 2019).

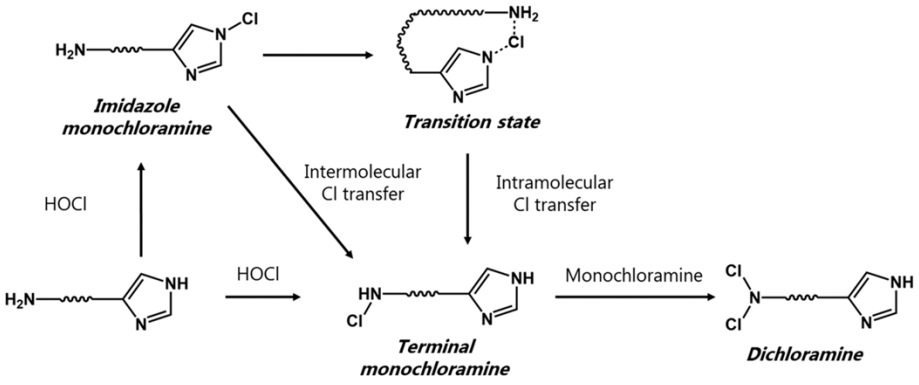

Figure 2.5. Carnosine reacts with hypochlorite (Carroll et al., 2019)

b) Carbonyl scavenging activity and inhibition of advanced glycation

Carnosine is also capable of quenching carbonyl compounds originating from various molecules. Carnosine can directly quench carbonyl compounds generated from lipid oxidation, for example, the n-6 PUFAs derived genotoxic and cytotoxic MDA (malondialdehyde) and 4-HNE (4-hydroxynonenal). This can occur through the nitrogen atom (amino group) from the β-alanine moiety of carnosine first reacting with the aldehyde groups, followed by an intramolecular Michael addition and further hydrolysis yielding the stable hemiacetal derivative (Carini et al., 2003; Aldini et al., 2002). In addition, carnosine can also bind di-carbonyls, such as glyoxal (GO) and methylglyoxal (MGO), which can be derived from lipids and sugars and further contribute to AGE formation (Wang & Ho, 2012; Aldini et al., 2021). Although studies related to the mechanism behind carnosine-di-carbonyl interaction are not as detailed as that of carnosine-4-HNE reaction, the formation of adducts between carnosine and di-carbonyls is still observed and can involve the nitrogen of amino group from the β-alanine moiety of carnosine binding with the carbonyl group of MGO (Aldini et al., 2021). The reaction of di-carbonyls with carnosine can contribute to the inhibition of AGE formation, considering otherwise these trapped di-carbonyls could interact with lysine, cysteine, arginine, and other side chains of protein and/or amino acids for the formation of different types of AGE (Aldini et al., 2021).

Carnosine can also show impacts on AGE formation through reducing the formation of Schiff bases, which is the early step of AGE formation. Schiff base formation can be controlled by the nucleophilic attack of carnosine, resulting in the de-glycation of

aldosamines, subsequently suppressing AGE formation (Szwergold, 2005). In addition, the protective effect of carnosine on enzymes (such as glyoxalase I and glutathione peroxidase) from the glycation-induced and oxidation-induced loss of enzyme activity can also lead to decreases in AGE levels (Seidler et al., 2001; Ghodsi & Kheirouri, 2018).

Apart from previously mentioned health concerns brought about by AGE, the AGE formation process can also generate free radicals, hence promoting oxidation (Rizzi, 2003). Therefore, carnosine, by quenching the intermediate compounds involved in AGE formation, could show its additional benefits to health. Furthermore, the capacity of carnosine to activate the Nrf2 pathway, which up-regulates not only antioxidation but also antiglycation enzymes (such as aldehyde dehydrogenase and carbonyl reductase) and interferes with the GO- and MGO-derived AGE formation as recently reviewed (Aldini et al., 2021), represents a very important potential of carnosine in vivo. Although the overall benefits of carnosine against AGE formation have been reported under certain conditions, there is still a need for further research to obtain a better understanding of non-enzymatic and/or enzyme-involved mechanisms, considering the numerous reactions and pathways involved (Ghodsi & Kheirouri, 2018; Aldini et al., 2021).

2.5.3 Potential ability of carnosine to inhibit nitrosamine formation

According to the mechanisms of nitrosamine formation shown in Figure 2.2, the metal ion chelating ability of carnosine could interfere with the formation of nitrosamine. Carnosine's ability to scavenge radicals, such as superoxide anion ($O_2^{\cdot -}$), may also indirectly contribute to decreasing the formation of nitrosamines, given that oxidation and

nitrosation can be related to each other. For instance, the peroxynitrite can be formed via the reaction $O_2^{\cdot-} + NO \rightarrow ONOO^-$; and this is then related to the generation of N_2O_3, a direct precursor of nitrosamine, via reactions $ONOO^- + H^+ \rightarrow NO_2 + OH$, $NO + NO_2 \leftrightarrow N_2O_3$, subsequently relating to the nitrosamine formation (Radi et al., 2000). In addition, the potential of carnosine to form carnosine-NO and carnosine-NO_2 adducts brought about by NO or NO_2 biding with the L-histidine moiety of carnosine (Nicoletti et al., 2007) could also reduce the amount of N_2O_3, thereby decreasing the amount of N_2O_3-mediated nitrosamine formations. Caruso et al. (2017) confirmed the formation of carnosine-NO adducts and reported the suppression of NO production in simulated macrophages.

On the other hand, carnosine could activate the NO-generating enzyme (endothelial nitric oxide synthase, eNOS) in different cells or in vitro through carnosine-induced Ca^{2+} mobilization (Alaghband-Zadeh et al., 2001; Takahashi et al., 2009). Such an increase in NO may raise the levels of the direct precursor of nitrosamine N_2O_3 ($NO + NO_2 \leftrightarrow N_2O_3$) and contribute to nitrosamine formation. The possible role of carnosine in nitrosamine formation could be related to its L-histidine moiety as an important source of secondary amine that may facilitate nitrosamine generation (Shephard et al., 1987; de La Pomélie et al., 2017), although a recent study has suggested the potential of histidine as an inhibitor of nitrosamine formation (Nanda et al., 2021). These conflicting results were obtained in studies carried out under different conditions that were focused on changes in the level of NO and did not provide direct information on the changes in the final nitrosamine levels.

Overall, available information on the impact of carnosine on nitrosamine formation is inadequate and inconclusive, suggesting the need for further investigations.

2.6 In vivo benefits of carnosine

Because of these multiple properties of carnosine, in vivo benefits of carnosine can be observed in many tissues and organs. For example, the pH-buffering and metal ion chelating abilities of carnosine can show benefits in skeletal muscles, considering the production of lactic acid and the importance of calcium ions for muscle (Culbertson et al., 2010; Schroder et al., 2008; Dutka & Lamb, 2004). Additionally, its antioxidant and enzyme regulating (such as activation of myosin ATPase) abilities contribute to attenuating muscle fatigue, enhancing the efficiency of muscle contraction, and improving exercise performance (Dutka & Lamb, 2004; Culbertson et al., 2010).

Furthermore, carnosine's antioxidant, anti-glycation as well as its other properties can protect different tissues against many diseases, as reviewed by Boldyrev et al. (2013). As previously mentioned, the antioxidant ability of carnosine prevents the oxidation of LDL, which is closely associated with atherosclerosis (Vasdev et al., 2007), and its ability to bind and remove oxidation-produced carbonyls (Barski et al., 2013) can contribute to the protection of the cardiovascular system. In the kidney, carnosine can reduce oxidative stress-related pro-inflammatory and profibrotic cytokine formation and decrease mitochondrial apoptosis and aberrant mesangial cell proliferation, bringing protections against many kidney-related problems, such as ischemia/reperfusion damage and diabetic nephropathy (Kilis-Pstrusinska, 2020). Similarly, carnosine can also protect the brain

from damage, sclerosis, apoptosis, and disorders, such as Alzheimer's disease and psychiatric disorders, and also improves cognitive functions, as reviewed by Schön et al. (2019).

Apart from the carnosine molecule, constituent amino acids of carnosine, if hydrolyzed, can show their health benefits. The β-alanine can act as a neurotransmitter (Schön et al., 2019; Tiedje et al., 2010); histamine generated from L-histidine can affect the neural activity of the renal sympathetic nerve and lowers the risk of ischemia; and both L-histidine and β-alanine benefit wound healing (Boldyrev et al., 2013).

Overall, carnosine shows its in vivo advantages in various tissues and is expected to benefit overall health. However, the acquisition of these benefits of carnosine in dietary conditions can require more studies, for example, that consider potential interference from interactions among bioactive compounds and changes in bioavailability.

2.7 Factors affecting the health outcomes of dietary carnosine

2.7.1 The effect of carbohydrates and reducing sugars as dietary pro-oxidants

Apart from meat, other food products are also consumed, providing different dietary components, including another macronutrient carbohydrate. For instance, the carbohydrates and reducing sugars from bread and beverages, which are parts of diets such as a typical Western diet, have potential effects on glycation, AGE formation, and related reactions.

Baking of bread is a well-known cooking process where the Maillard reaction plays an important role in generating baked aromas, browning, and AGE, even beneficial compounds, for example, fructose-tryptophan Maillard reaction product acting as an effective antioxidant against lipid oxidation (Morales & Jiménez-Pérez, 2001). In addition to bread, soft drinks can be considered as a direct source of reducing sugars; together with bread, they increase in vivo AGE formation, which is responsible for the increased oxidative stress and cell damage, for example, affecting activation of transcription factors, leading to impaired immune response, dyslipidemia, obesity, type 2 diabetes, and other metabolic diseases (Aragno & Mastrocola, 2017). Increased lipid and protein oxidation is also observed in the presence of reducing sugars. Sugars catalyze the reduction of Fe (III) to Fe (II), thereby contributing to the Fenton reaction, which can directly produce lipid alkoxyl from lipids (Carlsen et al., 2005) and generate hydroxyl radical attacking both fat and protein under a wide range of temperature from 4°C-110°C (Adriana & Estévez, 2013). Furthermore, the oxidation of sugars can also form reactive carbonyls, such as glyoxal, which subsequently contributes to downstream AGE formation and their effects (Adriana & Estévez, 2013; Twarda-Clapa et al., 2022).

Overall, in a typical Western diet, the increased AGE formation and oxidation by bread carbohydrates and by reducing sugars from beverages may generate reactive molecules that can interact with carnosine, such as ROS, Schiff bases, MDA, and glyoxal, as reviewed above. Consequently, there may be a change in the bioavailability of carnosine. Therefore, the bioavailability and the possible effect of carnosine in a Western meal

under the combined effect of dietary factors need to be further assessed for better estimation and understanding of the performance of carnosine as a meat constituent.

2.7.2 Role of other dietary antioxidants

In addition to dietary pro-oxidants, dietary antioxidants could also show their effects, which are particularly abundant in healthy diets, and could offer functional abilities similar to that of carnosine and may play a supporting role in decreasing oxidative stress and its impact on health conditions.

For better healthy conditions, the World Health Organization (2020) recommends adequate amounts and varieties of vegetables and fruits, selected sources of carbohydrates and lipids, and limited free sugars. Among different diets following the guidelines and directions for healthy eating, the Mediterranean diet is popular.

The Mediterranean diet is a typical diet with multiple health benefits against cardiovascular diseases, diabetes, and many other metabolic disorders, including decreasing cancer risk and mortality rates. (Willett et al., 1995; Buckland & Agudo, 2015). These benefits may be attributable to the characteristic diet components, which provide less toxic and more health-beneficial molecules, such as abundant plant-based foods (vegetables, fruits, grains, etc.) and olive oil.

a) Olive oil

Olive oil, as one of the most well-known elements in MD, provides relatively high concentrations of health-beneficial unsaturated fatty acids and other bioactive compounds (Alkhatib, 2020). For instance, oleic acid protects LDL from oxidation, subsequently contributing to atherosclerosis prevention, and oleuropein, as one of the major phenolic molecules, can play multiple roles, including antioxidant, anti-aging, anti-inflammatory, and protection against atherosclerosis and cancer (Alkhatib, 2020). It can be hypothesized that these health-promoting compounds from olive oil may have synergistic effects with carnosine and/or contribute to spare carnosine and increase its bioaccessibility for further in vivo benefits. Olive oil, on the other hand, could contribute to lipid oxidation by providing the substrates that could require more antioxidant activity from either or both carnosine and antioxidant components from olive oil (Covas et al., 2006). Overall, the presence of olive oil should be considered when studying the outcome of dietary carnosine in a meal.

b) Vegetables, fruits, and whole grains

Consumption of adequate vegetables and fruits is usually recommended for a better health outcome against cardiovascular, coronary, and many other chronic diseases and cancers (Slavin & Lloyd, 2012; Salas-Salvadó & Papandreou, 2020).

The widely distributed and abundant phenolic compounds, carotenoids, vitamins, dietary fibers, and their bioactive metabolites can account for in vivo chemoprevention. For instance, phenolic compounds show their health-beneficial effect by scavenging radicals,

reducing low-density lipoprotein (LDL) oxidation, preventing platelet aggregation, improving endothelial function in the coronary artery, and many other pathways, as reviewed by Lilamand et al. (2014). In addition, the capability of regulating kinases and gene expression, such as inducing cancer cell apoptosis, contributes to the anti-cancer properties of polyphenols and carotenoids from vegetables and fruits (Koyama et al., 2010; Milani et al., 2017). Vitamins, the essential micronutrients, are also found in plant foods, such as the well-known hydrophilic antioxidant ascorbic acid (Slavin & Lloyd, 2012). Its ability to reduce oxidation by being oxidized itself can protect against many oxidation-related problems, such as atherogenesis, diabetes, and certain cancers (Chambial et al., 2013). It is also important for the biosynthesis of L-carnitine and certain neurotransmitters, as well as the enhanced absorption of non-heme iron (NIH Office of Dietary Supplements, 2021).

In addition to antioxidants, dietary fibers from vegetables and fruits also show a health contribution. For instance, dietary fiber could support the healthy colonic microflora by providing prebiotics for obligate anaerobic bacteria (many of them are probiotics), which are important in keeping intestinal homeostasis, producing vitamin and anti-microbial factors, as well as engaging in immunological responses and multiple activities, subsequently maintaining and even improving the intestinal health (EI Enshasy et al., 2016). By providing prebiotics, dietary fiber could indirectly contribute to the health status of bones and even decrease the incidences of osteoporosis, attributing to the metabolic by-products of prebiotics that can benefit the growth of epithelial cells and the absorption of calcium (Praznik et al., 2014). In addition, dietary fibers also show their

benefits by affecting the peristalsis and delaying the feeling of hunger by retarding glucose transfer and intestine emptying progress (Praznik et al., 2014).

Besides vegetables and fruits, whole grains are also a source of dietary fiber and are favored in many healthy diets, including the MD. This kind of food also contains some antioxidants, such as phenolic compounds and vitamins (Yu et al., 2013), and shows beneficial properties along with dietary fiber, such as antioxidant and anti-inflammatory effects (Dominguez & Barbagallo, 2020). Considering carbohydrates are essential as one of the major nutrients, choosing whole grains, which are rich in antioxidants and dietary fiber, is considered a good choice (Castro-Quezada et al., 2020; Dominguez & Barbagallo, 2020).

Nevertheless, there is still inconclusive and incomprehensive information regarding the consumption of vegetables and fruits in daily diets. Many studies only focused on certain aspects, such as the health-beneficial molecules from plant-based foods. It has been reported by Bellavia et al. (2016) that high fruit and vegetable consumption showed no significant effect in reducing high mortality rates associated with high red meat consumption. Simultaneously, bioactive components from meat, such as the multifunctional carnosine, have attracted very limited attention from a dietary point of view, even though meat is an important element of diets (Altomare et al., 2013). Because carnosine has the potential to bring health benefits to meat-containing meals, according to the previously reported beneficial role of dietary carnosine (Boldyrev et al., 2013; Li et al., 2021), further studies are needed on the potential effect and interactions of different

bioactive compounds when present in the diet, including dietary carnosine, less beneficial components (carbohydrates and reducing sugars), various beneficial components (originating from vegetables, fruits, and whole grains) such as found in the Western diet and MD.

2.7.3 Digestion Process

During digestion, the mechanical movement and hydrolysis of large molecules will release smaller molecules to their surroundings, affecting their availability to exert their effects (Rein et al., 2013). In addition, the gastrointestinal tract provides conditions promoting various reactions. For instance, the acidic environment in the stomach facilitates the transformation of nitrates in vegetables to nitrites and also increases the generation of nitrosating agents, such as NO^+ and N_2O_3, subsequently contributing to nitrosamine formation (Toldrá, 2017). It also escalates oxidation, for example, in the presence of oxygen, low pH facilitates hydrogen peroxide formation, which is capable of initiating lipid oxidation (Morrissey et al., 1998). This acidic environment promotes the release of heme (from the myoglobin in meat) for the further catalysis of lipid oxidation and nitrosamine formation (Hargrove et al., 1996; Bastide et al., 2011). In addition, the acidic condition favors the release of reducing sugars from unreactive disaccharides and amino acids from proteins through hydrolysis (Perez-Locas & Yaylayan, 2010), promoting the formation of AGE (Hipkiss, 2018).

The digestion process can also liberate or generate health-beneficial molecules that may act against undesirable reactions (Nieva-Echevarría et al., 2017; Holland et al., 2020;

Rasera et al., 2023). Limited studies have been reported on the interactions among beneficial compounds found in food, for example, the well-known interaction between ascorbic acid and oxidized α-tocopherol whereby ascorbic acid restores the antioxidative capacity of α-tocopherol (Thomas & Stocker, 2000). Ascorbic acid can also contribute to oxidation under certain conditions owing to its ferric-reducing ability that can convert the oxidized product ferric ion to ferrous ion, which is able to initiate oxidation, and this maintenance of the iron ion cycle makes ascorbic acid a pro-oxidant (Rietjens et al., 2002). Nevertheless, these beneficial molecules can still interfere with reactions related to the generation of undesirable products, protecting some molecules from degradation and affecting the solubility, changing bioaccessibilities of both adverse and beneficial compounds, thereby expressing their activities during digestion (Rasera et al., 2023). Therefore, digestion needs to be addressed when assessing the outcome of the diet, which has numerous bioactive compounds.

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Connecting paragraph

Chapter 2 reviews the background knowledge on the health-related results, benefits, and concerns associated with meat consumption, the ways in which carnosine, a dipeptide originating from meat, could influence oxidative status, and the ways in which other dietary components and the digestion process could affect the health outcomes of dietary carnosine.

Considering the previously reported beneficial role of carnosine during meat digestion, carnosine has the potential to contribute to health benefits, particularly in the popular meat-containing pro-oxidative meals, such as burger meals. However, the presence of other ingredients consumed with these meals, such as reducing sugars found in beverages, can modulate potential glycation and oxidation reactions during digestion. This raises the question of how dietary carnosine could perform in the presence of other anti- and pro-oxidant factors. In Chapter 3, the study was carried out to evaluate functional properties of dietary carnosine during in vitro digestion of a burger meal system, including burger buns, pork patty with three levels of carnosine, with or without fructose and/or ascorbic acid (as the simulation of beverage and the representative of antioxidant supplement).

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Chapter 3 Dual effects of dietary carnosine during in vitro digestion of a Western meal model with added ascorbic acid

3.1 Abstract

The beneficial role of carnosine during in vitro digestion of meat was previously demonstrated and it was hypothesized that such benefits could also be obtained in a meal system. The current study, therefore, assessed carnosine effects on markers of lipid and protein oxidation and of advanced glycation end-products (AGEs) during gastric and duodenal in vitro digestion of a burger meal model. The model included intrinsic (low) and enhanced (medium and high) carnosine levels in a mix of pork mince and bread, with or without ascorbic acid (AA) and/or fructose as anti- and pro-oxidants, respectively. In the presence of either AA or fructose, a carnosine pro-oxidative potential during digestion was observed at the medium carnosine level depending on markers and digestive phases. However, free carnosine found at the high carnosine level exerted a protective effect reducing the formation of 4-hydroxynonenal in the gastric phase and glyoxal in both the gastric and duodenal phases. Dual effects of carnosine are likely concentration-related, whereby at the medium level, free radical production increases through carnosine's ferric-reducing capacity, but there is insufficient quantity to reduce the resulting oxidation, while at the higher carnosine level some decreases in oxidation are observed. In order to obtain carnosine benefits during meal digestion, these findings demonstrate that consideration must be given to the amount and nature of other anti- and pro-oxidants present and any potential interactions.

3.2 Practical Application

Carnosine, a natural compound in meat, is a multifunctional and beneficial molecule for health. However, both pro- and antioxidative effects of carnosine were observed during

digestion of a model burger meal when ascorbic acid was included at a supplemental level. Therefore, to obtain benefits of dietary carnosine during digestion of a meal, consideration needs to be given to the amount and nature of all anti- and pro-oxidants present and any potential interactions.

3.3 Introduction

Carnosine is a β-alanyl-L-histidine dipeptide naturally synthesized in the muscles of many vertebrates and is therefore found exclusively in muscle foods. Carnosine possesses many biochemical properties such as pH buffering, antioxidant capacity, metal chelation, and carbonyl and radical scavenging, along with anti-inflammatory and antiglycation capacities offering therapeutic potential for diseases involving oxidative stress (Boldyrev et al., 2013), such as diabetes (Peng et al., 2020), cardiovascular diseases (Menon et al., 2020) and neurological disorders (Caruso et al., 2021). The potential for increasing the muscle concentration of this multifunctional molecule in meat animals through breeding and nutrition is documented (D'Astous-Pagé et al., 2017; Robbins et al., 1977). D'Astous-Pagé et al. (2017) reported the variability in muscle carnosine content and carnosine-related gene expression between commercial swine breeds used in Canada to be important enough to be used for breeding for increased muscle carnosine content. They also showed that improved pork quality parameters were associated with higher muscle carnosine level. Supplementing the diet of broilers (Kralik et al., 2015) and pigs (Paniagua et al., 2023) with histidine to support animal growth as well as other important physiological functions, including muscle carnosine synthesis, efficiently increased muscle carnosine content and improved meat quality parameters (such as pH, color, and

water retention), without impairing the performance of the animals (Paniagua et al., 2023). Enhanced carnosine levels in meat have been shown to improve oxidative stability during the storage of raw (Das et al., 2006) and cooked products (Decker & Crum, 1993). Furthermore, it was inferred by Brownlee (2001) that diets containing carnosine and its related peptides might ameliorate pathologies associated with macromolecular glycation, such as diabetes and other health conditions. So far, however, most of the research on carnosine has been conducted on the isolated molecule (carnosine supplement), either in vitro or in vivo with animal or human subjects. As a result, no information is available on the role, benefits, and interactions of dietary carnosine when provided through and digested with its meat matrix in the presence of other foods or nutrients.

Using in vitro digestion, in our laboratory it was demonstrated that in the gastrointestinal environment, a twofold increase of carnosine in cooked ground pork compared with the inherent level in pig muscle reduced the formation of both lipid and protein oxidation markers and of N^{ε}-(1-carboxymethyl)-l-lysine (CML), a marker of advanced glycation end products (AGEs) (Li et al., 2021). In a meat-based meal context, the meat-related oxidation occurring during digestion can also be reduced by antioxidants from the accompanying food. For instance, using in vitro co-digestion of grilled turkey meat and a Mediterranean diet salad, Martini et al. (2020) reported a dose-dependent reduction of meat lipid hydroperoxides with increased amounts of the salad mixture attributed to increased phenolic compounds.

A common antioxidant that has long been recognized and used to improve the color and shelf life of meat while at the same time providing potential health benefits for consumers is ascorbic acid (AA) (Bauernfiend & Pinkert, 1970). However, the antioxidant effect of AA in meat appears only to occur at high concentrations as shown in ground beef (Benedict et al., 1975; Mitsumoto et al., 1991) and in model systems (Lee & Hendricks, 1997; Miller & Aust, 1989). In these studies, low levels of AA consistently led to increased lipid peroxidation. This antioxidant effect of high ascorbate level is attributed to its capacity to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions (Miller & Aust, 1989). However, in the presence of hydrogen peroxide, this ferric-reducing capacity can change the ratio of ferric and ferrous ions and maintain the iron ion cycle, initiating the oxidation chain reactions through free radical production (Miller & Aust, 1989). Similar to AA, carnosine can also reduce Fe^{3+} to Fe^{2+} (Mozdzan et al., 2005) and therefore could also have pro-oxidant potential given the presence of hydrogen peroxide during digestion. Yet, only antioxidant and antiglycation effects of carnosine were observed during in vitro digestion of pork with enhanced carnosine levels (Li et al., 2021). It is interesting to note that Van Hecke et al. (2016), adding AA before in vitro digestion, observed increased lipid peroxidation in high-fat (15%) beef digests, but not in low-fat digests (1%). And in an earlier study using ground beef with ≤5% fat content, Lee et al. (1999) found that carnosine was a more effective lipid peroxidation inhibitor than AA, but it had a synergistic antioxidant effect when the two were combined. Using a model system, these authors also observed that carnosine, in a dose-dependent manner, can protect AA by inhibiting its copper-catalyzed oxidation. The combined effect of carnosine and AA

during digestion of a Western meat-based meal system, however, has never been investigated.

With 10 billion burgers consumed each year in the United States alone (Waite et al., 2018), a burger meal represents a simple, but pertinent Western meal for use as a model system to study dietary interactions. Including AA as a part of a Western diet model system is also fitting given that AA is the world's leading antioxidant supplement (Wunsch, 2021), with available commercial concentrations ranging from 45 mg in multivitamins to more than 5000 mg per dose in powder form. Including AA in the model system would allow carnosine effects to be studied in the presence of a known antioxidant. Similarly, to assess the protective effect of dietary carnosine in the presence of a known pro-oxidant, fructose was included in the study given the ubiquitous presence of high-fructose sweetener as a chief candidate for the presumptive association between the Western diet and the occurrence of metabolic syndrome, type 2 diabetes, and nonalcoholic fatty liver disease, and as the potential source for the probable intestinal formation of AGEs (DeChristopher, 2017; Gugliucci, 2017).

The aim of this study was, therefore, to assess the functional properties of dietary carnosine during in vitro digestion of a burger meal model system consisting of a bun and pork patty with three levels of carnosine, and with or without added AA and/or fructose.

3.4 Materials and methods

3.4.1 Chemicals and reagents

All reagents and chemicals were of analytical or higher grade. All chemicals and enzymes were purchased from Sigma–Aldrich except ferrous sulfate heptahydrate (Thermo Fisher Scientific), and 4-hydroxyhexenal-d_3 (HHE-d_3; ≥99%) and 4-hydroxynonenal (4-HNE; ≥99%) (Cayman Chemical).

3.4.2 Burger meal model preparation

The combination of treatments used for in vitro digestion of the burger meal model is presented in Figure 3.1. The meat batters were prepared as detailed in Li et al. (2021). Briefly, subcutaneous fat and four entire longissimus dorsi muscles from a commercial pork processing plant in Saint-Henri, Quebec, Canada (Olymel S.E.C) were ground (6 mm plate) and mixed to attain 10% total fat content. Batters of three carnosine levels were prepared by adding 300 or 600 mg carnosine/100 g meat to the control (intrinsic 277 mg carnosine/100 g pork; LCar), providing the intermediate (577 mg carnosine/100 g ground pork; MCar) and high (877 mg carnosine/100 g ground pork; HCar) carnosine treatments. The addition of 300 and 600 mg carnosine/100 g meat was based on the natural occurrence of high levels (>1000 mg/100 g) of histidine-containing dipeptides reported in some meat and fish species with comparable muscle glycolytic profiles (Boldyrev et al., 2013). The batters were stuffed into polypropylene wide-mouth screw top containers (60 mL), which were then sealed in individual vacuum bags, heated to a core temperature of 90°C, and held 30 min at this temperature. The cooked batters were portioned (20 g) into plastic bags, vacuum packaged, and stored at −80°C until in vitro

digestion. Just prior to digestion, the cooked batters were thawed (room temperature, approximately 15 min). Fructose (1.34 g) and/or AA (0.09 g) were solubilized in 2 mL water representing the liquid portion accompanying the solid meal sample. The AA concentration was arbitrarily selected to represent 30% of the highest dose available in commercial supplements, and the concentration of fructose was based on the average level found in some popular commercial soft drinks (Walker et al., 2014). The cooked pork samples (4.5 g), commercial white bread hamburger bun (3.0 g), and the liquid portion (2 mL of water as a control or with added AA and/or fructose) were mixed by vortexing in a 50-mL polypropylene tube for in vitro digestion.

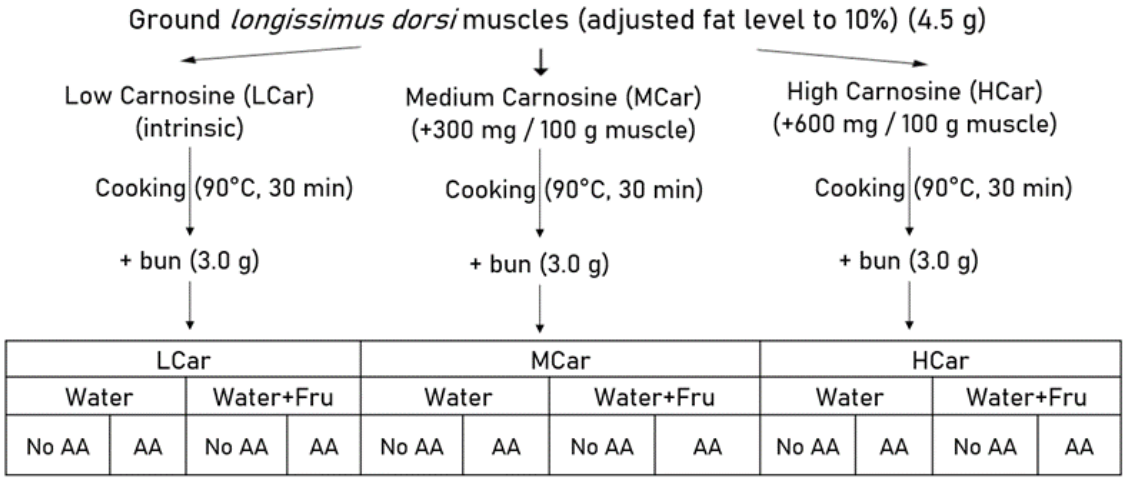


Figure 3.1. Preparation of the burger meal model (water: 2 mL; Fru: 1.34 g; AA: 0.09 g). Fru, fructose; AA, ascorbic acid.

3.4.3 In vitro digestion

The in vitro digestion method was described by Li et al. (2021) and is based on the general food digestion method of Versantvoort et al. (2005), which was adapted for the digestion of meat by Van Hecke et al. (2014). Briefly, the meal samples (7.5 g solid and 2

mL liquid) were sequentially incubated for 5 min with saliva (4 mL), 2 h with gastric juice (12 mL), and 2 h with 1 M bicarbonate buffer (pH 8.0, 2 mL), duodenal juice (12 mL), and bile (6 mL). Colonic digestion was not undertaken. The mediums for the saliva, gastric juice, and duodenal juice are described in Van Hecke et al. (2014), with modifications to the saliva medium. In order to respect the prescribed solid-to-liquid ratio of Versantvoort et al. (2005), only 4 mL of saliva medium was added to the sample mix given the prior addition of 2 mL of water. Therefore, to maintain the same final concentrations in the salivary phase, a saliva medium of 1.5 times the concentration of that of Van Hecke et al. (2014) was used. The enzymatic incubations were performed in quadruplicate. Two tubes of digested samples were taken after the gastric phase, and one after the duodenal. The digests were homogenized at 10,000 rpm for 1 min using a Polytron homogenizer (PT-MR 3100 with a PT-DA 12/2EC-B154 dispersing aggregate; Kinematica AG), and aliquots were removed to Eppendorf tubes and stored at −80°C pending chemical analyses.

3.4.4 Free carnosine measurement

Free carnosine measurement in the meat was measured by HPLC as described by Mora et al. (2007) with modifications (Li et al., 2021). Briefly, 0.01 N HCl (2 mL) was added to gastric or duodenal digest (1.5 g), mixed well, and centrifuged (10,621 × *g*, 4°C, 20 min). An aliquot of filtered (nylon, 0.45 µm) supernatant (250 µL) was mixed with acetonitrile (750 µL), held at 4°C for 20 min, and then centrifuged (10,621 × *g*, 4°C, 10 min). The supernatant was analyzed by HPLC. The HPLC system (Waters Alliance 2695 HPLC fitted with a W600 pump; Waters Corporation) achieved separation by a HILIC Silica

column (4.6 × 150 mm, 3 µm; Waters Corporation) preceded by a corresponding HILIC Silica pre-column (Atlantis 3.9 × 5 mm, 3 µm) at room temperature. Mobile phases consisted of solvent A containing 10 mM ammonium acetate (pH 5.5) in water/acetonitrile (25:75, v/v) and solvent B containing 10 mM ammonium acetate in water/acetonitrile (70:30, v/v). Elution was by means of a gradient of filtered solvents (0.22-µm nylon membrane filter). The gradient was as follows: initial eluant 100% B; 30% A and 70% B at 9.1 min; 100% A at 11.1 min and maintained at these conditions for 10.0 min. The separation was monitored using a photodiode array detector at a wavelength of 214 nm for carnosine. Peak areas were correlated to compound concentration by interpolation in the corresponding calibration curve.

3.4.5 Protein oxidation markers (carbonyls and free thiols)

Determination of protein carbonyls was based on a modified method of that description of Ventanas et al. (2006), which is based on the method of Oliver et al. (1987). Two aliquots of gastric or duodenal digest (200 µL) were each mixed with 20% trichloroacetic acid (TCA) (200 µL), then centrifuged (3000 × *g*, 5 min). One pellet was treated with 2 N HCl (2 mL) for protein concentration measurement and as a blank, and the other pellet with an equal volume (2 mL) of 10 mM 2,4-dinitrophenylhydrazine in 2 N HCl for carbonyl concentration measurement. Both samples were held at room temperature for 1 h, then 20% TCA (2 mL) was added. The precipitate was washed three times with freshly prepared ethyl acetate: ethanol (1:1, v/v; 2 mL), then fully dissolved in 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5, 2.5 mL), and centrifuged (2240 × *g*, 2 min) to remove insoluble fragments. Protein concentration was

calculated from absorption at 280 nm using BSA as standard. Carbonyls were determined at 365 nm using an adsorption coefficient of 22,000 $M^{-1} \cdot cm^{-1}$ and expressed as nmol/mg protein.

Free thiols were measured by the method described by Winterbourn (1990) using 2,2′-dithiobis (5-nitropyridine) (DTNP) as modified by Martinaud et al. (1997) and with further modifications. The protein level of digest was first determined using the biuret method (Gornall et al., 1949) and adjusted to 5 mg/mL with 200 mM phosphate buffer (pH 7.4). Then, urea buffer (8 M urea in 100 mM phosphate buffer, pH 8.0) was used to adjust the protein level to 1 mg/mL. An aliquot of 10 mM DTNP solution in ethanol (20 µL) was added to the protein solution (2 mL) and held for 1 h at room temperature. The absorbance of the solution at 386 nm was measured against a blank of protein at the same concentration without DTNP. The absorbance of diluted DTNP was subtracted, and the thiol concentration was calculated using an absorption coefficient of 14 $mM^{-1} \cdot cm^{-1}$. Results are expressed as µmol free thiol/mg protein.

3.4.6 Lipid oxidation markers

Measurements of hexanal, 4-HNE, glyoxal (GO), and methylglyoxal (MGO) were based on the study of Tsikas et al. (2016) using an Agilent 7890B gas chromatograph coupled to a 5977B quadrupole mass spectrometer (Agilent Technologies) with Ultimate Plus deactivated fused silica tubing (5 m × 0.25 mm; Agilent Technologies) and HP-5MS columns (30 m × 0.25 mm × 0.25 µm; Agilent Technologies). A first derivatization was undertaken by vortexing digest (200 µL), *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine

hydrochloride (OPFB) (170 µL of a 30 mg/mL solution), and HHE-d_3 (10 µL of a 20 ng/µL solution in ethanol) together and incubating the mix for 3 min in an ultrasonic bath. Extraction of the components of interest was undertaken by the sequential addition of methanol (500 µL), isooctane (1 mL), and concentrated sulfuric acid (six drops), after which the mix was vortexed for 1 min and centrifuged (774 × *g*, 5 min). Supernatant (800 µL) was transferred to vials through anhydrous sodium sulfate and glass wool and evaporated to dryness. A second derivatization was undertaken with the addition of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; 50 µL) incubated at 80°C for 1 h. Aliquots (1 µL) of the derivatized samples were injected with the following temperature program: held at 70°C for 3 min, increased to 80°C at a rate of 6°C/min, further increased to 280°C at a rate of 20°C/min and held for 15 min, and finally increased to 300°C at a rate of 20°C/min then held another 5 min. Helium (61 kPa, constant flow of 1 mL/min) was used as the carrier gas. The mass selective detector was operated in electron impact (EI) mode with ionization energy of 70 eV, and the dwell time for each ion was 5 ms in the selected-ion monitoring (SIM). For quantification, the following ions were chosen: 187 and 203 *m*/*z* (RT [retention time] 12.20 and 12.33 min for the internal standard HHE-d3); 239 *m*/*z* (RT 10.35 min for hexanal); 352 *m*/*z* (RT 13.62 min for 4-HNE); 448 *m*/*z* (RT 13.04 and 13.10 min for GO); and 265 and 462 *m*/*z* (RT 13.26 min for MGO).

3.4.7 N^{ε}-(1-Carboxymethyl)-L-lysine (CML)

An OxiSelect CML competitive enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs Inc.) was used for the measurement of CML as a marker of AGEs formation. Digested samples were diluted with PBS buffer as per the instructions. Results were

obtained from a CML-BSA standard curve (BioTek Epoch 2 microplate spectrophotometer; Agilent Technologies) and expressed as ng/mL digest.

3.4.8 Statistical analyses

Simple effects and all interactions of the three carnosine levels in the meat with or without AA and/or fructose were analyzed as a factorial arrangement with the MIXED procedure of SAS (SAS version 9.4, 2002–2012; SAS Institute Inc.). Heterogeneous variances were used when appropriate. Four repetitions were carried out on the entire protocol for a total of 48 observations (12 combinations of treatments × 4 repetitions) for each variable within each of the gastric and duodenal phases. A $p \leq 0.05$ was considered significant with a tendency defined as $0.05 < p < 0.10$. Interactions were further analyzed using partitioned analysis (slice option of the LSMEAN statement). Significant interactions are presented as figures.

3.5 Results and discussion

3.5.1 Carnosine levels during preparation and digestion

Before cooking, the free carnosine levels in LCar (277.43 ± 0.01 mg/100 g), MCar (445.92 ± 1.59 mg/100 g), and HCar (527.30 ± 5.09 mg/100 g) groups were significantly different from each other ($p < 0.05$). Incomplete recovery of carnosine was observed in the samples with enhanced levels, which indicates that some unmonitored reactions likely occurred between the dipeptide and the ground meat matrix upon mixing. After cooking, the carnosine levels in each of the LCar, MCar, and HCar groups remained significantly different from each other ($p < 0.05$) and were slightly higher (305.30 ± 8.61,

470.61 ± 14.74, and 543.08 ± 14.56 mg carnosine/100 g meat, respectively) than in the uncooked meat. Since carnosine is known to be heat stable under the cooking conditions used (Miwako et al., 2015), the increased concentrations after cooking could be explained by the concentrating effect of cooking loss, particularly fat. Using the same cooking procedure, Li et al. (2021) also observed increased carnosine levels after the cooking of ground pork irrespective of carnosine treatments.

The free (bioaccessible) carnosine levels in the gastric and duodenal digests for each of the treatments are reported in Tables 3.1 and 3.2, respectively. In the gastric phase, a significant carnosine × fructose interaction ($p < 0.0001$) was obtained. In the absence of fructose, a significant increase in bioaccessible carnosine in the digests was observed at enhanced carnosine levels compared to LCar, while no significant difference in free carnosine was found between the three carnosine treatments with added fructose (Figure 3.2). This effect of fructose on carnosine bioaccessibility could be due either to the capacity of fructose to nonenzymatically glycosylate carnosine as shown by Hipkiss et al. (1995) or, indirectly, to carnosine reduction of fructose-induced lipid oxidation as reported below. No other interactions or simple effects of AA on the bioaccessibility of carnosine in gastric digests were observed.

Table 3.1. Effects of carnosine level, fructose, and ascorbic acid on free carnosine, indicators of protein and lipid oxidation, and AGEs (least squares means with *SEM* in parentheses) during gastric in vitro digestion of a burger meal system.

	Treatments		Carnosine (μg/mL digest)	Protein carbonyls (nmol/mg protein)	Free thiols (nmol/mg protein)	Hexanal (ng/mL digest)	4-HNE (ng/mL digest)	GO (ng/mL digest)	MGO (ng/mL digest)	CML (ng/mL digest)
	Fructose (Fru)	Ascorbic acid (AA)								
LCar	No Fru	No AA	463.9 (25.6)	4.89 (0.24)	5.51 (1.11)	5395 (439)	331.9 (16.3)	75.6 (5.0)	77.7 (3.1)	180.0 (58.3)
		AA	484.6 (25.6)	4.29 (0.24)	7.96 (1.11)	4497 (439)	196.7 (10.2)	122.7 (5.0)	78.4 (3.1)	213.0 (58.3)
	Fru	No AA	97.4 (25.6)	6.04 (0.86)	5.61 (1.11)	5290 (439)	280.3 (11.4)	65.9 (5.0)	92.5 (5.3)	274.5 (58.3)
		AA	84.2 (25.6)	5.14 (0.86)	7.45 (1.11)	4807 (439)	181.6 (3.8)	108.6 (5.0)	101.6 (5.3)	180.5 (58.3)
MCar	No Fru	No AA	761.3 (81.4)	5.45 (0.84)	5.95 (0.72)	5075 (439)	316.8 (16.3)	83.5 (5.0)	82.0 (3.1)	127.9 (26.0)
		AA	741.5 (81.4)	5.43 (0.84)	6.11 (0.72)	6043 (439)	267.4 (10.2)	165.3 (5.0)	78.5 (3.1)	388.3 (129.1)
	Fru	No AA	76.1 (31.3)	4.65 (0.73)	6.40 (0.72)	6189 (439)	310.5 (11.4)	80.7 (5.0)	118.2 (5.3)	197.0 (26.0)
		AA	103.4 (25.6)	4.65 (0.73)	7.19 (0.72)	7217 (439)	313.6 (3.80)	158.7 (5.0)	139.7 (5.3)	278.4 (129.1)
HCar	No Fru	No AA	901.7 (81.4)	5.50 (1.97)	6.78 (0.35)	4807 (439)	314.4 (16.3)	66.6 (5.0)	66.5 (3.1)	97.1 (26.0)
		AA	961.8 (81.4)	6.41 (1.97)	6.32 (0.35)	4989 (439)	228.7 (10.2)	129.9 (5.0)	78.1 (3.1)	321.4 (129.1)
	Fru	No AA	123.2 (31.3)	4.43 (0.62)	4.70 (0.35)	5742 (439)	344.0 (11.4)	72.4 (5.0)	95.6 (5.3)	105.5 (26.0)
		AA	130.5 (25.6)	5.23 (0.62)	5.72 (0.35)	6376 (439)	274.9 (3.8)	135.9 (5.0)	108.4 (5.3)	432.4 (158.1)
p Values										
		Car	**<0.0001**	0.9194	0.2719	**0.0048**	**<0.0001**	**<0.0001**	**<0.0001**	0.7629
		Fru	**<0.0001**	0.6195	0.5769	**0.0042**	0.2302	0.2298	**<0.0001**	0.6445
		AA	0.6557	0.9590	0.0505	0.3566	**0.0001**	**<0.0001**	**0.0050**	**0.0113**
		Car × Fru	**<0.0001**	0.2089	0.0643	0.1705	**0.0011**	0.0575	**0.0004**	0.8276
		Car × AA	0.9013	0.5755	0.3265	**0.0363**	**<0.0001**	**0.0002**	0.7000	**0.0253**
		Fru × AA	0.8301	0.9171	0.5937	0.5468	**0.0162**	0.6508	**0.0153**	0.5061
		Car × Fru × AA	0.8096	0.9875	0.6425	0.9409	0.5463	0.9416	0.2643	0.5381

Note: Statistical significance at $p \leq 0.05$.

Abbreviations: 4-HNE, 4-hydroxynonenal; AA, ascorbic acid; AGEs, advanced glycation end products; CML, Nε-(1-carboxymethyl)-L-lysine; Fru, fructose; GO, glyoxal; HCar, high carnosine; LCar, low carnosine; MCar, medium carnosine; MGO, methylglyoxal.

The values in bold are the P values for each of the treatments or combination of treatments that were significantly different statistically.

Table 3.2. Effects of carnosine level, fructose, and ascorbic acid on free carnosine, indicators of protein and lipid oxidation, and AGEs (least squares means with *SEM* in parentheses) during duodenal in vitro digestion of a burger meal system.

	Treatments		Carnosine (µg/mL digest)	Protein carbonyls (nmol/mg protein)	Free thiols (nmol/mg protein)	Hexanal (ng/mL digest)	4-HNE (ng/mL digest)	GO (ng/mL digest)	MGO (ng/mL digest)	CML (ng/mL digest)
	Fructose (Fru)	Ascorbic acid (AA)								
LCar	No Fru	No AA	200.5 (48.3)	4.85 (0.25)	6.61 (0.58)	9913 (782)	83.8 (9.6)	66.6 (6.7)	116.8 (5.6)	148.9 (10.4)
		AA	173.6 (48.3)	4.64 (0.25)	12.59 (1.47)	11458 (782)	92.8 (9.6)	107.7 (6.7)	116.7 (5.6)	140.3 (10.4)
	Fru	No AA	103.7 (33.7)	4.26 (0.25)	7.46 (0.58)	8926 (782)	68.6 (9.6)	47.1 (6.7)	109.9 (5.6)	178.0 (32.5)
		AA	96.5 (33.7)	4.95 (0.25)	12.60 (1.47)	11074 (782)	85.9 (9.6)	81.8 (6.7)	105.3 (5.6)	166.8 (32.5)
MCar	No Fru	No AA	233.2 (77.3)	4.41 (0.55)	9.24 (0.58)	13145 (782)	107.0 (9.6)	48.2 (8.1)	123.0 (5.6)	141.6 (10.4)
		AA	286.8 (63.1)	5.17 (0.55)	11.97 (0.58)	20899 (3226)	134.7 (2.3)	171.9 (6.7)	117.0 (5.6)	123.5 (10.4)
	Fru	No AA	145.7 (40.3)	4.12 (0.55)	9.10 (0.58)	9367 (3226)	78.3 (2.3)	71.4 (6.7)	128.2 (5.6)	162.1 (32.5)
		AA	142.7 (40.3)	4.91 (0.55)	12.03 (1.37)	19614 (782)	122.5 (2.3)	154.0 (6.7)	105.3 (5.6)	133.0 (32.5)
HCar	No Fru	No AA	460.2 (101.5)	4.24 (0.34)	9.06 (1.47)	10729 (782)	69.6 (2.3)	62.1 (6.7)	97.4 (5.6)	146.2 (10.4)
		AA	404.1 (101.5)	4.81 (0.34)	13.40 (1.47)	23040 (782)	137.5 (21.9)	67.3 (6.7)	145.6 (5.6)	121.2 (10.4)
	Fru	No AA	211.5 (54.1)	4.07 (0.34)	7.30 (0.58)	9927 (782)	68.4 (2.3)	50.2 (6.7)	105.6 (5.6)	166.2 (32.5)
		AA	192.9 (54.1)	5.65 (0.34)	11.41 (0.58)	23672 (3226)	151.5 (21.9)	156.2 (6.7)	165.7 (5.6)	159.3 (32.5)
p Values										
		Car	**0.0126**	0.9925	0.5697	**0.0002**	**0.0037**	**<0.0001**	**0.0013**	0.5692
		Fru	**0.0013**	0.9074	0.4229	0.2463	0.2228	**0.0307**	0.8550	0.1072
		AA	0.7914	**0.0081**	**<0.0001**	**<0.0001**	**0.0001**	**<0.0001**	**0.0008**	0.2562
		Car × Fru	0.3551	0.4933	0.3323	0.5856	0.2976	**<0.0001**	**0.0185**	0.9023
		Car × AA	0.7905	0.1752	0.1930	**0.0010**	**0.0305**	**<0.0001**	**<0.0001**	0.9217
		Fru × AA	0.9979	0.1872	0.8121	0.4133	0.3229	0.1364	0.6240	0.9580
		Car × Fru × AA	0.8499	0.7507	0.9356	0.8799	0.9391	**<0.0001**	0.2059	0.9092

Abbreviations: 4-HNE, 4-hydroxynonenal; AA, ascorbic acid; AGEs, advanced glycation end products; CML, N^{ε}-(1-carboxymethyl)-L-lysine; Fru, fructose; GO, glyoxal; HCar, high carnosine; LCar, low carnosine; MCar, medium carnosine; MGO, methylglyoxal.

The values in bold are the P values for each of the treatments or combination of treatments that were significantly different statistically.

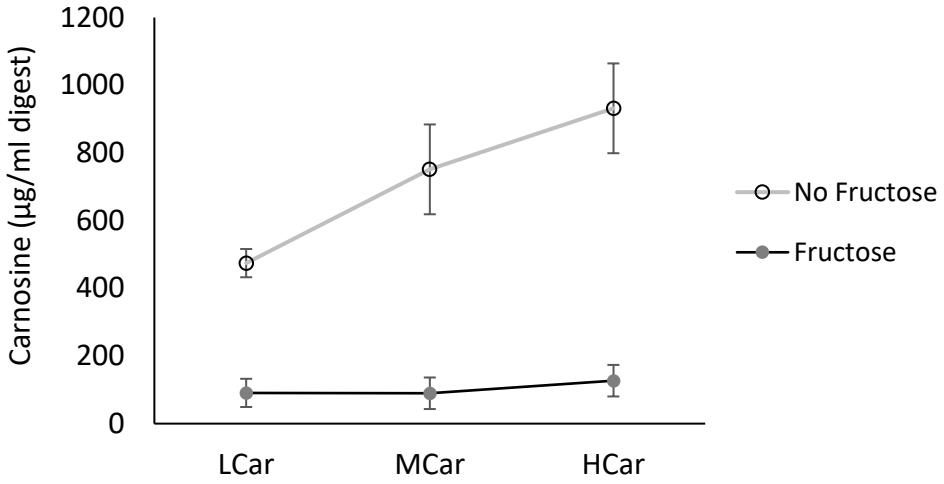


Figure 3.2. Interaction between carnosine and fructose on free carnosine concentrations (least squares means with 95% confidence intervals) in gastric digests ($p < 0.0001$).

The overall bioaccessibility of carnosine was lower in the duodenal phase than in the gastric phase irrespective of the treatments. A similar decrease in carnosine bioaccessibility from gastric to duodenal phases was also observed in an earlier study (Li et al., 2021) and was associated with reduced protein and lipid oxidation and AGEs formation by carnosine during in vitro digestion of pork. Continuous oxidation during meat digestion can generate radicals (Oueslati et al., 2016) and induce the formation of cytotoxic and genotoxic compounds, which can be substrates for carnosine to react with, for example, by scavenging hydroxyl radicals and/or forming 4-HNE-carnosine adducts, thereby resulting in less free carnosine in the duodenal phase (Li et al., 2021). No interactions of treatments were found in the duodenal phase, but simple effects of carnosine ($p = 0.0126$) and fructose ($p = 0.0013$) were obtained. Increased carnosine levels resulted in concomitant increased carnosine bioaccessibility in the digests, while

the presence of fructose reduced carnosine bioaccessibility. No effect of AA was observed ($p = 0.7914$).

3.5.2 Protein oxidation markers

Protein oxidation generally leads to the generation of protein carbonyls and the reduction of free thiols (Domínguez et al., 2021). These two markers are, therefore, widely used as indicators of protein oxidation, with a decrease in protein carbonyls and an increase in free thiols demonstrating an antioxidant effect (Domínguez et al., 2021). No significant effects of carnosine and fructose were observed for protein carbonyl and free thiol levels in either gastric or duodenal environments (Tables 3.1 and 3.2). Furthermore, no interactions of treatments were found in protein oxidation markers. Effects of AA were observed, acting as an antioxidant leading to increased free thiols in each of the gastric ($p = 0.0505$) and duodenal ($p < 0.0001$) digests, while simultaneously acting as a pro-oxidant leading to increased protein carbonyl formation in the duodenal digests ($p = 0.0081$). This unexpected dual effect of a single dose of AA on the stability of different fractions from a common protein source does not appear to have been previously reported. In fact, little information is available concerning the role of AA during the digestion of meat. Van Hecke et al. (2016) observed that duodenal digests of AA concentrations from 0 to 20 mg per 4.5 g meat sample increased lipid oxidation only in the high-fat beef digests (15%), and not in those of low-fat beef (1%). No effect on protein oxidation was observed. These findings suggest that in the current study, the ferric reduction capacity of a high AA concentration during digestion likely led to the pro-oxidative Fenton reaction due to the presence of hydrogen peroxide, increased free

radical production, and a favorable acidic environment (Miller &, Aust, 1989; Sotler et al., 2019) and resulted in protein carbonyl formation (Domínguez et al., 2021). However, detailed pathways and conditions converting AA from antioxidant to pro-oxidant specifically targeting protein oxidation have not yet been elucidated. The antioxidant effect of AA leading to increased free thiols is also little documented, but could result from its potential capacity to reduce protein disulfide as reported by Landino et al. (2006). These workers hypothesized that the presence of iron and other transition metals might play a role in determining the ability of AA to facilitate disulfide cleavage. This dual effect of a single dose of AA on different protein oxidation markers during meat digestion merits further study.

3.5.3 Lipid oxidation and AGE markers

Several indicators were chosen to assess the effect of dietary compounds on lipid oxidation-related reactions. Hexanal was measured being a saturated aldehyde that is widely used to follow lipid oxidation in meat (Shahidi, 1998), and 4-HNE, an unsaturated aldehyde, was measured as the typical product of the chain reactions of *n*-6 PUFA (polyunsaturated fatty acids) peroxidation. Observing changes in these two markers provides a relatively comprehensive view by assessing both general lipid oxidation and the more specific lipid peroxidation, which directly generates the cytotoxic and genotoxic unsaturated aldehydes. In addition to these two markers, determination of changes in GO and MGO levels can reflect the potential changes in both lipid oxidation and AGEs formation and provide information on how carnosine and other dietary compounds might exert their effects. Finally, CML is an AGE frequently used as a general marker of AGEs,

and its formation can therefore directly evaluate the potential effect of dietary compounds on AGEs formation.

a) Hexanal

As a general product of lipid oxidation in meat, any decrease or increase in the level of hexanal caused by dietary components provides a valuable assessment of their anti- or pro-oxidant effects, respectively. Increased formation from gastric to duodenal digests was observed (Tables 3.1 and 3.2) in agreement with the results reported by Li et al. (2021) on in vitro digestion of pork with or without carnosine enhancement. In both gastric ($p = 0.0363$) and duodenal digests ($p = 0.0010$), interactions between carnosine and AA showed significantly increased hexanal formation in MCar with added AA (MCar-AA) compared to LCar-AA ($p = 0.0019$ for gastric digests; $p = 0.0007$ for duodenal digests) (Figure 3.3). In the duodenal digest, the hexanal concentration was also significantly higher in HCar-AA than in LCar-AA ($p < 0.0001$). No significant differences were found between MCar-AA and HCar-AA. This additive pro-oxidant effect of carnosine and AA was more marked in the duodenal than gastric digests (Figure 3.3). As simple effects, AA addition led to increased hexanal formation in duodenal digests ($p < 0.0001$) irrespective of carnosine levels, and carnosine enhancement increased hexanal levels in both MCar and HCar duodenal digests compared to LCar samples ($p < 0.0001$) (Table 3.2). Fructose addition caused a slight increase in hexanal concentration ($p = 0.0042$) in the gastric digests. The increased hexanal observed in the MCar-AA groups contrasts with the results of Li et al. (2021) who, using the same in vitro digestion system, showed that equivalent enhanced

carnosine levels in pork resulted in reduced hexanal formation in gastric and duodenal digests of lean ground pork. As antioxidants, both AA and carnosine can reduce Fe^{3+} to Fe^{2+} as shown by Miller and Aust (1989) and Mozdzan et al. (2005), respectively, and would therefore, in the presence of hydrogen peroxide during digestion, synergistically stimulate the Fenton reaction and induce free radical production and lipid oxidation. In the case of carnosine, the exact mechanism by which it can reduce ferric ions is not clear, especially given that histidine is not a reducing agent (Seth & Mahoney, 2000). It is also possible that the 10% fat in the meat sample may have contributed to hexanal production by offering expanded lipid compartments allowing the migration of the produced reactive oxygen species (ROS) and their pro-oxidant activity to occur out of reach of hydrophilic antioxidants, such as AA and carnosine. This hypothesis was suggested by Van Hecke et al. (2016) as a potential explanation for the AA-induced hexanal production observed in high-fat beef digests.

Using an in vitro model system with isolated Low Density Lipoprotein (LDL), Lankin et al. (2015) showed that both glucose and fructose led to spontaneous increases in LDL oxidation. Fructose was, however, a stronger inducer of free radical processes attributed to the more active free radical intermediates formed during the autoxidation of ketoses than of aldoses and to a higher rate of accumulation of α-oxoaldehydes, such as MGO, in turn, promoting the formation of oxygen radicals. It is interesting to observe that the concentration of free carnosine in the digests was not influenced by AA but was significantly reduced in the presence of fructose alone ($p < 0.0001$ and $p = 0.0013$ for gastric and duodenal digests, respectively; Table 3.1) or in interaction with carnosine

levels in the meat samples (Figure 3.2). It is possible that fructose may have reacted with other unmonitored markers that could have reacted with carnosine, or even directly with carnosine itself, which can be nonenzymatically glycosylated by fructose as shown by Hipkiss et al. (1995).

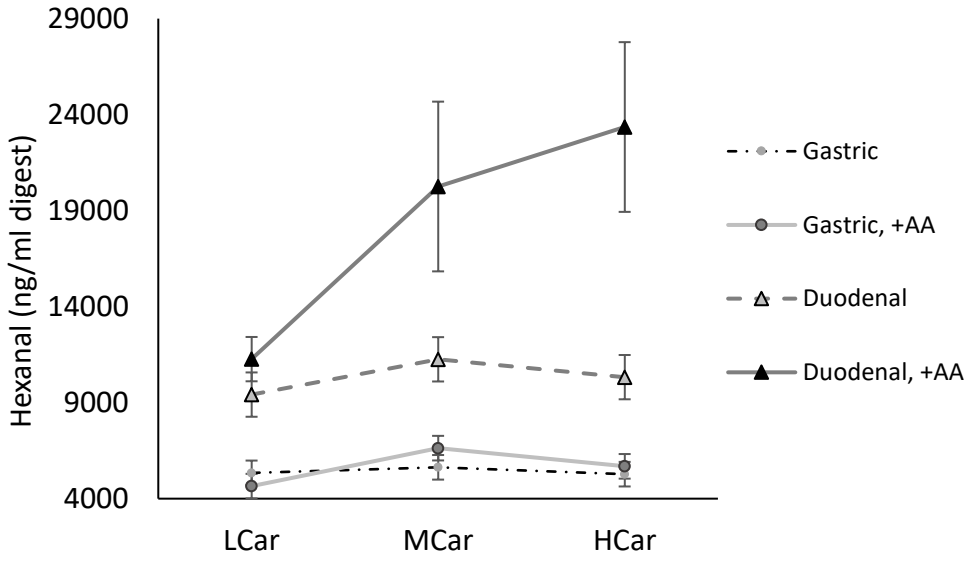
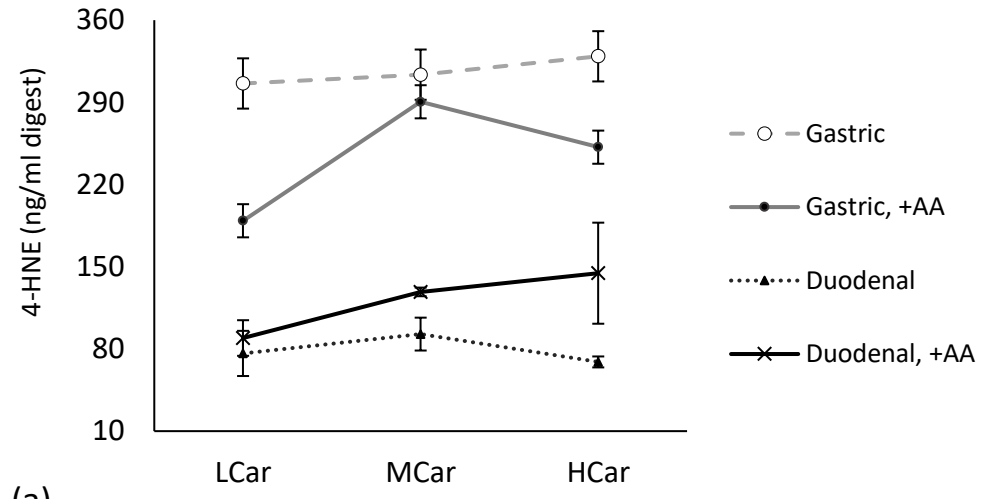


Figure 3.3. Interactions between carnosine and ascorbic acid (AA) on hexanal concentrations (least squares means with 95% confidence intervals) in gastric (p = 0.0363) and duodenal (p = 0.0010) digests.

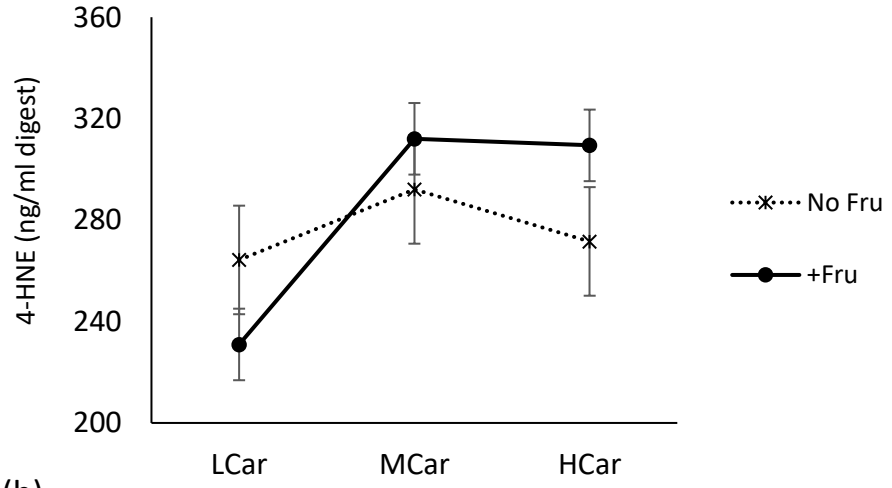
b) 4-Hydroxynonenal (4-HNE)

Effects of carnosine, AA, and fructose treatments on 4-HNE formation, a typical *n*-6 PUFA peroxidation product, are presented in Tables 3.1 and 3.2. Three significant interactions of the treatments on 4-HNE concentrations were found in the gastric digests. The carnosine × AA interaction (p = 0.0001; Figure 3.4a) showed that in the presence of AA, significantly less 4-HNE was found in both LCar ($p < 0.0001$) and HCar

($p = 0.0001$) compared to their counterparts without AA. Furthermore, both MCar-AA and HCar-AA gastric digests had higher 4-HNE concentrations than those of LCar-AA ($p < 0.05$). Without AA, no significant differences were observed among carnosine groups. It is suggested that the MCar-AA peak in 4-HNE concentrations represents a transition phase where a pro-oxidative effect of the combined treatments from LCar-AA to MCar-AA is followed by an antioxidant effect attributable to the additional carnosine in HCar-AA.



(a)



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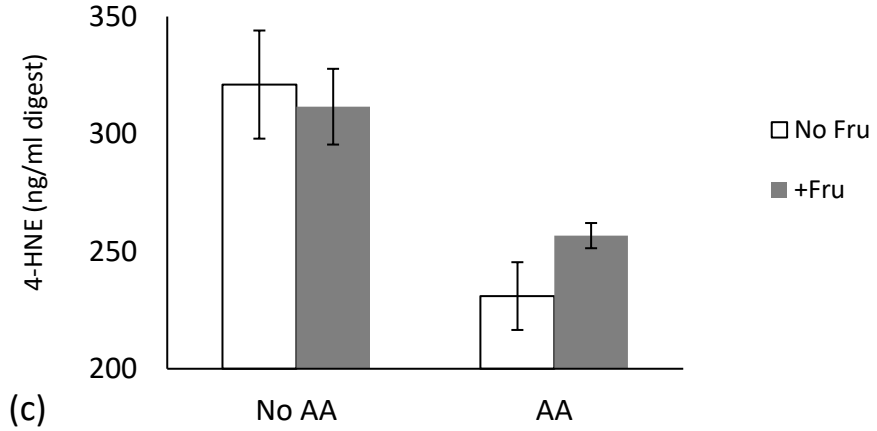


Figure 3.4. Interactions on 4-hydroxynonenal (4-HNE) concentrations (least squares means with 95% confidence intervals) between (a) carnosine and ascorbic acid (AA) in gastric (p = 0.0001) and duodenal (p = 0.0305) digests, (b) carnosine and fructose (Fru) in gastric digests (p = 0.0011), and (c) Fru and AA in gastric digests (p = 0.0162).

As previously indicated, both AA and carnosine can contribute to the reduction of Fe^{3+} to Fe^{2+} (Miller & Aust, 1989; Mozdzan et al., 2005) and lead, in the presence of hydrogen peroxide, to lipid oxidation. According to Buettner and Jurkiewicz (1996) and Miller et al. (1990), AA increases peroxide and hydroxyl radical formation, particularly at a balanced Fe^{2+}/Fe^{3+} ratio. Accordingly, a higher average concentration of hydrogen peroxide was observed in the digests with AA (12.07 mM) than without (2.22 mM); however, the ferrous and ferric ions were not measured. Nevertheless, in the presence of AA, significantly higher concentrations of 4-HNE in MCar digests suggest that a nearly balanced Fe^{2+}/Fe^{3+} ratio was established at this carnosine level, which may not be the

case in LCar. According to Buettner and Jurkiewicz (1996) and Miller et al. (1990), the predominance of either Fe^{3+} reduction or Fe^{2+} oxidation can reduce the rate and even stop the oxidation of lipids when any of the ions becomes marginal. In HCar-AA, the additional free carnosine might have, in addition to its ferric reduction capacity, contributed to the reduction of 4-HNE directly through its capacity to react with secondary products of oxidation or indirectly through its radical scavenging ability. Such multifunctional antioxidant activities of carnosine have been demonstrated during iron/ascorbate-induced phospholipid oxidation by Kansci et al. (1997).

The carnosine × fructose interaction ($p = 0.0011$; Figure 3.4b) showed that fructose decreased 4-HNE ($p = 0.0188$) in the LCar gastric digests, but increased it in the HCar digests ($p = 0.0103$). No significant effect of fructose was observed in MCar groups. Overall, fructose showed no simple effect on 4-HNE concentrations ($p = 0.2302$), nor were there any differences between MCar and HCar concentrations of 4-HNE, with or without fructose. Li et al. (2021) previously observed a reduction in 4-HNE formation following gastric digestion of pork with added carnosine compared to intrinsic carnosine levels. The effect of combined fructose and added carnosine on 4-HNE formation in this meal model can be explained by their capacities to contribute, at the levels used, to increased free radical production (Lankin et al., 2015; Mozdzan et al., 2005), as previously discussed. Alternatively, increased carnosine may provide an easily accessible substrate to be glycated by fructose, indirectly allowing increased 4-HNE formation through reduced availability of carnosine. This hypothesis is supported by the presence of fructose inhibiting the dose-dependent increase in bioaccessible carnosine, while this

increase was observed in the digests without fructose ($p < 0.0001$; Figure 3.2). Another potential mechanism explaining the antioxidant effect of fructose exerted in the LCar group could be its capacity to chelate iron and therefore suppress the iron-catalyzed formation of ROS as demonstrated by Valeri et al. (1997). However, there is no apparent explanation as to why this antioxidant effect occurs only in LCar digests. Finally, in the presence of fructose, an interaction ($p = 0.0162$) was observed whereby the antioxidant effect of AA was less effective at lowering 4-HNE levels (Figure 3.4c), in agreement with the oxidation capability of fructose (Lankin et al., 2015).

In the duodenal phase (Table 3.2), lower 4-HNE levels were found in the digests than in those of the gastric phase (Table 3.1). Although continuous oxidation during digestion would be expected to generate more oxidation products, this decrease in 4-HNE is in agreement with previous findings (Li et al., 2021) and is suggested due to the ability of 4-HNE to react further with other compounds (Steppeler et al., 2016). For example, 4-HNE could form adducts with nucleophilic amino acids, such as the thiolate site of cysteine (LoPachin et al., 2009). Given that the thiolate site is preferred in a basic environment, generation of the adducts between 4-HNE and protein would be more likely in the duodenal phase (LoPachin et al., 2009), explaining detection of lower amounts of 4-HNE molecule in the duodenal than gastric digests.

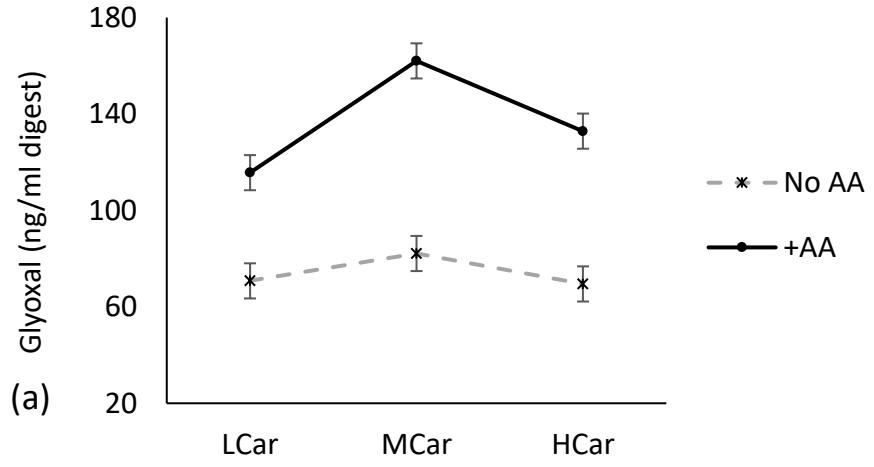
In the duodenal digests, a carnosine × AA interaction ($p = 0.0305$) was found (Figure 3.4a) such that higher concentrations of 4-HNE in MCar and HCar were observed in the presence of AA, than in its absence. A similar interaction was found for hexanal.

As simple effects, both enhanced carnosine levels ($p = 0.0037$) and the presence of AA ($p = 0.0001$) increased 4-HNE formation. With respect to the gastric digests, the loss of antioxidant effect observed in duodenal digests of HCar-AA compared to MCar-AA is not readily explainable. While contributing to increased levels of 4-HNE in the duodenal digests, AA did not reduce the amount of free or bioaccessible carnosine in the digests. This observation raises the question as to whether the emulsification process in the duodenal phase may have contributed to further reduction of potential antioxidant activity of the hydrophilic antioxidants, not only inside as hypothesized by Van Hecke et al. (2016), but also at the interface of the emulsified fat droplet.

c) Glyoxal (GO)

Levels of GO, a molecule that can be derived from both lipid oxidation and glycation and that directly contributes to CML formation (Nguyen et al., 2014), are shown in Tables 3.1 and 3.2. Decreased GO levels can represent reduced oxidation and AGEs formation. A carnosine × AA interaction ($p = 0.0002$) was found in gastric digests (Figure 3.5a). In the presence of AA, the GO concentration was highest in MCar digests ($p < 0.0001$ compared to LCar and $p = 0.0002$ to HCar), followed by the HCar digests ($p = 0.0123$ compared to LCar). In the absence of AA, statistical trends also suggest a tendency of higher GO concentrations in MCar digests than in those of both LCar ($p = 0.0985$) and HCar ($p = 0.0638$). As simple effects, carnosine addition resulted in the highest GO observed in the MCar digests ($p < 0.0001$), and AA addition strongly increased GO in all digests, irrespective of the carnosine level ($p < 0.0001$). The mechanism(s) behind the GO-promoting effect(s) of the treatments might be

multifactorial. For instance, in acidic environments such as in gastric digests, the presence of iron is known to reduce the stability of AA (Buettner & Jurkiewicz, 1996; Miller et al., 1990), and the importance of transition metal-catalyzed ascorbate oxidation on increased GO formation has been demonstrated by Shangari et al. (2007) using an in vitro cell-free system. The higher average hydrogen peroxide concentrations observed in the gastric digests with added AA (12.07 mM) compared to those without AA (2.22 mM) also supports AA-associated GO formation that can occur through increased lipid oxidation (Vistoli et al., 2013) following ferric reduction in the presence of AA and carnosine, and resultant radical formation in the presence of hydrogen peroxide, as discussed previously. On this basis, the GO decrease observed from MCar to HCar in the presence of AA could be explained by the antioxidant mechanisms offered by the additional carnosine at the HCar level, including its capacity to decrease reactive carbonyl species, such as GO and MGO as reviewed by Boldyrev et al. (2013).



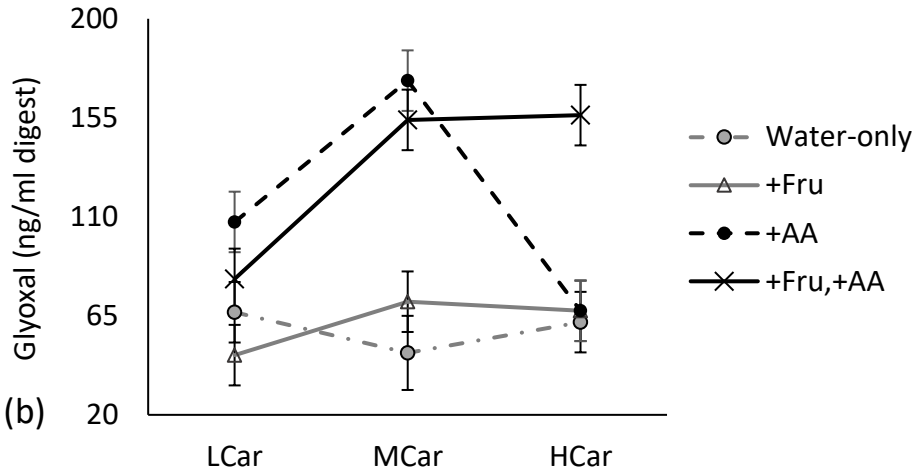


Figure 3.5. Interactions on glyoxal concentrations (least squares means with 95% confidence intervals) (a) between carnosine and ascorbic acid (AA) in gastric digests ($p = 0.0002$), and (b) among carnosine, AA, and fructose (Fru) in duodenal digests ($p < 0.0001$).

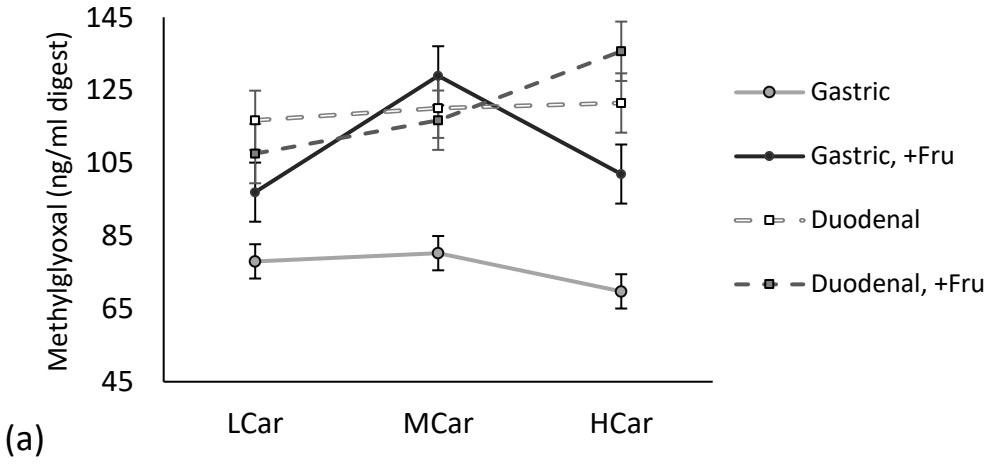
Irrespective of the treatments, the level of GO was reduced in the duodenal digests compared to the gastric digests (Tables 3.1 and 3.2). It is known that GO can react with amino acid residues (Zeng & Davies, 2005). However, given that GO formation prefers an acidic environment (Gurumayum et al., 2021), a lesser amount of GO is likely formed in the duodenum phase than in the more acidic gastric phase resulting in a net decrease in the GO concentration. A three-way interaction of the treatments was also found ($p < 0.0001$; Figure 3.5b) in the duodenal phase, although only carnosine × AA ($p < 0.0001$) and carnosine × fructose ($p < 0.0001$) interactions were significant. In the absence of fructose, AA caused a strong increase in GO in the digests of MCar compared

to LCar, but not in those of HCar where the lowest GO was observed. These latter findings are consistent with the antioxidant and antiglycation activity of the extra carnosine available at HCar as discussed for 4-HNE. However, the protective effect observed in the HCar-AA digests did not occur in the presence of fructose, showing the additive radical production capacity of the combined presence of fructose and AA, as discussed previously. This additive effect most likely overwhelmed the protective effect of carnosine, even at enhanced carnosine levels. In the absence of AA, no difference in GO was observed among carnosine treatments, irrespective of the absence or presence of fructose ($p > 0.05$). Finally, as simple effects, the addition of each of carnosine ($p < 0.0001$), AA ($p < 0.0001$), and fructose ($p = 0.0307$) increased GO concentrations.

d) Methylglyoxal (MGO)

Another indicator is MGO, a molecule that can be derived from both lipid oxidation and glycation (Nguyen et al., 2014), with decreased or increased levels brought about by dietary components demonstrating either their health advantages or disadvantages, respectively. Formation of MGO in the gastric digests was impacted by carnosine × fructose ($p = 0.0004$) and fructose × AA interactions ($p = 0.0153$). In the presence of fructose, the MGO concentration in the MCar treatment was significantly higher than that in LCar ($p = 0.0002$) and HCar ($p = 0.0007$) (Figure 3.6a). As already discussed, the pro-oxidative combination of fructose and an enhanced level of carnosine in MCar on the formation of MGO is likely a result of their additive capacity to increase free radical production leading to lipid peroxidation (Lankin et al., 2015; Mozdzan et al., 2005). In addition, MGO can be derived directly from fructose (Wang &

Ho, 2012). The significant decrease in MGO concentration from MCar to HCar in the presence ($p = 0.0007$) or absence ($p = 0.0128$) of fructose can be explained by the carbonyl quenching ability of carnosine (Boldyrev et al., 2013). The fructose × AA interaction (p = 0.0153; Figure 3.6b) showed that the effect of fructose on increased MGO was enhanced by the presence of AA, again suggesting their additive effect on radical formation as was observed for 4-HNE in gastric digests. As simple effects, the addition of carnosine ($p < 0.0001$), fructose ($p < 0.0001$), and AA ($p = 0.0050$) all contributed to increased MGO in the gastric digests.



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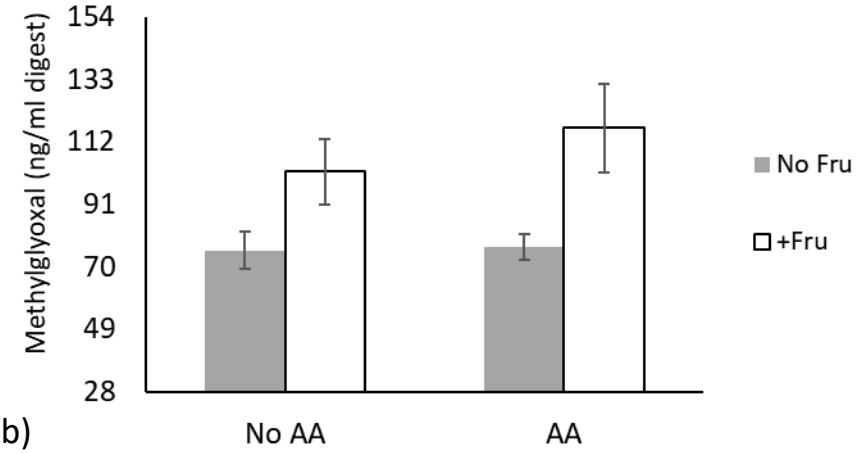

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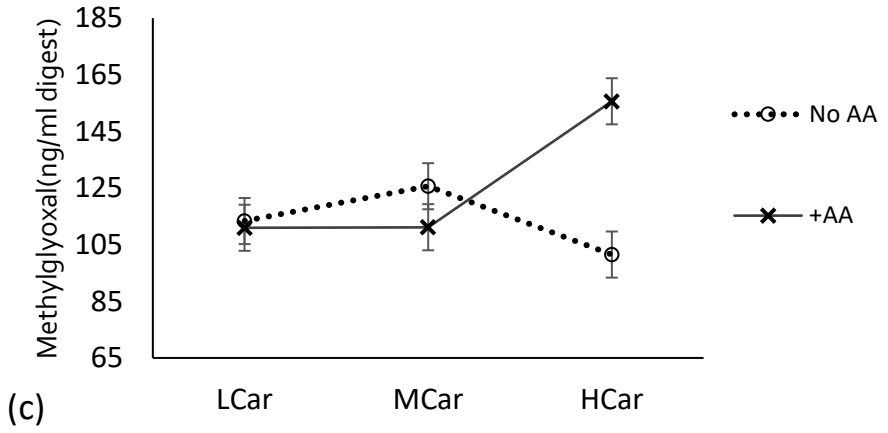

Figure 3.6. Interactions on methylglyoxal concentrations (least squares means with 95% confidence intervals) between (a) carnosine and fructose (Fru) in gastric ($p = 0.0004$) and duodenal ($p = 0.0185$) digests, (b) Fru and ascorbic acid (AA) in gastric digests ($p = 0.0153$), and (c) carnosine and AA in duodenal digests ($p < 0.0001$).

In the duodenal phase, interactions of carnosine × fructose ($p = 0.0185$) and carnosine × AA ($p < 0.0001$) were found (Figure 3.6a,c). For each of the two interactions, the highest levels of MGO were obtained in the HCar groups in the presence of either fructose ($p = 0.0009$) or AA ($p < 0.0001$). No differences were observed in MGO concentrations between LCar and MCar treatments, with or without fructose or AA. Compared to the observed carbonyl quenching activity of HCar in the gastric digests, the reduced efficacy of carnosine in the duodenal digest at the HCar level could relate in part to its reduced accessibility below an optimal level following its potential scavenging of both GO and MGO in the gastric digests. Despite remaining bioaccessible carnosine in duodenal digests, a similar reduction in its efficacy between successive digestive phases

was reported by Li et al. (2021) following in vitro digestion of carnosine-enriched pork. This effect might result from the selectivity of carnosine toward particular compounds. For instance, compared to the radical scavenging ability of carnosine dependent principally on the reactivity of its imidazole ring, carbonyl quenching requires both the imidazole ring of histidine and the contribution of β-alanine, which provides selectivity toward α,β-unsaturated carbonyls, such as 4-HNE (as reviewed by Vistoli, 2015). Unfortunately, there is a lack of scientific information on the effect of the environmental conditions (brought about by the digestion) on MGO, and further research involving additional markers that could not only validate the observed effect of carnosine and other dietary components but also identify how these dietary molecules exert their roles is needed.

e) N^{ε}-(1-Carboxymethyl)-L-lysine (CML)

Levels of CML, a commonly used marker of AGE formation from carbonyls of either lipids or reducing sugar, are presented in Tables 3.1 and 3.2. Contrary to the findings of Li et al. (2021) who reported an increase in CML from gastric to duodenal digests of carnosine-enriched pork, a decrease in CML was observed from the gastric to duodenal digests in the current study using the burger meal model (Tables 3.1 and 3.2). In this burger model, both the bun portion and the fat level (10%) in the meat patty proportionally reduced the amount of the meat fraction digested, which may account for the different CML levels in the two studies. However, this decrease in CML during digestion is consistent with the decreased GO concentrations, noting that CML is generated by the reaction between GO and lysine (Twarda-Clapa et al., 2022). A carnosine × AA interaction ($p = 0.0253$) was observed in the CML concentrations in the

gastric digests (Figure 3.7). The presence of AA increased CML formation in the HCar group ($p = 0.0289$), while in the absence of AA, the CML concentration was significantly lower in HCar than LCar ($p = 0.0403$). As a simple effect, AA at the level used in this study increased CML content ($p = 0.0113$), while no simple effect of added carnosine was observed ($p = 0.7621$). In the earlier study, it was reported that pork with additional 300 mg carnosine/100 g meat reduced CML in both gastric and duodenal digests (Li et al., 2021). Although the digestion of the bread moiety in the current study might have contributed to some background CML formation, the results suggest that CML production might mainly have occurred through GO formation from lipid oxidation as reported by Li et al. (2021). In addition, it cannot be ruled out that the high level of AA used may have directly contributed to increased CML, even though its formation and the Maillard reaction are not favored in acidic conditions (Ott et al., 2014), which are also known to reduce the stability of AA in the presence of iron (Buettner & Jurkiewicz, 1996). However, such formation, if any, would be minimal. According to Smuda and Glomb (2013), in the Maillard reaction, only 6% of AA following its oxidation can produce glycolic acid, the precursor of GO. And Liu et al. (2022), using Maillard reaction models at alkaline pH, demonstrated that addition of AA to a U-13C-Glu + Lys model can produce GO, MGO, CML, and N^{ε}-(1-carboxyethyl)-L-lysine (CEL), although AGEs production by AA was much lower than from glucose. It is therefore suggested that the AA-related increase in CML in the current study occurred mostly through lipid oxidation.

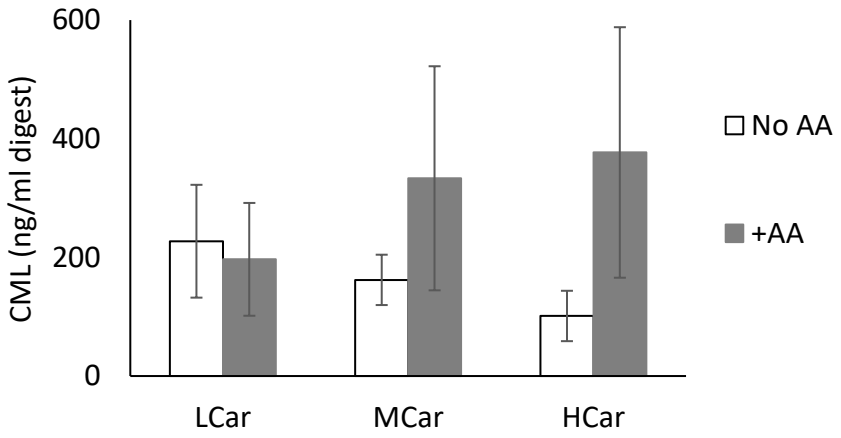

Figure 3.7. Interaction between carnosine and ascorbic acid (AA) on N^{ε}-(1-carboxymethyl)-L-lysine (CML) concentrations (least squares means with 95% confidence intervals) in gastric digests ($p = 0.0253$).

3.6 Limitations and implications of the study

In vitro models represent valuable alternatives to in vivo models for the study of oxidative processes, as reviewed by Bohn et al. (2018). However, in reducing ferric iron to its soluble and absorbable ferrous form, both AA and carnosine could also, depending on their concentrations, potentially contribute to a reduced rate and extent of the oxidative aspects reported in the present study. According to Seth and Mahoney (2000), the principal enhancers of iron absorption in the diet, other than AA, come from the digestion of animal tissues providing some of the most effective enhancers, cysteine- and histidine-containing peptides, a phenomenon known as the "meat factor." On that basis, carnosine, as an inherent muscle histidine-containing dipeptide, may very well be an important component of this "meat factor" responsible for iron absorption. This is supported by the results of Igarashi et al. (2004), who suggested that following the

administration of ferrous sulfate in the presence of carnosine to cannulated rats, carnosine may facilitate the velocity of iron absorption by enhancing iron solubility. Such a potential iron absorption role of carnosine during digestion deserves further in vivo investigation. This first report on the effects of carnosine in meat as part of a meat-based meal revealed important interactive effects involving carnosine and other meal compounds during digestion. These observations, apart from their nutritional significance, also underscore the importance of considering the administration of carnosine supplements, with or without a meal, for instance, in the context of clinical studies.

3.7 Conclusion

Carnosine, designated in the literature as a forgotten remarkable and enigmatic molecule due to its multiple chemical and biochemical properties, is exclusively present in meat and muscle food and could contribute to counteracting ailments associated with increased consumption of red meat and meat products, as shown by reduced markers of lipid and protein oxidation and of AGEs formation following in vitro digestion of pork enhanced in carnosine. Despite the availability of clinical studies demonstrating the benefits and limitations of the use of carnosine supplements, no study has yet been conducted to understand how carnosine in meat could interact in the presence of other dietary compounds, such as those occurring during the digestion of a meal.

The current study is the first to report the effects of dietary carnosine during digestion of a meat-based meal model and reveals a high reactivity of carnosine with the other redox-

active molecules contained in the meal. In the presence of AA and/or fructose, different interactive pro- or antioxidant effects of carnosine were observed on lipid oxidation-related markers hexanal, 4-HNE, GO, and MGO depending on the carnosine level in the meat, the markers, and the digestive phase. This was exemplified by the increased concentrations of lipid oxidation markers at the intermediate carnosine level (MCar) in both digestive phases and the decreased concentrations at the higher carnosine level (HCar) of 4-HNE in the gastric phase and GO in both the gastric and duodenal phases. The different interactions reported did not all follow the same pattern and could involve different pathways and or mechanisms. Although the pro- or antioxidant activity of dietary carnosine concentration appeared to be regulated by the overall redox potential of the diet, other factors, such as the lipid content and cooking conditions of the meat, have been reported to influence the protective efficacy of carnosine during digestion. Further research is therefore needed to understand how the concentration of dietary carnosine, the composition and preparation of the meal, and the presence and concentration of other redox-active compounds can, altogether, promote the beneficial interactive effects during digestion for the optimization of a healthier meat-based diet.

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Connecting paragraph

Chapter 3 reported how carnosine in meat, which is digested as part of a meat-based meal, performs in the presence of other redox-active compounds in the same meal. A pro-oxidant effect of carnosine in the presence of a high level of ascorbic acid (antioxidant supplement) was observed at the intermediate level of carnosine (MCar), with further enhancement in carnosine (high level of carnosine, HCar) showing an antioxidant potential.

Considering that the high level of ascorbic acid in this meal model was one of the predominant factors for the pro-oxidative effect of carnosine, it is possible that, when a different level of ascorbic acid is included for the simulation of beverages of a meal combo, a different effect of dietary carnosine during in vitro digestion of a burger meal combo might be obtained. In Chapter 4, therefore, the effect of carnosine as a meat constituent is assessed during in vitro digestion of a model of a burger meal combo that includes bun, pork patty with three levels of carnosine, and water solutions with or without fructose and/or ascorbic acid at dietary levels.

Chapter 4 will be submitted to the *Journal of Food Science*.

Chapter 4 Effect of dietary carnosine during in vitro digestion of a burger meal model including ascorbic acid as an antioxidant and fructose as a pro-oxidant

4.1 Abstract

Carnosine, as a meat constituent, was previously shown to possess health-beneficial properties during in vitro digestion of a burger meal model, although the presence of high (supplemental) level of ascorbic acid (AA) brought out some pro-oxidative effects of carnosine. In the present study, the effect of different levels of carnosine on lipid and protein oxidation and formation of glyoxal (GO) and advanced glycation end-products (AGEs) were assessed during gastric and duodenal in vitro digestion of a burger meal model, including intrinsic (low; LCar) and enriched (intermediate, MCar; high, HCar) carnosine in ground pork, with or without dietary levels of AA and/or fructose as anti /pro-oxidants in the simulated beverages, respectively. Increased levels of dietary carnosine (MCar and HCar) showed antioxidant activity, irrespective of the digestion phase and the presence of fructose and/or AA in simulated beverages. At a dietary level, AA generally acted as an antioxidant but promoted the formation of GO in gastric digests and malondialdehyde (MDA) and hexanal in duodenal digests. Fructose generally showed a pro-oxidant ability, but decreased MDA and GO concentrations in both digestion phases. Despite a higher bio-accessible carnosine level in HCar, the MCar treatment provided optimal antioxidant activity without further benefits obtained at the HCar level, showing a ceiling-like effect of carnosine benefits in vitro. These results demonstrate the health advantages of increased carnosine in meat during digestion of a meal despite the presence of other redox-active dietary compounds in the meal.

4.2 Practical Application

Carnosine, a multifunctional compound naturally present in meat, can bring considerable advantages to meat consumers. Increased dietary levels of carnosine in meat reduced oxidation and the formation of AGEs during digestion of a burger meal in the presence of dietary levels of either AA (as an antioxidant) or fructose (as a pro-oxidant). In the context of this study, optimal benefits of carnosine were attained at the intermediate enhancement level, which can be naturally obtained through breeding and nutrition.

4.3 Introduction

Carnosine (β-alanyl-L-histidine) is a naturally occurring dipeptide that is present exclusively in muscle of vertebrates and, hence, in meat and some fish. Carnosine possesses multiple health-beneficial properties, including antioxidant, anti-glycation, and anti-aging properties (reviewed by Boldyrev et al., 2013), and in vivo, carnosine supplements have been shown to reduce oxidative stress and inflammation (reviewed by Jukić et al., 2021). Many studies have also shown the therapeutic potential of carnosine supplements in the context of many chronic diseases (Jukić et al., 2021; Sale et al., 2013) and in improving exercise performance in healthy people (Sale et al., 2013; Varanoske et al., 2019). In addition, carnosine was shown to reduce lipid oxidation during the storage of raw (Kopec et al., 2020) and cooked meat (Das et al., 2006; Decker & Crum, 1993).

To gain the benefits of carnosine as a food constituent in diets, however, additional studies are required, for example, that take into consideration interference from interactions, likely of oxidoreduction nature, that can occur in the meat and meal matrix

during the digestion process (Marcolini et al., 2015; Li et al., 2021). Indeed, during in vitro digestion, enhanced levels of carnosine in pork were shown to reduce oxidation and AGEs formation brought about by high fat content and high cooking intensity, resulting in reduced carnosine bioaccessibility (Li et al., 2021). A recent study was undertaken on the effect of dietary carnosine during in vitro digestion of a burger meal system including pork with enriched carnosine levels and water solutions containing fructose and/or ascorbic acid (AA) (Li et al., 2024). A dietary level of fructose (simulating soft drinks in a burger meal combo) was included for its pro-oxidant potential (Franke et al., 2005; Lankin et al., 2015), while a supplemental level of AA was used because the antioxidant effect of AA in meat appears to only occur at high concentrations (Benedict et al., 1975; Mitsumoto et al., 1991). However, Li et al. (2024) observed that, depending on the level of carnosine, a combined presence of carnosine and AA induced an increase in oxidation markers in the digests, suggesting an additive pro-oxidant effect of the two compounds. This research, along with other studies reviewed by Solter et al. (2019), indicates that the dietary components that have redox activity may all take part in interactions and, depending on their concentrations, may result in the conversion of antioxidants to pro-oxidants, as shown for AA, phenolics, and other natural antioxidants (reviewed by Sotler et al., 2019) and for carnosine (Li et al., 2024). Such occurrences could be particularly true during the digestion process, considering the pro-oxidant environment that the digestive tract itself represents (Oueslati et al., 2016). Thus, a further study is warranted to determine the effect of dietary carnosine in a meal environment containing dietary concentrations of fructose and AA, which could be available in fruit juices and soft drinks in a burger meal combo and behave as anti- and pro-oxidant nutrients,

respectively. The objective of this study was, therefore, using a burger meal model with simulated beverages, to assess the effect of dietary carnosine during in vitro digestion in the presence of AA and/or fructose at levels found in commercial beverages as potential anti- and pro-oxidants.

4.4 Materials and methods

4.4.1 Chemicals and reagents

All reagents and chemicals were of analytical grade or higher. All chemicals and enzymes were purchased from Sigma-Aldrich (Saint Louis, MO, USA) except for the following: anhydrous acetonitrile (ACN), formic acid, and methanol (LCMS grade), purchased from Thermo Fisher Scientific (Waltham, MA, USA); L-carnosine-d_4 (≥ 99%), 4-hydroxyhexenal-d_3 (≥ 99%, HHE-d_3), 4-hydroxynonenal (≥ 99%, 4-HNE), and N^{ε}-(1-carboxymethyl)-L-lysine-d_3 (≥ 99%, CML-d_3), purchased from Cayman Chemical (Ann Arbor, MI, USA); and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (OPFB) and *N, O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), purchased from the American Division of the Tokyo Chemical Industry (Portland, OR, USA).

4.4.2 Burger meal model preparation

A burger meal was chosen for the model in this study in view of its wide availability and popularity. For instance, one fast-food brand with restaurants in more than 100 countries worldwide has been said to sell 75 burgers per second globally (Smith, 2023). As pork is the most commonly consumed protein food worldwide (United States Department of Agriculture, 2023), the burger meal model utilized bread and cooked pork containing

different levels of carnosine, together with fructose and/or AA in water solutions to simulate beverages (soft drinks and fruit juices) served in fast-food outlets.

To prepare the cooked pork, subcutaneous fat and four entire *longissimus dorsi (LD)* muscles obtained from a commercial pork processing plant (Olymel S.E.C, Saint-Henri, Quebec, Canada), were ground (6 mm plate) and mixed to attain a 10% total fat level. Batters containing three carnosine levels were achieved by adding 300 or 600 mg of carnosine per 100 g meat to the control (intrinsic 545 mg carnosine/100 g pork representing a low carnosine level; LCar), providing the intermediate (845 mg carnosine/100 g ground pork; MCar) and high (1145 mg carnosine/100 g ground pork; HCar) carnosine treatments. The addition of 300 and 600 mg of carnosine per 100 g of meat was based on the natural occurrence of high levels (>1000 mg/100 g) of carnosine and histidine-containing dipeptides in meat species having comparatively high muscle glycolytic potential (Boldyrev et al., 2013) and the demonstrated possibility of increasing and optimizing the carnosine level in pork through breeding and nutrition (D'Astous-Pagé et al., 2017; Paniagua et al, 2023). The prepared fresh pork was then cooked in polypropylene screw-top containers (60 mL), which were sealed in individual vacuum bags. The sealed containers were placed in a water bath, heated to a core temperature of 90°C, and held for 30 minutes at this temperature. The cooked pork was portioned (20 g), vacuum packaged, and stored at -80°C until in vitro digestion. Just prior to digestion, beverage-simulating solutions were prepared according to Table 4.1, and the pork was thawed at room temperature (approximately 15 min). Then, the cooked pork samples (4.5 g), crushed bread (3.0 g), and 2 mL of beverage-simulating solution were mixed by

vortexing (for 30 s) in a 50 mL polypropylene tube, following the combination of treatments shown in Figure 4.1.

4.4.3 In vitro digestion

The in vitro digestion method was that described by Li et al. (2024) and was based on the general food digestion method of Versantvoort et al. (2005), adapted by Van Hecke et al. (2014) for meat digestion. The modification for the salivary phase described by Li et al. (2024), was followed. Considering the volume of the beverage-simulating solutions (2 mL), only 4 mL of saliva medium was added to the sample mix in order to respect the solid-to-liquid ratio prescribed by Versantvoort et al. (2005). Therefore, a saliva fluid of 1.5 times the concentration of that employed by Van Hecke et al. (2014) was prepared to maintain the same final concentrations in the salivary phase. The other digestive fluids for the gastric and duodenal juices were prepared in the manner described in Van Hecke et al. (2014). After the preparation of digestive fluids, the meal samples (7.5 g of solid and 2 mL of liquid) were sequentially incubated for 5 minutes with saliva (4 mL), 2 hours with gastric juice (12 mL), and 2 hours with 1 M bicarbonate buffer (pH 8.0, 2 mL), duodenal juice (12 mL), and bile (6 mL). Colonic digestion was not carried out. The digest collected from each gastric and duodenal phase was homogenized (10,000 rpm, 1 min), and aliquots were transferred into Eppendorf tubes and stored at -80°C. The entire process was performed in quadruplicate.

4.4.4 Free carnosine measurement

Carnosine levels in cooked meat and digests were determined according to the LC-MS/MS method described by Han et al. (2019). To extract carnosine from meat, 100 mg of ground meat was extracted with 5 mL water by rotating together for 20 min (50 turns/min). Digests were diluted as follows: 0.1 mL of gastric digest was diluted to 1 mL with water, and 0.2 mL of duodenal digest was diluted to 1 mL with water as well. A 200 µL aliquot of meat extract or diluted digests was mixed with an internal standard of L-carnosine-d_4 (50 ng/µL, 200 µL) and water (0.6 mL), followed by purification using a pre-conditionned HLB column (Waters Corporation, Milford, MA, USA). The purified solution was diluted to 10% with acetonitrile and filtered using a nylon membrane filter (0.22 µm). These prepared samples were then injected in the LC-MS (Vanquish™ HPLC, LTQ XL Mass Spectrometer, HESI-II probe, Thermo Fisher Scientific, Waltham, MA, USA) with ACQUITY UPLC BEH AMIDE column (2.1 × 100 mm, 1.7 µm; Waters Corporation, Milford, MA, USA). The elution and operational parameters are presented in Table 4.2. The characteristic ions of 227 *m/z* and 231 *m/z* were used to quantify carnosine and carnosine-d_4, respectively.

4.4.5 Lipid oxidation markers and glyoxal

The GC-MS method used by Li et al. (2024) was used for the measurement of hexanal, 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and glyoxal (GO). The method employed an Agilent 7890B gas chromatograph coupled to a 5977B quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with Ultimate Plus deactivated fused silica tubing (5 m × 0.25 mm; Agilent Technologies, Santa Clara, CA,

USA) and HP-5MS columns (30 m× 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). Samples for injection were prepared by two derivatizations. Digest (0.2 mL) was mixed with the internal standard HHE-d_3 (10 µL of a 20 ng/µL solution in ethanol) and OPFB (0.17 mL of a solution of 30 mg/mL), followed by ultrasonication of the mix for 3 min. Then, the extraction was carried out by adding methanol (0.5 mL), isooctane (1 mL), and concentrated sulfuric acid (6 drops) to the mix, followed by centrifugation for 5 min (774 ×*g*). The supernatant (0.8 mL) was passed through anhydrous sodium sulfate and glass wool into vials and evaporated to dryness, then BSTFA (50 µL) was added to the vial and incubated for 1 hour at 80°C. Upon injection of 1 µL of this sample into the GC-MS, the following temperature program was applied: column held at 70°C for 3 min, increased to 80°C at a rate of 6°C/min, further increased to 280°C at a rate of 20°C/min and held for 15 min, and finally increased to 300°C at a rate of 20°C/min then held another 5min. Helium (61 kPa, constant flow of 1 ml/min) was used as the carrier gas. The mass selective detector was operated in electron impact (EI) mode with the ionization energy of 70 eV, and the dwell time for each ion was 5 ms in the selected-ion monitoring (SIM). The following characteristic ions were chosen for quantification: 187 and 203 *m/z* (RT (retention time) 12.20 and 12.33 min for HHE-d_3); 239 *m/z* (RT 10.35 min for hexanal); 448 *m/z* (RT 13.04 and 13.10 min for GO); 352 *m/z* (RT 13.48 and 13.63 min for 4-HNE); and 250 *m/z* (RT 13.76 and 13.79 min for MDA).

4.4.6 Protein oxidation markers (carbonyls and free thiols)

Protein carbonyls from digests were measured according to the method of Ventanas et al. (2006), which is based on the description of Oliver et al. (1987). Two aliquots of gastric

or duodenal digest (200 µL) were each mixed with 20% trichloroacetic acid (TCA) (200 µL), then centrifuged (3000 ×*g*, 5 min). One pellet was treated with 2 N HCl (2 mL) as a blank, which was also used for protein concentration measurement (absorption at 280 nm). The other pellet was treated with 10 mM 2,4-dinitrophenylhydrazine in 2 N HCl (2 mL) for carbonyl concentration measurement. Both samples were held at room temperature for 1 hour and mixed with 20% TCA (2 mL). The precipitate was washed three times with freshly prepared ethyl acetate: ethanol (1:1, v/v; 2 mL), dissolved in 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5, 2.5 mL), and centrifuged (2240 ×*g*, 2 min) to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using BSA as standard. Carbonyls were determined at 365 nm using an adsorption coefficient of 22,000 M^{-1} cm^{-1} and expressed as nmol/mg protein.

For the determination of free thiols, the colorimetric method using 2, 2'-dithiobis (5-nitropyridine) (DTNP) reagent was used, following the modifications from Winterbourn (1990) and Martinaud et al. (1997), respectively. Protein levels in digests were determined by the biuret method (Gornall et al., 1949) and adjusted to 5 mg/mL with 200 mM phosphate buffer (pH 7.4). A urea buffer (8 M urea in 100 mM phosphate buffer, pH 8.0) was then used to adjust the protein level to 1 mg/mL. For the measurement of free thiols, DTNP ethanol solution (10 mM, 20 µL) was added to the protein solution (2 mL) and held for 1 hour at room temperature. The absorbance of the mix at 386 nm was measured against a blank of protein at the same concentration without DTNP. The absorbance of the diluted DTNP solution was subtracted, and an absorption coefficient of

14 mM^{-1} cm^{-1} was used for the calculation of free thiols. Concentrations of free thiols are expressed as μmol/mg protein.

4.4.7 Bound CML (N^{ε}-(1-Carboxymethyl)-L-lysine) measurement

The same LC-MS/MS instrument and column used for carnosine quantification were also utilized for protein-bond CML (N^{ε}-1(carboxyethyl)lysine) determination, according to Nomi et al. (2020) and Sun et al. (2015). Briefly, 0.4 mL of the gastric digests or 0.8 mL of the duodenal digests were pre-incubated with 0.4 mL borate buffer containing 100 mM sodium borohydride (pH 9.2) at 4°C overnight. An aqueous TCA solution was then added to achieve a final concentration of 20%, and a pellet was obtained by centrifugation (865 ×*g*, 20 min). The pellet was mixed with CML-d_3 (100 ppm, 40 μL), followed by an acid hydrolysis using 6N HCl (0.5 mL) at 110°C for 24 hours. The hydrolysate was evaporated under a nitrogen stream to dryness (70°C), then reconstituted with water (0.5 mL) and purified with Sepra C18-E powder (50 μm, 65Å, Phenomenex, Torrance, CA, USA). The purified solution was diluted with ACN (0.3 mL purified sample in 0.7 mL ACN) and filtered (0.22 μm nylon filter). The filtered sample was injected into the LC-MS/MS with parameters presented in Table 4.2 for the acquisition and quantification of CML (*m/z* 205→*m/z* 129.9) and CML-d_3 (*m/z* 208→*m/z* 132.9).

4.4.8 Statistical analyses

The results were analyzed according to a complete 3 x 2 x 2 factorial (3 carnosine levels in burger meat, presence or absence of fructose in beverage, and presence or absence of

AA in beverage), with heterogeneous variances used when appropriate, by the MIXED procedure of SAS (SAS version 9.4, 2002 - 2012; SAS Institute Inc., Cary, NC, USA). 248 The entire protocol was repeated 4 times for a total of 48 observations (12 treatments 249 with 4 repetitions) for each of the gastric and duodenal phases. A $p \leq 0.05$ was considered significant, with a tendency defined as $0.05 < p < 0.10$. Significant statistical interactions were further analyzed using partitioned analysis (slice option of the LS Means statement). Significant interactions are presented as figures.

4.5 Results and discussion

4.5.1 Carnosine levels during preparation and digestion

In the current study, the carnosine concentrations in the pork LD muscle and in the digests were about twice the level observed in Li et al. (2024), which used a similar pork burger meal digestion model with the same carnosine enhancement treatments, but a higher level of ascorbic acid. The use of an internal standard in both HPLC in the study by Li et al. (2024) and LC-MS/MS in the present study, allows for the suggestion that the differences in carnosine concentrations measured in the meat and in the digests in these two burger studies likely originated from different intrinsic levels of carnosine in meat. As reviewed by Jukić et al. (2021), breeds, feeding, and carnosine measurement methods can all cause large variations in reported carnosine levels in beef. The authors also reported some potential suppression in serum carnosinase by beef components, which may affect the level of carnosine remaining in the muscle. The exact reason for the difference in muscle carnosine level reported in Li et al (2024) and in the present study is uncertain. However, since pigs from the same commercial crossbreed (Duroc x

Yorkshire-Landrace) were used in both experiments, the differences in the intrinsic levels of carnosine in the pork might be mainly related to the feeding of the pigs. The available literature reports concentrations of carnosine in pork LD varying from 270 to 500 mg/100g meat when measured by HPLC, (Mora et al., 2007; D'Astous-Pagé et al., 2017; Paniagua et al., 2023; Li et al., 2021; 2024) and from 400 to 550 mg/100g meat when measured by LC-MS (Yang et al., 2014; Enomoto et al., 2023).

As expected, the carnosine treatments used in the current study led to a gradual and significant increase in the carnosine concentration in the cooked meat, with statistically different levels in LCar, MCar, and HCar (Table 4.3). These differences among carnosine groups remained significant upon digestion in both gastric (Table 4.4) and duodenal digests (Table 4.5). Irrespective of digestion phases, digests from the MCar and HCar groups contained more carnosine than that of the LCar group ($p < 0.0001$), and digests from the HCar treatment also had more carnosine compared with that from the MCar group ($p < 0.0001$). In the gastric digests, the presence of fructose and AA in the simulated beverages had no effect on the free (bioaccessible) carnosine concentrations (Table 4.4). However, in the duodenal digests, the overall bioaccessible carnosine level was lower than in the gastric digests, irrespective of the treatments. This result is consistent with those obtained by Li et al. (2021; 2024) following in vitro digestion of carnosine-enriched meat and showed, in comparison with the gastric phase, the extended recruitment of carnosine in the duodenum to counteract the continuing formation of lipid and protein oxidation-related products, including CML (Li et al., 2021). No significant effect of AA was observed on the carnosine level. In contrast, fructose significantly

decreased the free carnosine concentrations in duodenal digests (p = 0.0171). The reduced amount of carnosine in the presence of fructose can be attributed to the capacity of this reducing sugar to promote both oxidation and glycation (Lankin et al., 2015; Gugliucci, 2017), engaging an increased amount of carnosine for the inhibition or suppression of the related reactions and resulting in reduced bioaccessible carnosine concentration.

4.5.2 Protein oxidation markers

Results for protein carbonyls as protein oxidation markers are presented in Tables 4.4 and 4.5. During both gastric and duodenal digestion, carnosine and fructose treatments had no effect on protein carbonyls formation ($p > 0.05$), and AA had no effect during duodenal digestion ($p > 0.05$). This absence of the effect of carnosine treatments on protein carbonyls ($p > 0.05$) during digestion is in accordance with the results by Li et al. (2024) using the same burger meal model conditions with a higher (supplemental) level of AA. However, during gastric digestion in current study, AA significantly ($p = 0.0449$) reduced the protein carbonyl concentrations from 4.05 nmol/mg protein in samples without AA, to 3.73 nmol/mg protein in groups with AA. This result is consistent with the generally agreed-upon role of AA as an antioxidant (Pehlivan, 2017) and is also in accordance with the reported reduction of protein carbonyls in plasma from healthy volunteers after vitamin C supplementation (Carty et al., 2000). These results, however, contrast with those of Li et al. (2024), who reported a pro-oxidant effect of AA leading to increased level of protein carbonyls. In this study, AA was used at a high concentration (equivalent to dietary supplementation) during the digestion of the same burger meal model as used

in the current study. The pro-oxidant effect of AA observed was associated with its capacity to reduce ferric ions, leading to increased peroxide production and radical formation, inducing, in turn, the oxidation of proteins (Rietjens et al., 2002; Falowo et al., 2014).

Results for the free thiols are presented in Tables 4.4 and 4.5. While no effect ($p > 0.05$) of carnosine and fructose was observed in the duodenal digests, in the gastric digests, a single effect of carnosine was obtained ($p = 0.0345$), and a trend was also reported for the effect of AA ($p = 0.054$). Compared to the MCar digests, a small but significant decrease ($p = 0.0102$) in the level of free thiols was measured in the HCar digests, suggesting a pro-oxidant effect of the highest level of carnosine. Neither MCar nor HCar was significantly different from the control (LCar) ($p > 0.05$). Li et al. (2021) previously reported higher levels of free thiols in the gastric digests of carnosine-enhanced meat than the control, showing an antioxidant effect of this dipeptide during the in vitro digestion of meat. However, during in vitro digestion of a burger meal model, the interactive effect of carnosine with either fructose or a high level of AA resulted in pro-oxidant activity of carnosine at the MCar and HCar levels depending on the markers (Li et al., 2024). Since protein oxidation, similar to that of lipid oxidation, can occur through a chain reaction involving the contribution of iron ions (Falowo et al., 2014), this pro-oxidant potential of carnosine on lipid and protein oxidation markers can be associated with its capacity to reduce ferric ions, as shown by Mozdzan et al. (2005), leading to increased peroxide production and radical formation (Li et al., 2024). The mechanism by which carnosine could directly or indirectly affect the free thiol level or interact with the disulfide bonds

in different environments is unclear. The trend reported for the effect of AA (p = 0.054) increasing the level of free thiols in the gastric digests became significant in the duodenal digests ($p < 0.0001$). This result is in agreement with the antioxidant activity of higher AA following in vitro digestion of a burger meal model enhanced in carnosine, as observed in Li et al. (2024).

4.5.3 Lipid oxidation markers

a) Hexanal

Results for hexanal are presented in Tables 4.4 and 4.5. In the gastric digests, a significant effect of carnosine was observed. Compared to the LCar digests ($p < 0.0001$), decreased hexanal was obtained in both MCar and HCar digests. A similar antioxidant activity of carnosine toward lipid oxidation was also reported by Li et al. (2021) during in vitro digestion of pork enhanced in carnosine. In the current study, however, despite the much higher level of bioaccessible carnosine in HCar than in MCar ($p < 0.0001$), no difference in hexanal level was found between the two enhanced groups, indicating that the benefits of carnosine toward lipid oxidation reached a plateau at the MCar level. A statistical trend for a fructose and AA interaction on hexanal level ($p = 0.0547$, Figure 4.2a) was obtained with the lowest level of hexanal observed in the digests containing both fructose and AA, likely due to the antioxidant capacity of AA since the presence of fructose had no effect irrespective of the presence or absence of AA. A single antioxidant effect of AA ($p = 0.0104$) was also reported and can be explained by its ability to reduce oxidized transition metal ions and scavenge free radicals (Pehlivan, 2017).

Contrary to the interaction of AA and fructose in the gastric phase, in the duodenal digests, the significant interaction of these two factors ($p = 0.0374$, Figure 4.2b) resulted in a pro-oxidant effect that led to increased hexanal levels. This interactive effect of fructose and AA in the duodenum could be partly explained by their significant single pro-oxidant effects (Table 4.5). No explanation of the single effect of AA becoming pro-oxidant under duodenal conditions is evident. However, a similar pro-oxidative effect of AA on increased hexanal, 4-HNE, and GO formation in the duodenal phase was reported by Li et al. (2024) during a previous in vitro digestion of a burger meal model and was attributed to the capacity of AA to initiate and stimulate lipid oxidation through the reduction of ferric to ferrous ions, thereby maintaining the Fenton reaction cycle (Rietjens et al., 2002), which would lead to increased lipid oxidation.

A carnosine and AA interaction ($p = 0.0268$, Figure 4.3) was also found in the duodenum. The highest level of hexanal was obtained in the LCar group in the presence of AA, demonstrating the pro-oxidant potential of AA, but, at the same time, the antioxidant capacity of increased dietary carnosine in a meal context. Furthermore, a single antioxidant effect of carnosine was also found ($p = 0.0098$) in the duodenum. The two carnosine-enhanced digests (MCar and HCar) contained significantly less hexanal than the control group (LCar). No significant difference in hexanal between MCar and HCar was observed, indicating, as reported in the gastric phase, that the antioxidant effect of enriched carnosine reached a plateau at the MCar level. With the statistically highest level of bioaccessible carnosine remaining in the HCar digests, a further decrease in hexanal would have been expected in the HCar digests, but this was not achieved. The

mechanism behind this potential ceiling effect of carnosine as an antioxidant is not clear, but could be associated with the redox potential of carnosine. In addition to mainly acting as an antioxidant, there might be sufficient carnosine remaining in the HCar digests to sustain the iron ion cycle in reducing the ferric to ferrous (as shown by Mozdzan et al., 2005), thereby contributing to the Fenton reaction and promoting some oxidation. Similar results were obtained by Decker et al. (1992) following an experiment aimed at assessing the effect of increased carnosine levels (1, 5, 10, and 25 mM) on lipid oxidation in the presence of transition metals using a phosphatidylcholine liposomes model. They observed that low levels of carnosine (≤10 mM) were more efficient in reducing lipid oxidation than levels in the 10-25 mM range. Overall, further research is warranted to explain this apparent ceiling effect of the antioxidant dietary carnosine concentration during digestion.

b) Malondialdehyde (MDA)

Results of the ANOVA for MDA in the gastric digests revealed a carnosine x AA interaction ($p = 0.0001$, Table 4.4, Figure 4.4a), showing the additive antioxidant effects of carnosine and AA contributing to decreased MDA in the carnosine-enhanced groups compared to the LCar ($p < 0.05$). In the presence of AA, the lowest MDA concentration was obtained in MCar digests, even though the HCar digests contained more MDA than in the MCar digests ($p = 0.0268$), emphasizing again the beneficial effect of carnosine being attained at the MCar level as discussed for hexanal. While no single effect of fructose was found in the gastric phase, an interaction between fructose and AA on MDA formation was also obtained ($p = 0.0040$, Table 4.4, Figure 4.5a). The highest MDA level

was found in samples without AA and fructose, which did not concur with the pro-oxidant capacity of fructose, but rather indicated an antioxidant role of both fructose and AA. Although the antioxidant effect of AA is largely documented (Pehlivan, 2017), and was observed as a single effect in the current study significantly reducing MDA formation ($p = 0.0025$), the effect of fructose on reducing lipid oxidation has seldom been observed. Instead, fructose is generally involved in glycation and can also contribute to oxidation, particularly in vitro (Semchyshyn et al., 2014). However, Morelli et al. (2003), reported that reducing sugars could exert radical scavenging activity by donating hydrogen atoms, yielding stable compounds (such as producing H_2O from the active hydroxyl radical). It appears that the effects of fructose may largely depend on the experimental conditions. As reviewed by Semchyshyn (2013), the debate on the biological role of fructose prevails, given the acknowledged adverse effect of long-term excessive fructose consumption but also the reported potential benefits of its short-term application. An interaction between carnosine and fructose ($p = 0.0534$, Figure 4.6) also supports the potential reducing effect of fructose. In the presence of fructose, LCar gastric digests had significantly lower levels of MDA than in its absence. As a single effect ($p < 0.0001$, Table 4.4), carnosine enhancement reduced MDA formation. Compared to the LCar digests, less MDA was found in MCar and HCar gastric digests, without being significantly different from each other ($p = 0.1194$), as also observed for hexanal.

The interactions between fructose x AA and carnosine x AA observed in the gastric phase still prevailed in the duodenal digests. However, contrary to what was observed in the

gastric phase for the fructose x AA interaction, the highest level of MDA was found in the samples containing both AA and fructose, indicating their synergistic pro-oxidant effects in the duodenal phase ($p = 0.0002$, Figure 4.5b). Individually, effects of each of AA ($p < 0.0001$) and fructose ($p = 0.0001$) increased MDA formation (Table 4.5). Such a pro-oxidative effect of fructose has been observed in meat by Villaverde and Estévez (2013). The oxidizing effect of AA could be due to its capacity to reduce ferric ions (Rietjens et al., 2002), as previously mentioned, for the hexanal formation in duodenal digests. However, this pro-oxidative effect of AA, which was observed in all carnosine treatments, was counteracted by increased carnosine level, resulting in the LCar-AA digests containing the highest level of MDA, as a result of the carnosine x AA interaction ($p = 0.0033$, Table 4.5, Figure 4.4b). As a single effect ($p = 0.0006$), carnosine-enhanced groups had less MDA than in the LCar duodenal digests ($p < 0.05$), with no further benefits observed in the HCar group compared to the MCar group, similar to the gastric phase. Reduced MDA in both gastric and duodenal digests was expected in the carnosine-enhanced groups, considering the radical scavenging property of carnosine and its ability to bind with MDA (as reviewed by Boldyrev et al., 2013). However, further reduction in MDA in HCar samples did not occur, as a plateau was attained in both digests, similar to the findings reported for hexanal formation in the previous section. There is little knowledge regarding the mechanisms underlying this plateauing effect of carnosine as an antioxidant, with the hypothetical explanation relating to the pro-oxidant potential of carnosine, as mentioned in the previous hexanal section, reiterating the need for more studies.

c) 4-Hydroxynonenal (4-HNE)

Results of 4-HNE in the gastric digests are presented in Table 4.4. A single effect of carnosine ($p = 0.0007$) indicated that gastric digests from the MCar and HCar groups contained significantly less 4-HNE than the LCar digests, in accordance with previous findings where antioxidant activity was observed in carnosine-enriched pork during in vitro digestion (Li et al., 2021). Similar to the results for hexanal and MDA, no significant difference in 4-HNE level was found between the MCar and HCar digests ($p = 0.8063$), despite the significantly higher free carnosine remaining in the HCar group. A significant interaction between fructose and AA ($p < 0.0001$, Figure 4.7) was also found in 4-HNE formation in the gastric phase, resulting in the highest level of 4-HNE being observed in the digests without both AA and fructose, suggesting their synergistic antioxidant effects when combined. The single effects of each AA ($p < 0.0001$) and fructose ($p < 0.0001$) confirmed their antioxidant abilities, decreasing the formation of 4-HNE. As reported above for MDA, although the antioxidant effect of AA is well-known, the hypothetical explanation for the antioxidant effect of fructose is based on its potential radical scavenging ability, despite that its biological significance is still debated (Morelli et al., 2003).

In the duodenal phase, the concentrations of 4-HNE were lower than the limit of quantification. This could be explained by the potential further reaction of 4-HNE forming adducts with other compounds in the duodenum (Steppeler et al., 2016), considering the more favored alkaline environment (LoPachin et al., 2009) of the

duodenal phase for such reactions, and the diluting effect of the added digestive juice used for duodenal digestion as reported earlier by Li et al. (2021).

4.5.4 Glyoxal (GO)

Levels of glyoxal, a molecule derived from both glycation and lipid oxidation (Nguyen et al., 2014), are presented in Tables 4.4 and 4.5. In the gastric phase, no significant effect of carnosine was observed ($p > 0.05$). However, an interaction between fructose and AA ($p = 0.0159$, Figure 4.8) was obtained. The presence of fructose and the absence of AA reduced the level of GO in gastric digests. This result reveals a beneficial effect of fructose since, in its absence, a pro-oxidant effect of AA significantly increased the level of GO compared to the digests without AA. Single effects of each of fructose and AA support this result. Fructose decreased the level of GO ($p = 0.0013$), while AA increased GO level ($p = 0.0061$). This promoting effect of AA in gastric digests could partially be attributed to the pro-oxidative ability of AA indirectly contributing to GO formation through the promotion of lipid oxidation (Rietjens et al., 2002; Nguyen et al., 2014) or to AA itself being oxidized, participating the formation of Schiff base fragmentation product (glycolaldehyde alkylamine), and further contributing to the generation of GO as reviewed by Nguyen et al. (2014). In the duodenal phase, no effects of carnosine ($p > 0.05$) and AA ($p > 0.05$) were observed. Fructose reduced the glyoxal level by 23.15% when compared with duodenal digests without fructose ($p < 0.0001$), similar to the effect observed in gastric digests. There is no clear explanation of how fructose lowered the levels of GO in both digestion phases. Considering that GO could be generated from oxidation, these decreases in GO by fructose could be related to its antioxidant capacity

attributed to its potential radical scavenging activity (Morelli et al., 2003), as mentioned above.

4.5.5 N^{ε}-(1-Carboxymethyl)-L-lysine (CML)

The results of CML, an indicator of AGEs, in the gastric and duodenal phases are shown in Tables 4.4 and 4.5. In the gastric phase, no effects of treatments were found ($p > 0.05$). In the duodenal phase, no effect of carnosine was observed ($p > 0.05$). However, as single factors, AA decreased the level of CML ($p = 0.0387$), while fructose increased it ($p = 0.0003$). This observation of AA is consistent with the beneficial role of AA towards the reduction of AGEs by competing with glucose for protein-binding and its antioxidant ability, as reviewed and suggested by Mazumder et al. (2019). The promoting effect of fructose on CML formation is supported by the potential of the sugar to glycoxidation during in vitro digestion (Semchyshyn, 2014) and its important contribution to glycation and AGEs formation in vivo (Takeuchi et al., 2010). Although CML could be generated from GO (Aldini et al., 2021), the changes in CML observed in both digestion phases were inconsistent with those observed in GO. This discrepancy in GO and CML results may be explained by the fact that GO is not the only reactant for CML formation. For instance, aldoses/ketoses can first react with proteins, yielding Schiff base, and then generate Amadori/Heyns products through re-arrangements and subsequent oxidation, leading to CML production, as reviewed by Nguyen et al. (2014). The decrease in CML level from the gastric to duodenal phase is also inconsistent with the increase in GO between the two digestion phases. There is no clear explanation for this observation other than the possible CML formation from pathways not involving GO.

4.6 Conclusion

Carnosine, a multifunctional dipeptide naturally present in muscle, has been reported for its benefits to the quality of the meat itself and, more importantly, for its health benefits when used as a supplement. Therefore, carnosine, when consumed through its meat matrix, should also provide, during digestion, potential health benefits for meat consumers. The current study effectively demonstrated the health-beneficial effect of dietary carnosine during in vitro digestion of a typical Western meal model containing a dietary level of AA and fructose as anti- and pro-oxidants, respectively. Irrespective of anti- or pro-oxidative interactions observed among dietary compounds, the enhancement of carnosine in meat consistently reduced oxidation and glycation.

This study confirmed that health benefits could be acquired from the consumption of a meal that includes abundant dietary carnosine. Furthermore, the study showed that the maximum benefit of carnosine enhancement was obtained at the intermediate carnosine concentration used, which corresponds to an obtainable level in muscle through the breeding and nutrition of pigs, thereby suggesting that obtaining benefits of dietary carnosine in meat could readily be achieved.

4.7 References

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Table 4.1. Preparation of beverage-simulating solutions in the burger meal model

Beverage	Water (mL)	Fructose (Fru; g)[1]	Ascorbic acid (AA; mg)[2]
Water	2.00	-	-
Water + Fru	2.00	1.34	-
Water + AA	2.00	-	6.20
Water + Fru + AA	2.00	1.34	6.20

[1]the absolute amount of fructose is proportionally reduced according to its content in soft drinks by the weight of the simulated burger (7.5 g) in the current model

[2]the absolute amount of ascorbic acid is proportionally reduced according to the content of ascorbic acid in juice by the weight of the simulated burger (7.5 g) in the current model

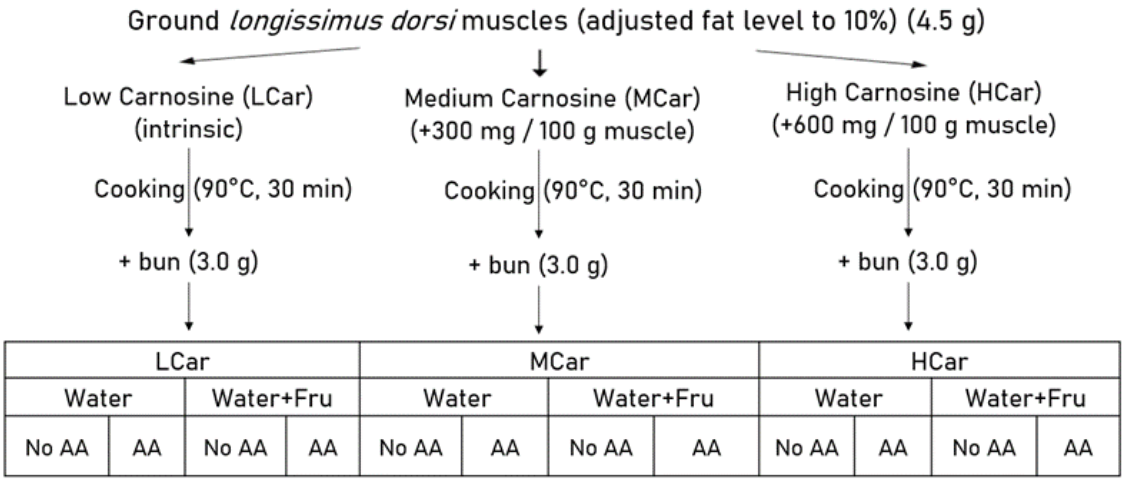

Figure 4.1. Preparation of the burger meal model (Water: 2 mL; Fru: 1.34 g fructose; AA: 6.20 mg ascorbic acid)

Table 4.2. LC–MS/MS analytical conditions for carnosine and CML measurements

	L-Carnosine and L-Carnosine d_4	CML and CML-d_3
Mobile phase A	ACN:H_2O 80:20, 10 mM ammonium acetate, 0.1% ammonium hydroxide	ACN:H_2O 80:20, 0.1% formic acid
Mobile phase B	ACN:H_2O 30:70 10 mM ammonium acetate, 0.1% ammonium hydroxide	ACN:H_2O 30:70, 0.1% formic acid
Flow rate (mL/min)	0.250	0.275
Gradient of solvent B (min,%B)	(0,0); (3,50); (6,50); (7,80); (12,80); (13.5,0); (20,0)	(0,25); (5,80); (8,80), (10,25); (18,25)
Spray voltage (kV)	4	4
Capillary	11 V, 300 °C	-7V, 250°C
Sheath gas (arbitrary unit)	30	25
Auxiliary gas (arbitrary unit)	5	5

Table 4.3. Carnosine concentrations (LS Means with SEM[1] in parentheses) in cooked meat[2]

				***p* Values**		
Phase	**LCar**	**MCar**	**HCar**	**LCar & MCar**	**LCar & HCar**	**MCar & HCar**
Cooked meat (mg/100g meat)	544.88 (23.87)	722.33 (23.87)	985.86 (23.87)	**0.0012**	**<0.0001**	**<0.0001**

[1]all SEM values within the same marker are expected to be the same when the analysis is preformed with the assumption of homogeneity of variances

[2]LCar: Low carnosine; MCar: Medium carnosine; HCar: High carnosine.

Table 4.4. Effects of carnosine level, fructose (Fru), and ascorbic acid (AA) on free carnosine (Car), indicators of protein and lipid oxidation and AGEs (LS Means with SEM[1] in parentheses) during in vitro gastric digestion of a burger meal system[2]

	Treatments[3]		Carnosine (µg/mL digest)	Protein carbonyls (nmol/mg protein)	Free thiols (nmol/mg protein)	Hexanal (ng/mL digest)	4-HNE (ng/mL digest)	MDA (ng/mL digest)	GO (ng/mL digest)	CML (ng/mL digest)
	Fructose (Fru)	Ascorbic acid (AA)								
LCar	No Fru	No AA	647.9 (26.6)	4.44 (0.27)	4.50 (0.53)	1940 (99)	179.5 (9.3)	771.4 (29.1)	120.9 (7.1)	87.61 (6.69)
		AA	663.7 (26.6)	3.80 (0.27)	5.35 (0.53)	1973 (99)	91.87 (9.3)	733.3 (29.1)	141.1 (7.1)	92.69 (6.69)
	Fru	No AA	698.6 (47.4)	4.05 (0.27)	6.05 (0.53)	1975 (99)	89.04 (3.5)	625.0 (29.1)	114.3 (7.1)	90.26 (3.57)
		AA	641.8 (47.4)	3.80 (0.27)	5.82 (0.53)	1762 (99)	52.52 (3.5)	735.4 (29.1)	110.8 (7.1)	92.08 (3.57)
MCar	No Fru	No AA	921.1 (26.6)	4.03 (0.27)	6.04 (0.53)	1571 (99)	161.4 (9.3)	582.1 (29.1)	123.1 (7.1)	84.82 (6.69)
		AA	941.2 (26.6)	3.83 (0.27)	6.60 (0.53)	1468 (99)	54.43 (9.3)	405.1 (29.1)	139.5 (7.1)	84.88 (6.69)
	Fru	No AA	906.4 (47.4)	4.00 (0.27)	5.35 (0.53)	1721 (99)	72.84 (4.0)	577.7 (29.1)	99.3 (7.1)	85.48 (3.57)
		AA	938.8 (47.4)	3.75 (0.27)	6.04 (0.53)	1288 (99)	41.37 (3.5)	434.5 (29.1)	116.1 (7.1)	89.71 (3.57)
HCar	No Fru	No AA	1165.0 (26.6)	4.08 (0.27)	4.17 (0.53)	1557 (99)	159.1 (10.7)	573.5 (29.1)	96.3 (8.2)	86.74 (6.69)
		AA	1117.8 (26.6)	3.60 (0.27)	5.77 (0.53)	1503 (99)	65.86 (10.7)	469.6 (29.1)	127.3 (7.1)	89.54 (6.69)
	Fru	No AA	1215.4 (47.4)	3.70 (0.27)	4.93 (0.53)	1753 (99)	64.52 (4.0)	531.9 (29.1)	114.6 (7.1)	95.53 (3.57)
		AA	1180.3 (47.4)	3.57 (0.27)	5.09 (0.53)	1592 (99)	45.88 (3.5)	556.0 (29.1)	106.1 (7.1)	91.75 (3.57)
	p Values									
		Car	**<0.0001**	0.3326	**0.0345**	**<0.0001**	**0.0007**	**<0.0001**	0.1042	0.3940
		Fru	0.4225	0.3414	0.6510	0.8202	**<0.0001**	0.4653	**0.0013**	0.3270
		AA	0.4855	**0.0449**	0.0546	**0.0104**	**<0.0001**	**0.0025**	**0.0061**	0.5867
		Car × Fru	0.3703	0.9105	0.1036	0.2594	0.3821	0.0534	0.0929	0.8387
		Car × AA	0.5482	0.8365	0.7447	0.3904	0.4687	**0.0001**	0.7077	0.8698
		Fru × AA	0.8291	0.4670	0.1986	0.0547	**<0.0001**	**0.0040**	**0.0159**	0.7618
		Car × Fru × AA	0.5750	0.8122	0.5488	0.7250	0.4086	0.3440	0.1574	0.7711

[1]all SEM values within the same marker are expected to be the same when the analysis is preformed with the assumption of homogeneity of variances

[2]Statistical significance at $p \leq 0.05$.

[3]LCar: Low carnosine; MCar: Medium carnosine; HCar: High carnosine.

Table 4.5. Effects of carnosine level, fructose (Fru), and ascorbic acid (AA) on free carnosine (Car), indicators of protein and lipid oxidation and AGEs (LS Means with SEM[1] in parentheses) during in vitro duodenal digestion of a burger meal system[2]

	Treatments[3]		Carnosine (µg/mL digest)	Protein carbonyls (nmol/mg protein)	Free thiols (nmol/mg protein)	Hexanal (ng/mL digest)	MDA (ng/mL digest)	GO (ng/mL digest)	CML (ng/mL digest)
	Fructose (Fru)	Ascorbic acid (AA)							
LCar	No Fru	No AA	373.9 (26.2)	3.09 (0.22)	4.57 (0.83)	2166 (223)	552.5 (32.4)	185.4 (12.8)	30.68 (4.01)
		AA	384.8 (26.2)	3.08 (0.12)	5.37 (0.83)	3072 (223)	838.1 (32.4)	178.6 (21.5)	27.04 (1.43)
	Fru	No AA	366.2 (10.3)	2.75 (0.22)	3.15 (0.20)	2242 (223)	557.8 (54.4)	170.8 (10.4)	35.86 (2.02)
		AA	341.0 (10.3)	3.08 (0.12)	4.80 (0.83)	3590 (223)	1093 (54.4)	152.7 (5.6)	32.44 (0.87)
MCar	No Fru	No AA	546.1 (37.8)	3.21 (0.22)	3.69 (0.83)	1994 (93)	524.3 (43.3)	194.9 (12.8)	28.98 (4.01)
		AA	531.3 (37.8)	2.86 (0.12)	5.87 (0.83)	2324 (93)	616.6 (43.3)	207.5 (21.5)	25.48 (1.43)
	Fru	No AA	487.3 (14.2)	3.02 (0.22)	3.24 (0.20)	2308 (93)	543.1 (9.8)	146.8 (10.4)	36.41 (2.02)
		AA	489.9 (14.2)	2.88 (0.12)	7.03 (0.83)	2800 (93)	829.3 (9.8)	140.4 (5.6)	34.57 (0.87)
HCar	No Fru	No AA	653.8 (30.9)	2.93 (0.22)	5.16 (0.83)	2079 (91)	539.8 (30.4)	208.9 (12.8)	32.61 (4.01)
		AA	700.2 (30.9)	3.22 (0.12)	6.42 (0.83)	2300 (91)	717.4 (30.4)	199.0 (21.5)	28.80 (1.43)
	Fru	No AA	648.4 (30.3)	2.92 (0.22)	3.65 (0.20)	2194 (91)	524.5 (33.2)	139.4 (10.4)	37.00 (2.02)
		AA	630.1 (30.3)	2.80 (0.12)	5.72 (0.83)	2961 (91)	810.9 (33.2)	152.3 (5.6)	34.62 (0.87)
	p Values								
		Car	**<0.0001**	0.9719	0.3421	**0.0098**	**0.0006**	0.9461	0.4747
		Fru	**0.0171**	0.1323	0.1798	**0.0004**	**0.0001**	**<0.0001**	**0.0003**
		AA	0.9849	0.9870	**<0.0001**	**<0.0001**	**<0.0001**	0.7473	**0.0387**
		Car × Fru	0.7660	0.8665	0.3034	0.9223	0.1737	0.1102	0.5886
		Car × AA	0.8290	0.2423	0.2255	**0.0268**	**0.0033**	0.6872	0.9683
		Fru × AA	0.3560	0.8382	0.2063	**0.0374**	**0.0002**	0.8791	0.6945
		Car × Fru × AA	0.5940	0.2883	0.9082	0.3572	0.4259	0.5365	0.9739

[1]all SEM values within the same marker are expected to be the same when the analysis is preformed with the assumption of homogeneity of variances

[2]Statistical significance at $p \leq 0.05$.

[3]LCar: Low carnosine; MCar: Medium carnosine; HCar: High carnosine.

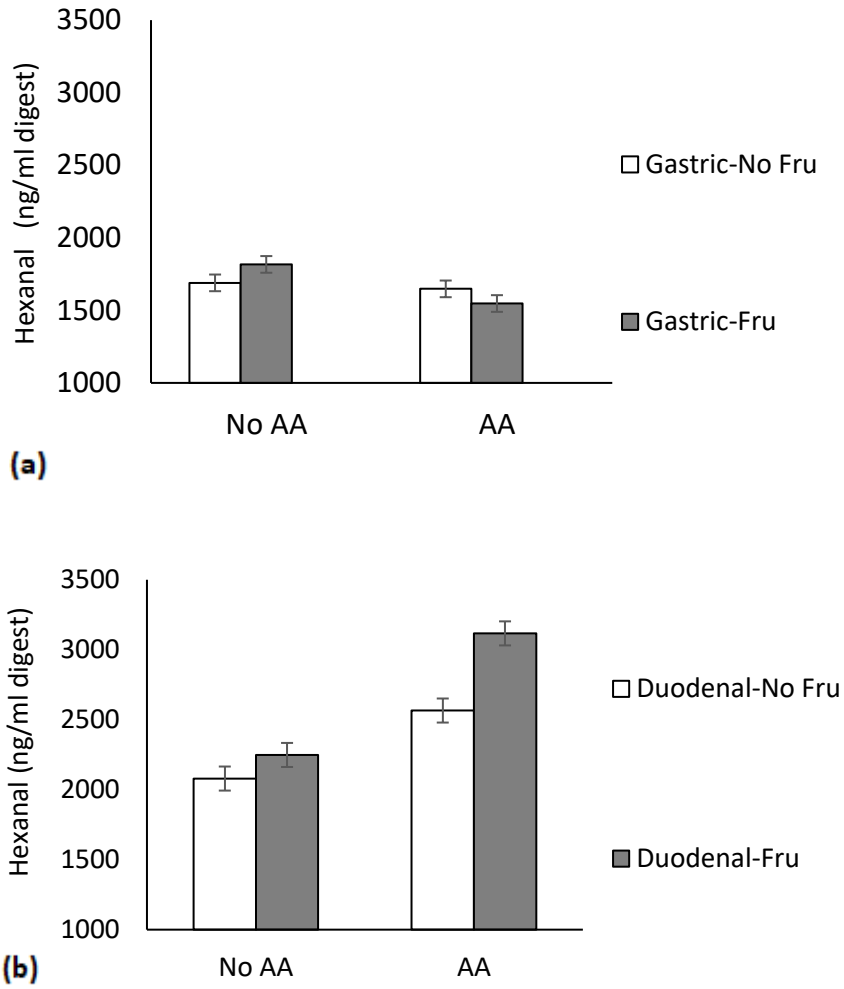

Figure 4.2. Interactions between ascorbic acid (AA) and fructose (Fru) on hexanal concentrations (LS Means ± SEM) in (a) gastric (p=0.0547) and (b) duodenal (p=0.0374) digests.

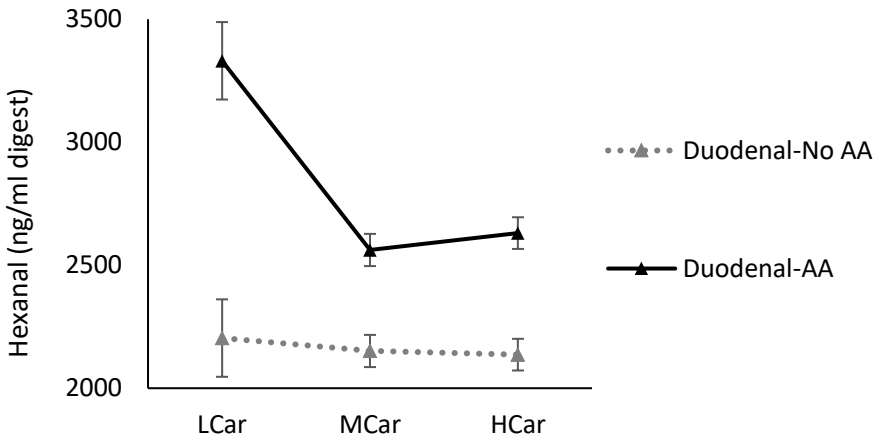

Figure 4.3. Interactions between carnosine and ascorbic acid (AA) on hexanal concentrations (LS Means ± SEM) in duodenal digests (p=0.0268).

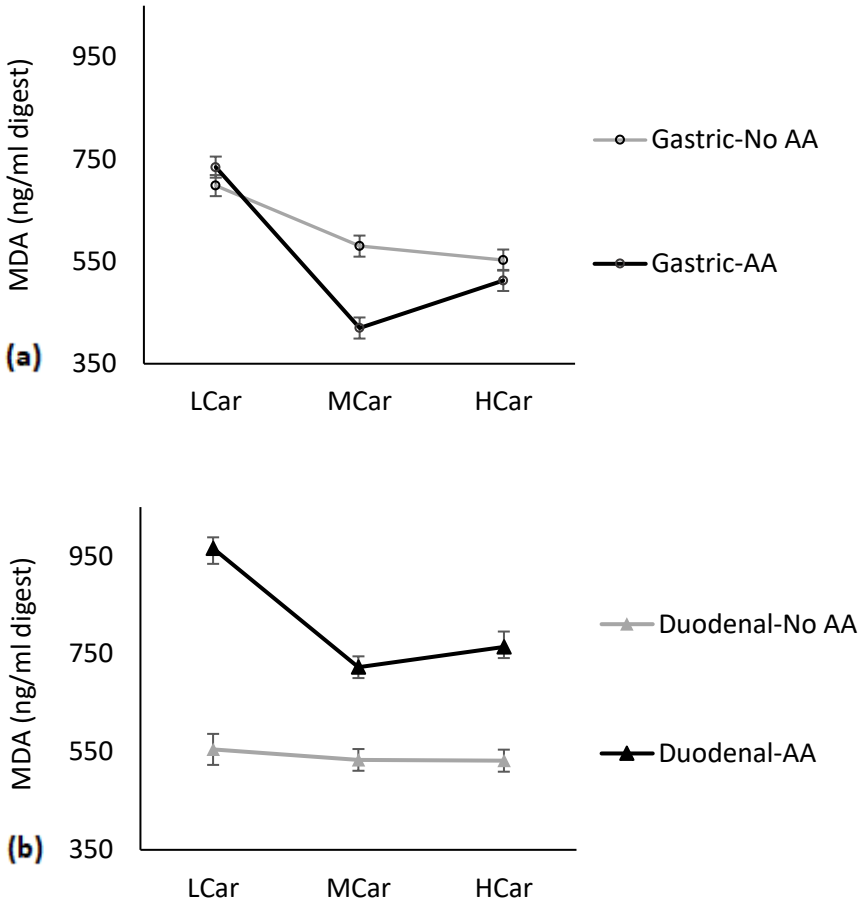

Figure 4.4. Interactions between carnosine and ascorbic acid (AA) on MDA concentrations (LS Means ± SEM) in (a) gastric (p=0.0001) and (b) duodenal (p=0.0033) digests.

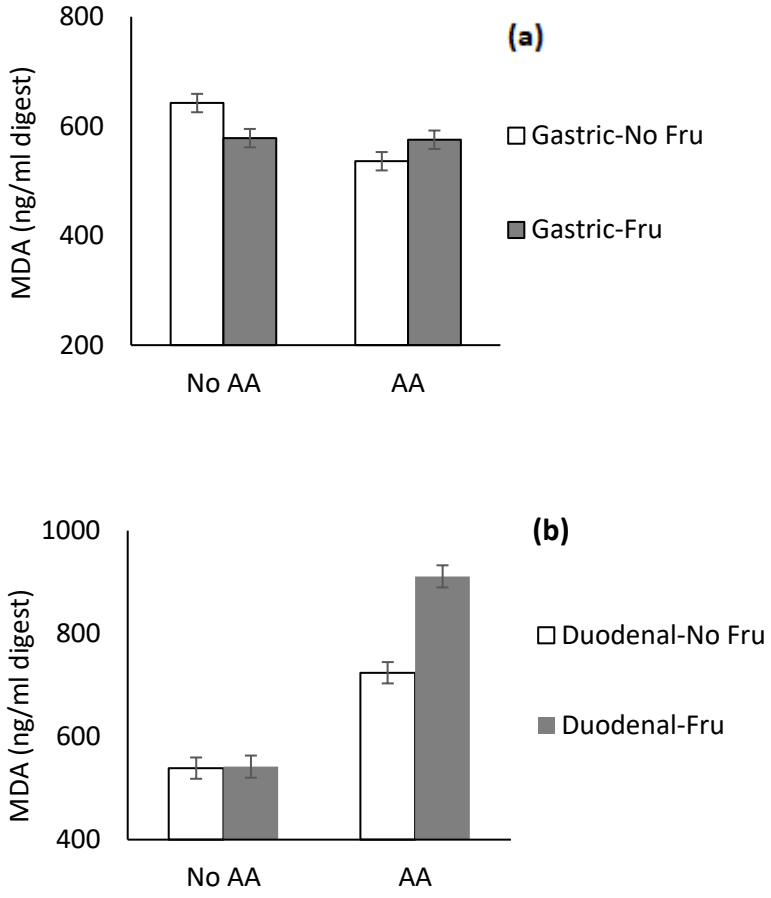

Figure 4.5. Interactions between ascorbic acid (AA) and fructose (Fru) on MDA concentrations (LS Means ± SEM) in (a) gastric (p=0.0040) and (b) duodenal (p=0.0002) digests.

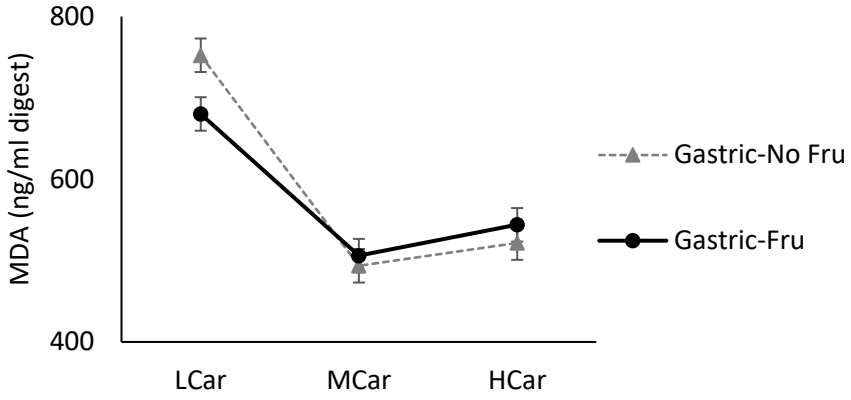

Figure 4.6. Interactions between carnosine and fructose (Fru) on MDA concentrations (LS Means ± SEM) in gastric digests (p=0.0534).

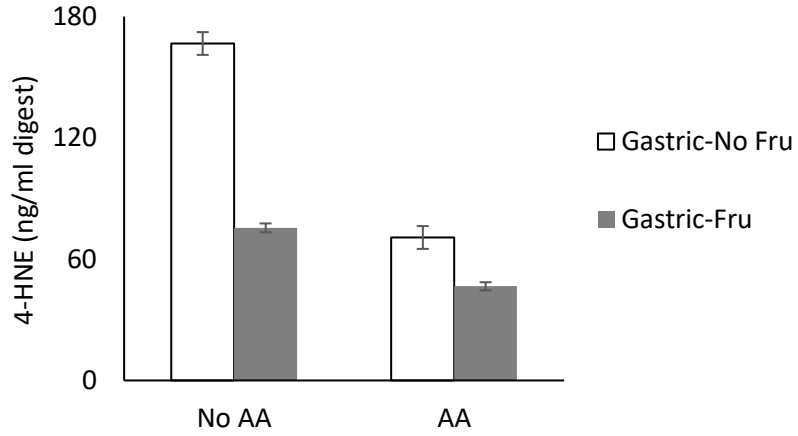

Figure 4.7. Interactions between ascorbic acid (AA) and fructose (Fru) on 4-HNE concentrations (LS Means ± SEM) in gastric digests (p<0.0001).

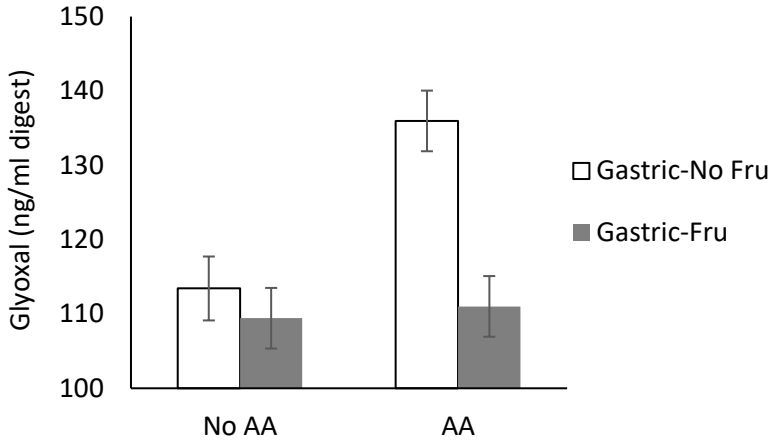

Figure 4.8. Interactions between ascorbic acid (AA) and fructose (Fru) on glyoxal concentrations (LS Means ± SEM) in gastric digests (p=0.0159).

Connecting paragraph

In Chapter 4, carnosine enhancement at both intermediate and high levels displayed antioxidant capacity, irrespective of the presence of other anti- and/or pro-oxidants. However, no additional benefits during in vitro digestion were obtained at the high level of carnosine. Considering the results from Chapters 3 and 4, it became imperative to consider other anti-/pro-oxidants present in the same meal when studying the effect of dietary carnosine in order to obtain the expected health benefits of carnosine in meat adapted to a healthier meal.

The Mediterranean diet contains multiple health-beneficial compounds that could possibly interact with carnosine to mitigate the oxidation occurring during the digestion of meat-containing healthy meals. In Chapter 5, therefore, the bioaccessibility and effect of dietary carnosine in the meat was assessed during in vitro digestion of a meat-containing Mediterranean meal consisting of lean pork with three levels of carnosine, whole grain bread, vegetables (tomato and onion), and olive oil.

Chapter 5 will be submitted to *Journal of Agricultural and Food Chemistry.*

Chapter 5 Effect of dietary carnosine during in vitro digestion of a pork-based healthy meal model

5.1 Abstract

The effect of dietary carnosine was assessed during the in vitro digestion of a healthy meal model, based on the Mediterranean diet and consisting of lean pork enriched with two levels of carnosine, whole grain bread, tomato, onion and olive oil. Lipid and protein oxidation markers were measured in gastric and duodenal digests, and the total antioxidant capacity was measured in hydrophilic and lipophilic extracts of digests from both phases. Carnosine enrichment of meat increased its bio-accessibility, simultaneously increasing the total antioxidant capacity and globally reducing oxidation during the digestion of meal. An increase in malondialdehyde content in gastric digests with an intermediate level of carnosine suggests that, in the absence of significant changes in the ferric-reducing antioxidant capacity, mechanisms other than the ferric-reducing capacity of carnosine may account for this pro-oxidant outcome. Overall, this study confirmed the beneficial properties of carnosine in a healthy meal.

5.2 Introduction

Nowadays, a large portion of the world population is suffering from diet-induced chronic diseases, such as type 2 diabetes, obesity, and cardiovascular diseases (1). For example, it is estimated that about 60 percent of adults in the United States have one or more of such diseases, with unhealthy eating habits being one of the major causes (2). Although complex mechanisms are involved in the increased incidences of diet-related diseases, free radical formation is considered to be critical in this respect, with unhealthy diets providing accelerated oxidation catalyzed by metal ions, generating substrates for oxidation and glycation (1, 3). In addition to the potential absorption of oxidation

products, reactive species can eventually enter the blood circulation, attack target molecules, and raise oxidative stress, interfering with cell signaling and other pathways leading to chronic diseases (1, 3). It is, therefore, recommended to avoid unhealthy eating habits. A healthy diet includes a variety of vegetables and fruits, limited free sugars and salts, as well as limited and selected sources of carbohydrates and lipids (4).

According to guidelines and directions for healthy eating, the Mediterranean diet (MD), which includes olive oil as the main source of lipids and an abundance of plant foods, including whole grains (5), is a viable choice (2), and its health benefits have been confirmed in preventing chronic diseases, as shown by numerous studies (6-7). Nevertheless, existing studies in this area have focused only on limited aspects, such as, beneficial effects of isolated compounds of plant origin (for example, carotenoids and oleuropein from tomatoes), and the impact of olive oil on oxidation, inflammation, and cancer (8-9). Even though meat is an important element of daily diets and is certainly part of the MD (10), bioactive molecules from meat have only attracted limited attention, if at all. For example, Martini et al. (11) reported on the benefits of plant-based phenolic compounds for the reduction of hydrogen peroxide and lipid oxidation from meat after in vitro digestion. In this study, a Mediterranean vegetable salad, including olive oil, with grilled turkey was co-digested, but carbohydrates, as one of the three major dietary components, as well as the presence and effect of carnosine, an important functional and beneficial molecule found in meat, were not taken into account.

Carnosine (β-alanyl-L-histidine) is naturally synthesized and abundant in the skeletal muscle of many mammals and, thereby, is exclusively available in meat, such as beef and pork, and in some fish species (12). The biochemical and physiological properties of carnosine in supplement (isolated molecule) form have been studied in animals and humans, among others, as reviewed by Boldyrev et al. (12). For instance, carnosine possesses pH buffering, metal chelating, antioxidant, anti-glycation, and anti-aging properties (12). These properties are manifested in diverse ways, ranging from reducing lipid oxidation during the storage of raw (13) and cooked meat (14-15) to improving exercise performance in healthy people and displaying therapeutic potential against many chronic diseases (16-18).

However, whether the benefits of carnosine demonstrated in these studies with the isolated molecule (carnosine supplement) would be acquired through the consumption of meat is subject to the interactions that can occur in the meal matrix during digestion. For instance, dual effects of carnosine as a meat constituent were observed during the in vitro digestion of two different burger-based meal systems that included antioxidants and pro-oxidants (19-20). Although carnosine in meat showed some health-beneficial properties, dietary carnosine in the presence of ascorbic acid (AA) at a supplemental level was found to have a pro-oxidative effect (19). However, in another burger-based meal system, the health-beneficial properties of carnosine in meat were demonstrated irrespective of interactions between carnosine and other anti- and pro-oxidants at dietary levels (20). These studies, along with other studies reviewed by Sotler et al. (21), indicate that the presence and concentrations of bioactive components, such as carnosine, AA, phenolics,

and other dietary antioxidants, in a meat-containing MD may all contribute to unexpected pro-oxidant effects. In addition, the digestion process can provide an environment favoring multiple oxidative reactions and hence interactions (22), and in certain cases, changes in the environment may cause antioxidants to act as pro-oxidants (21). Nevertheless, carnosine in its meat matrix can still be expected to present its potential health benefits in a MD meal, but the combined effect of dietary carnosine and other antioxidants in the same meal during digestion requires attention and further studies. A potential consequence of the abundant amount of antioxidants present in the MD is a sparing effect on carnosine during digestion, making more of it available for absorption and thereby enhancing its potential in vivo health benefits. However, no in vitro digestion studies have been performed to ascertain whether such effects occur.

The objective of the current study was, therefore, to determine the accessible level and effect of carnosine (in its meat matrix) during in vitro digestion of a pork-containing MD model consisting of lean ground pork, tomato, onion, whole grain bread, and olive oil.

5.3 Materials and methods

5.3.1 Chemicals and reagents

All reagents and chemicals were of analytical grade or higher. Methanol (⩾ 99.9%), anhydrous acetonitrile (LC-MS grade), formic acid (LC-MS grade), 37% hydrochloric acid, and ferrous sulfate heptahydrate were purchased from Thermo Fisher Scientific (Waltham, MA, USA); L-carnosine-d_4, 4-hydroxyhexenal-d_3 (≥ 99%, HHE-d_3) and 4-hydroxynonenal (≥ 99%, 4-HNE) from Cayman Chemical (Ann Arbor, MI, USA); and

O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (OPFB) from American Division of the Tokyo Chemical Industry (Portland, OR, USA). All other chemicals and enzymes were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

5.3.2 Mediterranean diet model preparation

The MD model used was based on that of Martini et al. (11) with modifications. Longissimus dorsi muscles were purchased from a commercial pork processing plant in Quebec, Canada (Olymel S.E.C). Lean tissue was ground (6 mm plate), and carnosine levels were adjusted by adding 300 or 600 mg carnosine/100 g meat to the control (intrinsic 277 mg carnosine/100 g pork; LCar), providing medium (577 mg carnosine/100 g pork; MCar) and high (877 mg carnosine/100 g pork; HCar) carnosine treatments. The meat was transferred into polypropylene wide-mouth screw top containers (60 mL), which were then sealed in individual vacuum bags, cooked in a water bath to a core temperature of 71°C and held at this temperature for 5 min. The cooked meat was removed from the containers, portioned (20 g) into bags, vacuum packaged and stored at -80°C. Just prior to digestion, the cooked meat was thawed (room temperature, approximately 15 min).

Tomatoes, red onions, cold-extracted extra virgin olive oil (President's Choice, Loblaw Companies Ltd, Canada), and whole wheat bread (Gadoua Boulangerie Ltée, Laval, QC, Canada) were purchased in a local supermarket. Just prior to digestion, each of the bread, onion, and tomato was finely ground, independently, in a domestic blender (approximately 10 s). Cooked pork (1.6 g), oil (0.16 mL), and ground bread (0.64 g),

tomato (3.2 g), and red onion (0.4 g) were added to a 50 mL polypropylene tube and submitted to in vitro digestion.

5.3.3 In vitro digestion

The in vitro digestion method used was that described by Li et al. (23), which was based on the general food digestion method of Versantvoort et al. (24) adapted for the digestion of meat by Van Hecke et al. (25). The medium for the saliva, gastric, and duodenal juices was prepared as described by Van Hecke et al. (25). Tubes containing meal samples (6.0 g) were sequentially incubated for 5 min with saliva (6 mL), 2 h with gastric juice (12 mL), and 2 h with 1 M bicarbonate buffer (pH 8.0, 2 mL), duodenal juice (12 mL) and bile (6 mL). Colonic digestion was not carried out. This enzymatic incubation was performed in quadruplicate. Two tubes of digested samples were taken after the gastric phase, and one after the duodenal. The digests from each digestion phase were homogenized at 10,000 rpm for 1 min using a Polytron homogeniser (PT-MR 3100 with a PT 3012/2 T dispersing aggregate, Kinematica AG, Littau, Switzerland), and aliquots (1.5 mL) were transferred to 2 mL conical tubes and stored at -80°C until chemical analyses.

5.3.4 Free carnosine measurement

Carnosine concentrations in cooked meat and digests were measured by LC–MS/MS as described by Li et al. (20), which was adapted from Han et al. (26). Carnosine in cooked meat was extracted by mixing water (5 mL) with meat (0.1 g) in a capped 15 mL polypropylene tube, rotating for 20 min (50 turns/min), and collecting the liquid portion.

Extraction was not required for the digests. Instead, the gastric digests were diluted 10x with water, and duodenal digests diluted 5x with water. Meat extract (200 µL) or diluted digest (200 µL) was mixed with L-carnosine-d_4 (200 µL, 50 ng/µL) and water (600 µL). The mixture was passed through an Oasis HLB solid phase extraction column (Waters Corporation, Milford, MA, USA) previously conditioned according to the recommended instructions of the manufacturer. The extract was filtered using a nylon membrane syringe filter (0.22 µm). The sample (0.3 mL) was then diluted with acetonitrile (0.7 mL), and 5 µL was injected into a Vanquish™ HPLC coupled with a LTQ XL Mass Spectrometer (equipped with HESI-II probe, Thermo Fisher Scientific, Waltham, MA, USA) with an ACQUITY UPLC BEH AMIDE column (2.1 × 100 mm, 1.7 µm; Waters Corporation, Milford, MA, USA). Mobile phases consisted of solvent A comprising 10 mM ammonium acetate (0.1% ammonium hydroxide) in water/acetonitrile (20:80, v/v) and solvent B comprising 10 mM ammonium acetate (0.1% ammonium hydroxide) in water/acetonitrile (70:30, v/v). Elution was done with a gradient of: initial eluant 0% B; 50% B at 3 min; 80% B at 7 min; 0% B at 13.5 min and maintained for another 6.5 minutes at a constant flow rate of 0.25 mL/min. The chromatographic column was held at 35°C, the HESI-II probe at 275°C, and the capillary at 300°C. A spray voltage of 4 kV was employed. The sheath gas was at 30 arbitrary units, and the auxiliary gas at 5 arbitrary units. The protonated molecular ions were 227 *m/z* for carnosine and 231 *m/z* for carnosine-d_4.

5.3.5 Protein oxidation markers (carbonyls and free thiols)

Protein carbonyls were determined using a DNPH (2,4-dinitrophenylhydrazine) method from Ventanas et al. (27) as modified by Li et al. (19). In brief, digests (200 µL) were mixed with 20% trichloroacetic acid (TCA, 200 µL) and centrifuged (3000 ×*g*, 5 min). One pellet was treated with 2 N HCl (2 mL) for protein concentration measurement and used as a blank. The DNPH solution (2 mL of 10 mM DNPH in 2 N HCl) was mixed with the other pellet for the measurement of carbonyl concentration. After holding both samples at room temperature for 1 hour, 20% TCA (2 mL) was added, forming a precipitate, which was washed three times with freshly prepared ethyl acetate: ethanol (1:1, v/v; 2 mL), and fully dissolved in 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5, 2.5 mL). Centrifugation (2240 ×*g*, 2 min) was carried out to remove insoluble fragments if present. Protein concentration was calculated from absorption at 280 nm using BSA as the standard. Carbonyls were determined at 365 nm using an adsorption coefficient of 22,000 M^{-1} cm^{-1} and expressed as nmol/mg protein.

Free thiols were measured by a modified 2, 2'-dithiobis(5-nitropyridine) (DTNP) method (19) based on that of Martinaud et al. (28). The biuret method (29) was used to determine the protein concentrations in digests. The protein content of the digests was adjusted to 5 mg/mL with 200 mM phosphate buffer (pH 7.4) and further adjusted to 1 mg/mL with urea buffer (8 M urea in 100 mM phosphate buffer, pH 8.0). The samples (2 mL) were then mixed with 10 mM DTNP solution in ethanol (20 µL) and held for 1 h at room temperature. Absorbance of solutions at 386 nm was measured against a blank of protein at the same concentration without DTNP. The absorbance of diluted DTNP was

subtracted from the sample absorbance, and the thiol concentration was calculated using an absorption coefficient of 14 mM^{-1} cm^{-1} (28). Results are expressed as µmol free thiol/mg protein.

5.3.6 Lipid oxidation markers

Lipid oxidation products hexanal, 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA) were measured using the GC-MS method adapted by Li et al. (23) from that of Tsikas et al. (30). The GC-MS was comprised of an Agilent 7890B gas chromatograph coupled to a 5977B quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with Ultimate Plus deactivated fused silica tubing (5 m × 0.25 mm) and HP-5MS columns (30 m× 0.25 mm × 0.25 µm).

Briefly, digests (200 µL) were mixed (vortexed for 30s) with OPFB (170 µL of a 30 mg/mL solution) and HHE-d_3 (10 µL of a 20 ng/µL solution in ethanol), then sonicated for 3 min in an ultrasonic bath. Extraction of the targeted components was undertaken by the sequential addition of methanol (100 µL), isooctane (1 mL), and concentrated sulfuric acid (6 drops), after which the mix was vortexed for 1 min and centrifuged (774 ×*g*, 5 min). The supernatant (800 µL) was transferred to vials through anhydrous sodium sulfate and glass wool and evaporated to dryness. Samples were dissolved in *N, O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 50 µL) and incubated at 80°C for 1 h.

The GC–MS analysis was undertaken as described by Li et al. (19), with changes in the selected ions. Prepared samples (1 µL) were introduced into the instrument and selected-

ion monitoring (SIM) was used. For quantification, the following ions were chosen: 187 and 203 *m/z* (retention time, RT, of 12.20 and 12.33 min for the internal standard HHE-d_3); 239 *m/z* (RT 10.35 min for hexanal); 352 *m/z* (RT 13.62 min for 4-HNE); and 250 *m/z* (RT 13.76 and 13.79 min for MDA).

5.3.7 Total antioxidant capacity

Hydrophilic and lipophilic antioxidant extracts were obtained by the method of Rodríguez-Roque (31) with modified solvent volumes. Gastric digest (1.5 mL) and methanol (1.5 mL) were mixed, and the methanol layer was retained as the hydrophilic extract. After removing this hydrophilic layer, the remainder was mixed with tetrahydrofuran (1 mL) to obtain the lipophilic extract. The duodenal digest (1.5 mL) was treated with methanol (1 mL) to obtain the hydrophilic extract and tetrahydrofuran (1.5 mL) to obtain the lipophilic extract.

The preparation of working solutions and detailed steps of determinations for each of the assays, DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and FRAP (ferric reducing antioxidant power) were conducted according to Rodríguez-Roque (31) and Xiao et al. (32). Briefly, a concentrated DPPH methanol solution (0.5 mg/mL) was prepared and then diluted with methanol to achieve an absorbance of 0.7–0.8 at 515 nm as a working solution. Solvent blank (methanol) or digest extract (0.2 mL) was added to DPPH working solution (1.8 mL) and held for 30 min at room temperature. Absorbances of the blank and samples were obtained at 515 nm, and the inhibition rate was calculated based on the decreased

absorbance (the difference between the blank and sample) divided by the absorbance of the blank.

An ABTS mother solution, containing 3.5 mmol/L of ABTS and 1.225 mmol/L of potassium persulfate, was prepared with acetate buffer (pH 4.5) and stored in the dark at room temperature, overnight (12–16 h). An ABTS working solution was then obtained by dilution with acetate buffer (pH 4.5) to achieve an absorbance of 0.737–0.743 at 734 nm (32). Digest extract (10 µL) and working solution (200 µL) were mixed and held in the dark at room temperature for 7 min. Absorbance was obtained at 734 nm.

A working solution of FRAP, containing both 9.09 mmol/L of TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) and 1.82 mmol/L of $FeCl_3$, was prepared according to Xiao et al. (32) and warmed to 37°C for 1 h. The FRAP working solution (180 µL) was mixed with the digest extract (5 µL) and incubated in the dark at 37°C. The absorbance at 593 nm was read after a 15-minute incubation period.

For all three techniques (DPPH, ABTS, and FRAP), Trolox equivalent standard curves were applied to the measurement.

5.3.8 Statistical analyses

Statistical analyses of all data were carried out by the ANOVA procedure of SAS version 9.4 (SAS, 2002-2012; SAS Institute Inc., NC, USA) at 3 carnosine levels, with the entire

protocol being repeated four times. A $p \leqslant 0.05$ was considered significant. The results are presented as least squares means (LS Means) with standard error of the mean (SEM).

5.4 Results and discussion

5.4.1 Carnosine levels during digestion of the healthy meal model

The average intrinsic carnosine level (LCar) in cooked pork was 465.63 ± 37.17 mg/100 g meat. Before digestion, carnosine enhancement in MCar significantly raised the level of carnosine to 710.57 ± 37.17 mg/100 g meat giving 244.94 mg/100 g meat more than the intrinsic group ($p < 0.0001$), while further carnosine enhancement in the HCar group increased its level to 1009.58 ± 37.17 mg/100 g meat, which was significantly ($p < 0.0001$) higher than both MCar and LCar groups.

Carnosine enrichment was also observed in the digests from both phases (Table 5.1). In the gastric phase, carnosine level in MCar digests was significantly increased to 566.15 µg/mL ($p = 0.0003$) compared to the LCar digests (365.06 µg/mL). Furthermore, the level of carnosine in the HCar digests reached 744.44 µg/mL, which was significantly higher than that found in both LCar ($p < 0.0001$) and MCar ($p = 0.0006$) digests. In the duodenal phase, the MCar group still presented higher concentrations of free carnosine (286.51 µg/mL) compared to the Lcar group (201.52 µg/mL; $p < 0.0001$). The highest concentrations of free carnosine were observed in the HCar treatment (400.51 µg/mL) as compared to the LCar ($p < 0.0001$) and the MCar group ($p < 0.0001$). Irrespective of the treatments, there was a decrease in the level of carnosine from the gastric to the duodenal phase, as previously reported by Li et al. (19, 23). This decrease may be related to the

involvement of carnosine in reduction of the oxidation occurring in the gastrointestinal tract (GIT).

In addition, the progressive and significant increases in free carnosine concentrations from LCar to MCar to HCar in both digests suggest the potential for benefits brought about by carnosine-enhanced meat in the GIT, given carnosine's antiproliferative effect on gastric and colorectal cancer cells (33-34). Considering the documented in vivo health advantages of carnosine (12), the more free carnosine remains in the duodenal digests and becomes accessible for absorption, the more benefits the carnosine-enhanced meat may bring to consumers. Therefore, the higher levels of bio-accessible carnosine in MCar and HCar groups than the control (LCar) demonstrate the in vivo health potential of enhancement of carnosine levels in meat. The MD-based healthy meal system used in the current study retained higher percentage of free carnosine in the duodenal digests when compared with the other burger-based meal systems (19-20). This difference emphasizes potential health benefits of an MD-based meal by sparing more carnosine for its in vivo benefits.

Table 5.1. Effect of carnosine-enriching treatments on free carnosine concentrations during in vitro digestion of a pork-containing healthy meal[1]

Phases	Low Carnosine (LCar) (μg/mL digest)	Medium Carnosine (MCar) (μg/mL digest)	High Carnosine (HCar) (μg/mL digest)	SEM	*p* Values
Gastric	365.06^{c}	566.15^{b}	744.44^{a}	21.46	**<0.0001**
Duodenal	201.52^{c}	286.51^{b}	400.51^{a}	7.30	**<0.0001**

[1]Values with different letters in the same row differ significantly ($p \leq 0.05$)

5.4.2 Lipid and protein oxidation-related indicators

As shown in Table 5.2, carnosine treatments had limited effect on the concentrations of protein oxidation-related markers (protein carbonyl and free thiols). The only significant difference was found in the duodenal phase, where the HCar treatment led to higher levels of free thiols when compared with the MCar ($p < 0.0001$) and LCar ($p < 0.0001$) groups. This finding supports the beneficial antioxidant potential of carnosine towards oxidation as reported by Li et al. (23) during in vitro digestion of carnosine-enriched pork.

In contrast to the protein oxidation markers, carnosine enrichment of meat caused significant differences in lipid oxidation products in the gastric digests while no effects were observed in the duodenal phase. In the gastric phase, no significant changes in the concentrations of hexanal or 4-HNE were observed in the MCar treatment compared to the LCar group. However, decreased levels of hexanal ($p = 0.0022$) and 4-HNE ($p = 0.0112$) were observed in HCar digests when compared to the MCar digests, substantiating the antioxidant role that carnosine can have in various environments, including the digestion process (12, 23).

In the case of MDA in gastric digests, an unexpected increase ($p = 0.0195$) was observed in the MCar treatment (300.56 µg/mL) compared to the LCar treatment (244.53 µg/mL). A trend of increased hexanal levels was also observed in MCar compared to LCar gastric digests ($p = 0.0737$). These results can be attributed to the pro-oxidative potential of carnosine, as observed by Li et al. (19) in the study on in vitro digestion of a burger meal

model in the presence of AA at a supplemental level. In the HCar gastric digests, however, MDA concentration was significantly lower than those in both MCar (p = 0.0195) and LCar ($p < 0.0001$) digests, indicating an antioxidant property of carnosine at the higher concentration in the current study. Although MDA was not measured in the study of Li et al. (19), a potential pro-oxidant effect of carnosine at the MCar level was observed in the measurement of other markers. For instance, in the presence of a high level of AA, the increased level of carnosine in meat significantly promoted oxidation and led to more 4-HNE at the MCar level during digestion of the meal, while the further increase of carnosine in the HCar group significantly reduced the level of 4-HNE compared to the MCar treatment. The most likely explanation for the dual effects of carnosine in the context of Li et al. (19) and particularly the pro-oxidative effect can be due to the ferric-reducing capacity of both carnosine and AA contributing to the regeneration of ferrous ions in the presence of hydroperoxide, and subsequently leading to the initiation of lipid oxidation.

Overall and irrespective of carnosine treatments, hexanal and MDA decreased from the gastric to the duodenal phase, with 4-HNE decreasing to a level below the quantification limit in the duodenal phase. Li et al. (23) reported similar decreases in 4-HNE and MDA levels from gastric to duodenal phases in a study on in vitro digestion of carnosine-enhanced pork and in 4-HNE levels during in vitro digestion of burger meal models (19-20). These decreases from gastric to duodenal phases could be explained by the dietary antioxidants (including carnosine) being released during digestion and exerting their antioxidant abilities, along with carnosine directly binding to oxidation products, such as

4-HNE (12). Furthermore, lower concentrations of lipid oxidation products were found in the current study employing an MD-based healthy diet model than previously reported for the other two burger-based meal systems (19-20). Considering the potential intestinal absorption of these lipid oxidation products and their health implications (35), the lower levels of oxidation products observed in duodenal digests of the current MD-based model underscore its suitability as a healthy diet system and demonstrate the value of a healthy diet.

Table 5.2. Effect of carnosine-enriching treatments on lipid and protein oxidation markers during in vitro digestion of a pork-containing healthy meal[1]

Oxidation marker	Digestion phase	Carnosine treatment			SEM	*p* Value
		Low (LCar)	Medium (MCar)	High (HCar)		
Hexanal (ng/mL digest)	Gastric	1589.68^{ab}	1891.37^{a3}	1311.98^{b}	83.91	**0.0029**
	Duodenal	1049.62	1071.37	1044.58	34.73	0.8479
4-HNE (ng/mL digest)	Gastric	128.25^{ab}	152.22^{a}	100.84^{b}	9.66	**0.0142**
	Duodenal[2]	-	-	-		
MDA (ng/mL digest)	Gastric	244.53^{b}	300.36^{a}	172.06^{c}	11.61	**0.0001**
	Duodenal	134.54	136.71	123.53	8.75	0.5444
Protein carbonyls (nmol/mg protein)	Gastric	5.31	5.00	4.74	0.19	0.1700
	Duodenal	3.62	3.52	3.71	0.20	0.8205
Thiols (nmol/mg protein)	Gastric	5.08	5.09	5.36	0.27	0.7198
	Duodenal	2.58^{b}	2.25^{b}	4.22^{a}	0.15	**<0.0001**

[1]Values with different letters in the same row differ significantly ($p \leq 0.05$).
[2]Not quantified.
[3]Indicates a trend compared with the LCar group in the same row ($0.05 < p < 0.10$).

5.4.3 Total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) was monitored for direct information on the potential benefits brought about by different levels of carnosine during digestion. Given that carnosine is hydrophilic, and the Mediterranean salad provides both hydrophilic and

lipophilic antioxidants, such as ascorbic acid phenolic compounds and carotenoids, respectively (11), both hydrophilic and lipophilic antioxidant extracts are necessary for a comprehensive assessment of the total antioxidant capacity of digests. Furthermore, it is recommended in the literature that a combination of different assays be employed for more comprehensive determination when analysing a complex food-based matrix (36). Given that hydrogen atom transfer (HAT) and electron transfer (ET) are the two major mechanisms underlying the TAC assays, widely used methods based on mixed mechanisms (36) were chosen in the current study. The DPPH (1,1-diphenyl-2-picrylhydrazyl) technique relies more on the ET, while the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay relies more on the HAT mechanism (37). Furthermore, both DPPH and ABTS methods can target radical scavenging ability, which is a well-known property of carnosine (12, 36). The ferric reducing/antioxidant power (FRAP) assay was used because the ferric-reducing ability of carnosine and ascorbic acid can contribute to their pro-oxidant potential (19, 38-39). The DPPH, ABTS, and FRAP assays were all used for the determination of both hydrophilic and lipophilic extracts of gastric and duodenal digests (11, 31, 36).

Results of the TAC of digests from each gastric and duodenal phase are presented in Table 5.3. Using the DPPH method the significantly high level of TAC in HCar in hydrophilic extracts from both gastric and duodenal digests was consistent with the antioxidant activity of carnosine given that carnosine is highly soluble in water. Carnosine has been reported to react with DPPH radical (40), and this histidine-containing peptide could act as an antioxidant mainly through ET-based pathways (41).

No significant differences were observed in the lipophilic extracts of gastric digests. A significantly lower TAC level in the HCar lipophilic extracts in the duodenal phase was observed.

The ABTS assay showed changes brought about by carnosine, not only in the antioxidant ability of the hydrophilic extracts, but also of the lipophilic extracts. Carnosine treatments had no effect in the gastric digests. However, significantly higher levels of TAC in the HCar group were observed compared with the LCar and MCar groups in the hydrophilic duodenal extracts, and with the MCar group in the lipophilic duodenal extracts. Even though carnosine is not a lipophilic antioxidant, it is possible that carnosine exerted its antioxidant activity at the water–lipid interface, subsequently contributing to interruption of the chain reactions of lipid oxidation in the lipid phase (42). Furthermore, the ABTS technique, which involves both HAT and ET mechanisms, can better assess the antioxidant capacity of HAT-based antioxidants compared to the other two techniques that rely more on the ET mechanism (DPPH and FRAP) (36). Accordingly, higher TAC levels were reported by the ABTS technique compared to the other two TAC methods used in the current study. Additionally, considering histidine's ability to donate protons, it is plausible that carnosine as a histidine-containing peptide might also contribute to higher levels of TAC through the HAT mechanism. However, more studies are needed to validate this possibility and determine to what extent this mechanism could contribute.

The FRAP method is only based on the ET mechanism and focuses on the ferric-reducing ability of the antioxidant. No significant changes in TAC levels determined by this

method were observed in gastric digests. In the duodenal phase, no significant differences were observed in lipophilic extracts, whereas the TAC determination for the hydrophilic extracts showed significantly higher antioxidant capacity/ferric-reducing capacity (p = 0.0009) of the HCar group compared to the MCar and LCar groups. This observation in duodenal digests is consistent with the antioxidant effect of carnosine and supports the beneficial property of this dipeptide in the meat matrix when co-digested with an antioxidant-rich meal.

The lack of significant changes in MCar gastric digests by the FRAP method does not support the hypothetical explanation for the increased MDA levels in the MCar treatments, relating the contribution of ferric-reducing capacity to the pro-oxidant potential of carnosine. In addition, the carnosine treatment caused no changes in hydroperoxide concentrations in either phase (not shown). These observations suggest the complexity of conditions in healthy meal digests, where the redox cycle between ferrous and ferric ions could contribute to Fenton-like reactions, but this might not be the only acting mechanism. For instance, phenolic compounds and carotenoids present in the meal containing onions and tomatoes can all have pro-oxidative potentials through different mechanisms (11, 43-45). When acting as antioxidants, carotenoids scavenge free radicals and can be converted into reactive carotenoid radicals, contributing to the overall oxidation process (45). The pro-oxidative effect of phenolic compounds can also be related to benzoquinone formation, which is unrelated to ferric-reducing ability (43). These bioactive compounds may also interact with each other. For instance, ascorbic acid (AA) can eliminate the very reactive carotenoid radical cations formed when radicals are

scavenged by carotenoids showing antioxidant activity (45) and may, therefore, reduce the possibility of carotenoids to exert pro-oxidative potential. In turn, the resulting consumption of AA may lower the possibility of AA taking part in other unfavorable reactions, such as promoting lipid peroxidation (46). However, these complex interactions among phytonutrients and the interaction of dietary carnosine with other bioactive compounds require further studies.

Table 5.3. Effect of carnosine-enriching treatments on total antioxidant capacity during in vitro digestion of a pork-containing healthy meal[1]

Types	Carnosine Treatment[2]	DPPH (Trolox equivalent µmol/mL digest)		ABTS (Trolox equivalent µmol/mL digest)		Ferric reducing power (Trolox equivalent µmol/mL digest)	
		Gastric	Duodenal	Gastric	Duodenal	Gastric	Duodenal
Hydrophilic	LCar	0.71^{b}	0.24^{b}	1.35	0.56^{b}	0.52	0.26^{b}
	MCar	0.62^{b}	0.23^{b}	1.31	0.51^{b}	0.55	0.24^{b}
	HCar	0.86^{a}	0.33^{a}	1.49	0.72^{a}	0.63	0.41^{a}
SEM		0.04	0.01	0.08	0.04	0.03	0.02
p Values		**0.004**	**0.0005**	0.286	**0.0078**	0.1334	**0.0009**
Lipophilic	LCar	0.23	0.31^{a}	0.39	0.33^{ab}	0.17	0.13
	MCar	0.22	0.34^{a}	0.38	0.30^{b}	0.18	0.15
	HCar	0.25	0.22^{b}	0.32	0.37^{a}	0.20	0.15
SEM		0.01	0.01	0.02	0.02	0.01	0.01
p Values		0.2322	**0.0003**	0.1141	**0.0486**	0.0835	0.569

[1]Values with different letters in the same column within the same type of extract differ significantly ($p \leq 0.05$).
[2]Low carnosine (LCar), medium carnosine (MCar), high carnosine (HCar).

5.5 Conclusion

Carnosine-enriched meat in a healthy meal reduced the level of oxidation and increased the total antioxidant capacity during digestion in the gastrointestinal tract. In addition, when compared to the effect of carnosine enhancement in burger meal models reported by Li et al. (19-20), where the antioxidant activity of carnosine was realized at the expense of its bio-accessibility, the decreased oxidative environment provided by the healthy meal spared some carnosine from acting as an antioxidant and therefore increased the level of free carnosine for absorption and potential further in vivo benefits. In the gastric phase, a high carnosine level (HCar) decreased lipid oxidation and increased the antioxidant capacity in the lipid environment. This HCar treatment also controlled protein oxidation and raised the antioxidant ability in both aqueous and lipid environments in the duodenal phase. However, the medium carnosine level (MCar) promoted MDA formation in the gastric digests, and the HCar treatment decreased the antioxidant ability of lipophilic extracts against DPPH radical in the duodenal phase, altogether suggesting a pro-oxidant potential of carnosine in some conditions with different oxidation level as previously observed by Li et al. (19-20) with burger meal models. The significantly higher levels of MDA with no significant difference in the ferric-reducing capacity (FRAP) observed in MCar gastric digests suggests that the ferric-reducing capacity of carnosine contributing to Fenton reaction may not be the only mechanism in accelerating lipid oxidation in the current study. Further studies are, therefore, required to understand how the level of carnosine and in the presence of other antioxidants, can promote MDA formation.

The benefits brought about by carnosine in a healthy meal represent an incentive to produce carnosine-rich meat, which can be achieved by feeding practices for example through increased levels of L-histidine in pig feed (47).

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Connecting paragraph

In addition to various dietary factors that could influence the effect of dietary carnosine during the digestion of a meal containing fresh meat, the presence of nitrite additives frequently utilized in processed meat products is an additional factor that warrants examination. These additives are a source of carcinogenic nitrosamines that represent an important health concern associated with the consumption of processed meat products. There are conflicting suggestions in the literature on the potential of carnosine to inhibit nitrosamine formation. In Chapter 6, therefore, the direct effect of different levels of carnosine on nitrosamine formation was assessed in a monophasic aqueous system and a lipid-water biphasic system, in order to collect fundamental knowledge and information from a simulated gastric environment as the groundwork for further research on the potential effect of carnosine on nitrosamine formation under dietary conditions.

Chapter 6 is submitted to *Journal of the Science of Food and Agriculture.*

Chapter 6 Effect of carnosine on nitrosamine formation in gastric-simulated aqueous and lipid environments

6.1 Abstract

Background: Nitrite salts are frequently utilized as meat additives to improve the quality and safety of processed meat products. However, these salts are associated with the formation of carcinogenic nitrosamines. Given its potential regulating effect on the formation of intermediate molecules, such as nitric oxide, it is hypothesized that carnosine, a meat constituent possessing antioxidant activity and other multiple health benefits, could dampen the formation of nitrosamines. The current study therefore assessed the effect of carnosine on nitrosamine formation in both a monophasic aqueous system, and a biphasic water-lipid system simulating a gastric environment.

Results: In the monophasic system, relatively high levels of carnosine were required to significantly reduce the formation of different species of nitrosamine compared with the control (no carnosine). While higher levels of some nitrosamines were generated in both phases of the biphasic system, low carnosine concentrations significantly suppressed nitrosamine formation in the aqueous phase, while in the lipid phase, intermediate levels of carnosine were required. At higher carnosine levels, further reduction in nitrosamines was observed in the lipid phase.

Conclusions: This study demonstrates the capacity of carnosine to decrease nitrosamine formation in aqueous and lipid environments and suggests the potential of dietary carnosine to lower the risks associated with the consumption of processed meat products.

6.2 Introduction

Processed meat products, such as bacon and sausages, are consumed on a daily basis by many North American[1] and European consumers[2]. Consumption of processed meat products has been associated with various health issues such as oxidative stress-related diabetes and cardiovascular diseases[3]. Different types of cancer have also been linked with carcinogenic nitrosamines induced by the use of nitrite salts[4-8]. The general mechanism by which nitrite salts (nitrite) can lead to the formation of harmful nitrosamines was detailed by Toldrá[9] as presented in Figure 5.

Nitrite is an important and frequently used ingredient in many meat products as a color- and flavor-improving agent and as a preservative owing to its antimicrobial and antioxidant functions[10]. Considering its versatility, nitrite as a meat additive is presently irreplaceable with a single chemical, in particular, for its important contribution to food safety since it can protect consumers against Clostridium botulinum, which produces lethal neurotoxin[11]. It is, therefore, important to be able to suppress nitrosamine formation in order to reduce the risks associated with the use of nitrites while also maintaining the advantages. Among a variety of compounds decreasing nitrosamine formation, those naturally found in the diet, such as ascorbic acid, can easily be utilized with few safety risks[12-13]. Carnosine (β-alanyl-L-histidine), a naturally occurring molecule in meat, has been suggested to have the potential to reduce nitric oxide[14], and therefore by deduction, could also potentially reduce nitrosamine formation.

The hypothesis that carnosine could affect nitrosamine formation is based on indirect observations. For instance, as part of its antioxidant activity, carnosine could potentially lower the levels of nitrosating agents, such as, peroxynitrite through its superoxide scavenging capacity. Superoxide is one of the precursors of peroxynitrite[14-15] which can generate nitrogen dioxide radicals that could take part in the formation of N_2O_3, a direct precursor of nitrosamines[15]. Furthermore, the L-histidine moiety of carnosine could potentially form adducts with nitric oxide (NO) or nitrogen dioxide (NO_2), subsequently reducing the formation of nitrosamine precursors, NO^+ and N_2O_3 [16]. And, it was also shown by Caruso et al.[17] that carnosine could suppress NO generation in vivo, which may contribute to the control of nitrosamine formation in vivo. Other researchers, however, have suggested that carnosine may enhance nitrosamine formation in vivo by stimulating the activity of NO synthase, an inference based on the changes brought about by carnosine on NO formation[14], which is not a direct precursor of nitrosamine. Information on the potential of carnosine to reduce nitrosamine formation, let alone its effects during the digestion process, is inconclusive and has never been clearly demonstrated. The current study, therefore, aimed to determine the effect of different carnosine concentrations on the formation of several nitrosamines in an aqueous model and, given that the stomach environment has been suggested to contribute to nitrosamine formation[18], in a lipid-water model simulating a gastric environment.

6.3 Materials and methods

All chemicals used for the models and sample extractions were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA) except for N-nitrosodimethylamine-d_6 (NDMA-d_6;

CDN Isotopes, Pointe-Claire, Quebec, Canada) and QuEChERS products, Bond Elut EMR—Lipid dSPE, and Bond Elut EMR—Lipid Polish tubes (Agilent Technologies, Santa Clara, California, USA).

6.3.1 Monophasic system for assessment of carnosine's inhibiting potential

The monophasic (aqueous) model was based on that of Combet et al.[19] with the following modifications: a) seven secondary amines that could contribute to the formation of seven detectable nitrosamines previously reported in foodstuffs[20] were selected, b) higher levels of each secondary amine precursor and a longer incubation time were used to form sufficient nitrosamines for quantification, based on Hinuma et al.[18] and data from a pre-test, and c) the sodium nitrite used was equivalent to the amount observed in digestive fluids[21]. Similar to Combet et al.[19,22], no digestive enzymes were used since nitrosamine formation is a non-enzymatic chemical process and carnosine hydrolysis only occurs through the activity of carnosinase, which has only been reported in tissue and serum in humans[14]. A control (no carnosine) and seven carnosine levels (60, 120, 200, 300, 500, 1000 and 1300 µg/mL) were used, covering the range of possible carnosine levels that can be found in meat digests, given that meat is a natural source of this functional molecule.

Briefly, 10 mM of each precursor secondary amine (dimethylamine (DMA), dibutylamine (DBA), diethylamine (DEA), dipropylamine (DPA), pyrrolidine (PYR), morpholine (MOR), and piperidine (PIP)), 1 mM EDTA, 1 mM potassium thiocyanate, 300 µM sodium nitrite, and different levels of carnosine were prepared in 0.1 M HCl (pH

1.5; 3 mL; a simulated gastric pH) in a 5 mL sealed snap cap polypropylene tube and incubated for 4 h in a water bath at 37°C (50 rpm)[19].

6.3.2 Aqueous-lipid model for assessment of carnosine's inhibiting potential

Based on the method of Combet et al.[19,22], glycerol tributyrate was chosen as the representative triacylglycerol for the lipid phase, and the ratio between water and lipid phases was 10:1 to simulate that in the stomach[19]. A control (no carnosine) and 5 levels of carnosine were used (120, 300, 500, 1000 and 1300 µg/mL).

The aqueous phase (3 mL) of the biphasic model was prepared following the same protocol as for the monophasic aqueous model described above, modifying the carnosine concentrations to 1.1 times those of the aqueous model (0, 132, 330, 550, 1100, and 1430 µg/mL) given that the final total volume of this water-lipid system was 3.3 mL. Glycerol tributyrate (0.3 mL) containing 10 mM of each precursor secondary amine (DMA, DBA, DEA, DPA, PYR, MOR, and PIP) was prepared as the lipid phase. The two phases were mixed in a 5 mL snap cap polypropylene tube and incubated for 4 h in a water bath at 37°C (50 rpm).

6.3.3 Analysis of nitrosamines in the monophasic model

The sample extraction method of Combet et al.[19] was followed with two modifications. The only internal standard used was 0.01% NDMA-d_6, and the extract was concentrated to 50 µL final total volume after evaporation for better quantification. In brief, sample (1 mL) and internal standard (10 µL) were mixed with 0.08 M HCl saturated sodium

chloride solution containing 5% sulphamic acid (0.5 mL). Two consecutive extractions with dichloromethane/diethyl ether (45:55 v/v, with 0.0025% 2,6-di-tert-butyl-4-methylphenol to avoid interference from oxidation during extraction) were carried out. The top layer was collected and evaporated to 50 µL under a gentle nitrogen stream before being injected into the GC/MS. Calibration curves were prepared with different concentrations of the targeted nitrosamines in 0.1 M HCl (pH 1.5; 1 mL) and mixed with the internal standard following the same extraction and evaporation processes as for the samples. The targeted nitrosamines were N-nitrosodimethylamine (NDMA), N-nitrosodibutylamine (NDBA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR) and N-nitrosopiperidine (NPIP). All samples and calibration curves were undertaken in duplicate. Some adjustments to the GC/MS instrument were also made based on the information from Combet et al.[19] and Sieira et al.[23]. The GC/MS system comprised an Agilent 7890B gas chromatograph coupled to a 5977B quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a VF-WAXms column (60 m× 0.32 mm × 0.50 µm, Agilent Technologies, CA, USA) in electron impact ionization and single ion monitoring modes. The oven temperature was programmed as follows: 37°C for 1 min then increased to 220°C at 8°C/min where it was held for 7 min. Helium was used as carrier gas at a flow rate of 1 mL/min. The ions selected for different molecules were 74 *m/z* (NDMA, RT (retention time)=14.77 min), 80 *m/z* (NDMA-d_6, RT=14.76 min), 102 *m/z* (NDEA, RT=16.18 min), 70 and 130 *m/z* (NDPA, RT=18.53 min), 158 *m/z* (NDBA, RT=21.37 min), 114 *m/z*

(NPYR, RT=22.11 min), 100 *m/z* (NPIP, RT=22.55 min) and 116 *m/z* (NMOR, RT=23.24 min).

6.3.4 Analysis of nitrosamines in the biphasic model

The two phases of the model were separated and the samples from the aqueous phase were extracted and analyzed as for the samples from the monophasic model (section 2.3 above). Samples from the lipid phase were mixed with 0.01% of NDMA-d_6 in acetonitrile (2 µL for each 200 µL lipid sample) and the nitrosamines were extracted and purified using a modified EMR—Lipid QuEChERS technique from Agilent[24]. In brief, 200 µL of this mixture was mixed with 10 mL of water by vortexing for 30 s, then incubated for 30 min. Acetonitrile (10 mL) was added and treated with buffered QuEChERS (9.75 g for method EN 15662). The following steps were as described by Sheshadri et al.[24] using two series of products, Agilent Bond Elut EMR—Lipid dSPE material and Agilent Bond Elut EMR—Lipid Polish material, to obtain the final extracts, which were concentrated three-fold by evaporation and then analyzed by GC/MS. The oven temperature program was as follows: 55°C for 1.5 min, then increased to 200°C at 12°C/min, held for 13 min, and then increased to 220°C at 25°C/min and held for 7 min. Although ions for different nitrosamines were all the same, retention times differed as follows: RT=10.91 min for NDMA; RT=11.02 min for NDMA-d_6; RT=11.92 min for NDEA; RT=13.46 min for NDPA; RT=15.55 min for NDBA; RT=16.34 min for NPYR; RT=16.33 min for NPIP; and RT=17.41 min for NMOR.

6.3.5 Statistical analyses

Statistical analysis of the data was carried out with the one-way ANOVA procedure followed by the Tukey honestly significant difference (HSD) test for multiple comparisons (SAS version 9.4, 2002-2012; SAS Institute Inc., NC, USA) with 8 carnosine levels (including the blank) for the aqueous model and 6 carnosine levels for each of the aqueous and lipid phases of the biphasic model. The entire protocol was repeated four times. Differences were considered significant at $P \leq 0.05$. All results are presented as LS Means with SEM.

6.4 Results and discussion

6.4.1 Effect of carnosine on nitrosamine formation in the monophasic system

Results from the aqueous model are shown in Table 6.1. Although the amount of each of the seven types of secondary amines used was the same, the corresponding nitrosamines formed to different extents. Indeed, NMOR reached the highest concentrations, ranging from about 85 to 850 times that of the other measurable nitrosamines, NDMA, NPYR, NPIP and NDBA. Signals for NDEA and NDPA were observable, but too small to be quantified.

Since unprotonated amines are more prone to nitrosation, the ammonium ion pKa values of the secondary amines in such an acidic environment can partially explain the differences in nitrosamine formation—the less basic the amines and, therefore, the smaller the pKa values, the higher the rate of formation will be, yielding more nitrosamines when incubated in the same environment[25]. Among the seven amines used

in the current study, MOR, the secondary amine generating NMOR which was found at the highest nitrosamine levels, possesses the lowest pKa (values reported by Perrin[26], Table 6.2). Furthermore, the basic DMA with an intermediary pKa value led to the third highest level of the nitrosamines, NDMA, and DEA, DPA, PIP and PYR alkylamines, with pKa values from 11.0 to 11.3[26], produced the lowest amounts of corresponding nitrosamines. However, the second highest nitrosamine level, NDBA, is derived from the alkylamine DBA that shares (with PYR) the highest pKa value of 11.3. Given that DBA, DMA, and DPA possess similar pKa values, proton affinity cannot explain the differing levels of nitrosamines generated and further research is required.

Irrespective of the different concentrations of the various nitrosamines, it was generally observed that less nitrosamine was generated with the addition of carnosine. Among the five quantified nitrosamines, NDMA, NPYR and NPIP are usually found in meat products[27]. The NDMA has one of the simplest nitrosamine structures and has been widely studied, not only in meat-related research, but also in medicine and other fields[28]. In the current study, only the highest carnosine level (1300 µg/mL) caused a significant decrease in the final NDMA concentration. Such high levels of carnosine were observed in the meat gastric digests following in vitro digestion of pork enhanced in carnosine[21], of which the enhancement corresponded to levels found in different types of meat[14]. Although the overall decrease in NDMA was minor, with no more than 0.011 µg/mL, during the four-hour incubation, carnosine in the system was still efficient in showing its potential for lowering this nitrosamine formation. Likewise, the highest carnosine level (1300 µg/mL) also significantly suppressed NPYR and NPIP formation compared with

the control group. Furthermore, carnosine levels of 120, 500 and 1000 µg/mL resulted in a significant decrease ($P < 0.05$) in NPIP formation, and 1000 µg/mL in NPYR formation compared to the control.

The efficiency of carnosine to reduce nitrosamine formation was also observed for nitrosamines whose concentrations were in the µg/mL range. For instance, carnosine at 500 and 1300 µg/mL exerted an inhibiting effect on the formation of NDBA ($P < 0.05$) and 1300 µg/mL, on NMOR generation. This high level of carnosine can be achieved during the digestion of carnosine-rich meat[21]. Overall, in this aqueous environment where carnosine is well dissolved, carnosine showed a capacity to inhibit the formation of different types of nitrosamines, consistent with the capacity of carnosine to form carnosine-NO and carnosine-NO2 adducts as reported by Nicoletti et al.[16].

6.4.2 Effect of carnosine on nitrosamine formation in the biphasic system

Data from the biphasic aqueous-lipid model are reported in Table 6.3. Except for NDPA, whose peak was below the quantification limit, nitrosamines from all other secondary amines studied were detected in each of the water and lipid phases of this biphasic system. In both the aqueous and lipid phases of the biphasic system, most of the measured nitrosamines were at higher levels than those observed in the monophasic aqueous system. The levels of nitrosamines observed in the biphasic model confirmed that the amounts of sodium thiocyanate, sodium nitrite and different secondary amines used were sufficient for nitrosamine formation and suggest that the low levels of

nitrosamines observed in the aqueous model were not caused by an insufficient amount of reactants.

With the exception of NMOR, the lipid phase provided a better environment for the formation of all nitrosamines, leading to significantly higher levels ($P < 0.0001$). Similar observations were found by Combet et al.[19] who reported that the monophasic aqueous model had less NDMA, NDEA and NPIP than the water phase from the biphasic model, and there were more NDMA and NPIP formed in the lipid phase than in both the monophasic and the aqueous phase from the biphasic model.

Compared to a monophasic aqueous environment, the higher concentrations of nitrosamines in the biphasic system could be mainly attributed to better solubilities of nitric oxide and oxygen in the lipid environment and higher reaction rates (up to 300 times higher) for the production of N_2O_3, which reacts with secondary amines to form nitrosamines[29]. The higher nitrosamine levels found in the lipid phase of the biphasic system may, therefore, be partially explained by this difference in N_2O_3 generation. The affinity of different nitrosamines for water or lipid needs to be considered, as Combet et al.[19] pointed out. For instance, being more hydrophilic than NDBA[30], there might be less NMOR in the water phase for the potential diffusion into the lipid phase, resulting in lower NMOR than NDBA concentrations in lipid samples. This effect of solubility might also explain the concentrations of NDEA and NDBA in both water and lipid phases (according to the solubility reported by the European Medicines Agency[30]). As a result of the alkalinity of precursor amines, more NDEA than NDBA was observed in the aqueous

environment. However, the higher lipophilicity of NDBA than NDEA[30] might explain why more NDBA was observed in the lipid phase.

Although the biphasic system favored nitrosamine formation, carnosine exerted a beneficial effect by showing decreasing trends in some and significant decreases in other types of nitrosamines. There were some decreases in NMOR and NDBA, the nitrosamines of highest concentrations in both phases. Indeed, a decreasing trend and a significant decline in NDBA were obtained with the 1300 µg/mL treatment of carnosine in the aqueous and lipid phases, respectively. This high level of carnosine (1300 µg/mL) showed a tendency towards reduction of NMOR in the lipid phase ($P = 0.079$). Given the lower concentration of the other four types of nitrosamines, lower levels of carnosine were required to significantly reduce their formation ($P < 0.05$).

In the aqueous phase, all carnosine treatments significantly impeded NDMA and NDEA formation compared with the control group. Interestingly, even though the five carnosine levels studied here represent a relatively large range of concentrations (120 to 1300 µg/mL), the impact on the formation of these two nitrosamines did not differ with carnosine concentration ($P > 0.1$). Similarly, carnosine treatments from 300 to 1300 µg/mL all led to less NPYR than samples containing no carnosine, but increasing carnosine concentration did not further decrease the level of NPYR ($P > 0.1$). The carnosine treatments containing 500 µg /mL and 1300 µg/mL significantly suppressed NPIP formation compared with the control group ($P < 0.05$). No significant differences were observed among the NPIP levels with added carnosine.

Although carnosine is a water-soluble molecule, its effect was also observed in the lipid phase. Different carnosine treatments led to less nitrosamine formation than the control group, and also brought about differences in concentrations within each of NDMA, NDEA, NPYR, and NPIP. Carnosine treatments from 300 to 1300 μg/mL decreased the level of NDMA in the lipid phase compared with both the control ($P < 0.0001$) and the 120 μg/mL group ($P < 0.05$), which were not significantly different from each other. The highest carnosine level (1300 μg/mL) even further reduced the amount of NDMA when compared with the treatment containing 300 μg/mL of carnosine ($P < 0.05$). These observations not only confirmed the ability of carnosine to decrease nitrosamine formation, but also indicated a greater capacity of carnosine to inhibit NDMA formation at a higher carnosine level. For NDEA, higher carnosine levels (1000 and 1300 μg/mL) were required to achieve significantly lower concentrations than the control group ($P < 0.0001$). The 1300 μg/mL treatment also led to significantly less NDEA than the 120 μg/mL and 300 μg/mL treatments ($P < 0.05$). The observations in NPYR and NPIP among different carnosine levels also supported a dose-dependent nitrosamine-reducing property of carnosine. Significant decreases (compared with the control group) in both NPYR and NPIP levels were obtained with the 500 μg/mL, 1000 μg/mL, and 1300 μg/mL treatments. Furthermore, compared to that at 500 μg/mL carnosine treatment, the production of NPYP and NPIP was further decreased when the carnosine concentration was increased to 1300 μg/mL ($P < 0.05$), again demonstrating the potential dose-dependent response of carnosine on nitrosamine formation in a lipid environment.

Since carnosine is a hydrophilic antioxidant, its effect on nitrosamine concentrations in the lipid environment is likely attributed to carnosine reducing the amount of nitrosating agents in the aqueous phase. Fewer nitrosating agents available in the aqueous phase would result in fewer being available in the lipid phase for further reactions. For instance, when carnosine forms carnosine-NO and carnosine-NO_2 complexes in the aqueous environment[16], the balance ($N_2O_3 \leftrightarrow NO + NO_2$) shifts increasing the consumption of N_2O_3 and reducing its availability to participate in nitrosamine formation. Simultaneously, since NO is prone to migrate to the lipid phase, the binding of NO by carnosine in water may lead to less NO diffusing into the lipid phase and, therefore, decrease the availability of NO for further reaction with oxygen, yielding less N_2O_3 [29] for nitrosamine formation in the lipid phase.

Interestingly, instead of occurring in the aqueous phase (where carnosine could act directly), it was in the lipid phase that the dose-dependent nitrosamine-reducing potential of carnosine was mainly observed. The hypothetical explanation could be related to nitrosating agents other than the NO-derived N_2O_3 in the water phase. For instance, the NaSCN included in the acidic model could easily react with HNO_2 and generate the nitrosating agent ON-SCN ($HNO_2 + H^+ \rightarrow H_2NO_2^+$; $H_2NO_2^+ + SCN^- \rightarrow$ ON-SCN + H_2O)[31]. This ON-SCN, in such a situation (containing different nitrosating agents), could be more electrophilic than N_2O_3 and possess a higher reaction rate for nitrosamine formation[31]. Therefore, the ability of carnosine to react with NO could only partially affect the nitrosamine generated by NO^+/N_2O_3, with little control in the nitrosamine derived from the reactions between ON-SCN and secondary amines. More studies

supporting this hypothetical explanation and providing information about the mechanisms involved are necessary.

The current study directly demonstrates the inhibitory ability of carnosine on nitrosamine formation, and suggests that dietary carnosine may also bring this benefit in the digestive environment. Considering that the carnosine levels in meat can be enhanced through breeding and nutrition[32-33], it is expected that beneficial effects of carnosine might be obtained by the consumption of a meal that contains carnosine-rich meat. However, the models used in the current study were relatively simple and there is still a lack of information on the complex environment associated with daily diets that may interfere with the possible carnosine nitrosamine-reducing ability. For instance, oxidation and nitrosation are related to each other. The formation of oxidative peroxynitrite ($O2^{\bullet-}$ + NO → $ONOO^-$) might leave fewer nitrosating agents, such as nitric oxide, for nitrosamine formation[34-35]. Based on the results reported in this study and the potential mechanisms involved, muscle carnosine could directly contribute to reducing nitrosamine formation in meat products, even during their consumption, thus warranting further study.

6.5 Conclusion

Although absorption of nitrosamine from food is limited, increased endogenous nitrosamines due to a high intake of nitrite has been associated with an increase in cancer risk, particularly gastric and oesophageal cancers. The results of this study demonstrate, for the first time, the potential of dietary carnosine to reduce the formation of nitrosamines in a simulated gastric environment. Additional studies are required to

evaluate the potential interactions that could occur between nitrite, carnosine, and other molecules, such as additional antioxidants and secondary amines generated during the production of processed meat products, as well as the global effects of cooking, diet composition, and digestion processes, all of which could impact the bioaccessibility of carnosine and, ultimately, its ability to inhibit nitrosamine formation. Nevertheless, the preliminary findings reported here show potential health benefits of carnosine toward the reduction of nitrosamine formation in the gastrointestinal environment.

6.6 References

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$$NO_2^- + H^+ \leftrightarrow HNO_2$$

$$2\ HNO_2 \leftrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \leftrightarrow NO + NO_2$$

$$HNO_2 + H^+ \rightarrow H_2NO_2^+ \leftrightarrow NO^+ + H_2O$$

$$H_2NO_2^+ + X^- \rightarrow XNO + H_2O$$

$$NO + M^{n+} \rightarrow NO^+ + M^{(n-1)+}$$

$$XNO + R_2NH \rightarrow R_2N\text{-}NO + HX$$

$$NO^+/N_2O_3 + R_2NH \rightarrow R_2N\text{-}NO$$

Where X^- represents catalyzing ions such as SCN^-, I^-, Br^-, and Cl^-; and $M^{n+}/M^{(n-1)+}$ represent transition metal ions

Figure 5. Nitrosamine formation as detailed by Toldrá[9]

Table 6.1. Effect of carnosine on the formation of nitrosamines (LS Mean with SEM) in a monophasic aqueous model

Carnosine levels	Nitrosamine concentrations †				
	NDMA (ng/mL)	NPYR (ng/mL)	NPIP (ng/mL)	NDBA (µg/mL)	NMOR (µg/mL)
Control (0µg/mL)	60.337[a]	25.427[a]	18.421[a]	0.184[a]	15.636[a]
60µg/mL	58.936[ab]	24.665[ab]	15.736[ab]	0.162[ab]	14.974[ab]
120µg/mL	56.412[ab]	22.785[ab]	15.038[b]	0.146[ab]	13.867[ab]
200µg/mL	55.872[ab]	22.381[ab]	15.157[ab]	0.145[ab]	14.183[ab]
300µg/mL	56.228[ab]	22.177[ab]	15.282[ab]	0.146[ab]	13.681[ab]
500µg/mL	53.410[ab]	21.921[ab]	14.048 [b]	0.141[b]	13.580[ab]
1000µg/mL	52.284[ab]	21.033[b]	13.167[b]	0.148[ab]	13.982[ab]
1300µg/mL	49.399[b]	21.266[b]	13.208[b]	0.130[b]	12.871[b]
SEM	3.000	1.000	1.000	0.010	0.500
P values	**0.0077**	**0.0138**	**0.0028**	**0.0156**	**0.0226**

†Values with different superscripts within the same column denote significant differences ($P \leq 0.05$).

Table 6.2. The pKa values of the secondary amines used in the current study[26]

Secondary amine ammonium ion	pKa values
Dibutylamine (DBA)	**11.3**
Diethylamine (DEA)	**11.0**
Dimethylamine (DMA)	**10.7**
Dipropylamine (DPA)	**11.0**
Morpholine (MOR)	**8.3**
Piperidine (PIP)	**11.1**
Pyrrolidine (PYR)	**11.3**

Table 6.3. Effect of carnosine on nitrosamine formation (LS Mean with SEM) in the aqueous and lipid phases of the biphasic model

Phases	Carnosine levels	Nitrosamine concentrations (µg/mL) †					
		NDMA	NDEA	NDBA	NPYR	NPIP	NMOR
Aqueous	Control (0µg/mL)	0.195^{a}	0.238^{a}	0.178	0.204^{a}	0.179^{a}	13.673
	120µg/mL	0.146^{b}	0.173^{b}	0.158	0.180^{ab}	0.143^{ab}	13.109
	300µg/mL	0.147^{b}	0.173^{b}	0.156	0.154^{b}	0.139^{ab}	13.140
	500µg/mL	0.143^{b}	0.168^{b}	0.154	0.151^{b}	0.136^{b}	13.062
	1000µg/mL	0.144^{b}	0.156^{b}	0.141	0.137^{b}	0.137^{ab}	12.912
	1300µg/mL	0.122^{b}	0.145^{b}	0.136	0.135^{b}	0.133^{b}	12.479
	SEM	0.008	0.015	0.010	0.011	0.010	0.402
	P Values	**0.0002**	**<0.0001**	0.0970	**0.0019**	**0.0283**	0.4898
Lipid	Control (0µg/mL)	2.609^{a}	1.084^{a}	10.416^{a}	1.353^{a}	2.054^{a}	8.982
	120µg/mL	2.395^{a}	1.006^{ab}	9.304^{ab}	1.211^{ab}	1.704^{ab}	7.763
	300µg/mL	2.003^{b}	0.962^{ab}	8.734^{ab}	1.131^{ab}	1.770^{ab}	7.783
	500µg/mL	1.842^{bc}	0.869^{abc}	8.782^{ab}	1.113^{bc}	1.540^{bc}	7.624
	1000µg/mL	1.740^{bc}	0.788^{bc}	8.805^{ab}	1.014^{bcd}	1.215^{cd}	7.506
	1300µg/mL	1.516^{c}	0.612^{c}	7.822^{b}	0.863^{d}	1.079^{d}	7.265
	SEM	0.115	0.080	0.532	0.055	0.087	0.412
	P Values	**<0.0001**	**<0.0001**	0.0637	**<0.0001**	**<0.0001**	0.1169

†Values with different letters in the same column differ significantly ($P \leq 0.05$).

Chapter 7 General discussion and conclusion

Muscle foods, or meat products, can play an important role in the nutritional status of individuals, providing high-quality proteins, minerals, vitamins, and essential amino acids. Health concerns regarding the consumption of meat are well-known and widely accepted by the general public; these include increasing the risk of cardiovascular diseases, obesity, and cancers through the promotion of oxidation, glycation, and other adverse reactions, such as nitrosamine formation. Carnosine, as a natural multifunctional molecule in meat, has the potential to counteract such adverse reactions and even address related health concerns.

To study the effects of dietary carnosine under conditions of daily diets while considering the digestion process and interferences that may be caused by the variety of compounds usually present in a genuine meal matrix, meat-based unhealthy (pro-oxidative) and healthy (less oxidative) meal models were employed in the research presented in this thesis. Despite the observed pro-oxidative potential of carnosine in the presence of AA at a supplemental level in a burger meal model, the findings reported in the current dissertation demonstrate that increased carnosine content in the meat matrix is generally associated with health benefits. This indicates that the consumption of carnosine-enriched meat is beneficial to health, and consuming mildly cooked lean fresh meat, together with adequate health-beneficial phytochemicals from food, can support better the health. In addition to previous studies conducted with fresh meat, the research performed using simple matrices provided evidence of the ability of carnosine to reduce nitrosamine formation in gastric-simulated aqueous and lipid environments, suggesting the possibility

of carnosine providing similar benefits in processed meat products and even during digestion.

7.1 Effect of different meal models

Meal models were designed to simulate the oxidative environments of daily diets that are expected to provide different levels of oxidation. A burger meal model including a supplemental level of ascorbic acid (AA) and a model of a burger meal combo were used as representatives of a typical Western diet, possessing relatively high levels of oxidizing potential. A model of a Mediterranean meal was used in the current dissertation as an example of a healthy meal to provide a less oxidative environment. The different designs of these models led to differences in oxidation-related markers.

In comparison among the three models mentioned above, no obvious differences were observed in protein oxidation. Conversely, in lipid oxidation, the burger meal with AA at a supplemental level had higher amounts of lipid oxidation products than the burger meal combo, which included AA at a dietary level. Furthermore, higher concentrations of lipid oxidation products were found in the two burger meal models compared to the model of a Mediterranean meal as a representative of a healthy diet. Considering that lipid oxidation products in these three models were measured by the same GC-MS method, these observations demonstrate that the model of burger meal with AA at a supplemental level provided the most oxidative environment, followed by the burger meal combo as the second most oxidative environment, with both of these models based on burger meals (unhealthy meals) being more oxidative than the model of a Mediterranean meal (healthy

meal). Altogether, these meal models successfully met their purpose and fulfilled the expectation that unhealthy meals could be more oxidized than healthy meals, thereby emphasizing the benefits of choosing healthy diets. Similarly, whereas the levels of di-carbonyls in the two burger meal models were comparable, the levels of di-carbonyls in the Mediterranean meal model were much lower, even too low to be quantified, demonstrating again the value of the healthy diet.

Given that carnosine is expected to interact with products of oxidation, measurement of its bioaccessibility after digestion of each of the meal models should also reflect their respective oxidative environments. The accessible carnosine in duodenal digests of the burger meal with a supplemental level of AA was around 40% to 55% of the level of carnosine in the undigested cooked meat, irrespective of carnosine-enhancing treatments. Similarly, the level of carnosine remaining in duodenal digests of the burger meal combo was roughly 64% of its level in the undigested cooked meat. In contrast, in the case of the Mediterranean meal model, nearly 100% of the carnosine in the undigested cooked meat was retained in the duodenal digests. These observations demonstrated, again, that the healthy diet provided a relatively less oxidative environment and might have the additional benefit of keeping more of the carnosine present in the undigested meal available to be absorbed and provide further in vivo protective effects.

Overall, when the dietary components in a meal exerted their effects, a healthy meal including adequate amounts of plant-origin antioxidants and mildly cooked lean fresh

meat could efficiently provide a less oxidative environment, whereby the health-beneficial carnosine in meat could impart additional advantages to the healthy diet.

7.2 Influence of other dietary components and dietary carnosine

Despite the similarity of the two models based on burger meals, AA and fructose included in these two models did not show the same effects during digestion. In the burger meal with AA at a supplemental level, fructose at a dietary level generally showed a pro-oxidant effect as expected, and the antioxidant AA also usually acted as a pro-oxidant. The relatively lower level of AA in the burger meal combo mainly acted as an antioxidant and fructose in this burger meal combo showed beneficial effects. Altogether, these different effects of AA and fructose could contribute to the lower oxidation level of the burger meal combo, consistent with lower concentrations of lipid oxidation markers observed in the burger meal combo than in the burger meal model with AA at a supplemental level.

The enhancement of carnosine in these two burger meal models possessing different oxidation levels did not demonstrate the same effect. In the model of burger meal including AA at a supplemental level, enhancement of carnosine levels led to pro-oxidant results in the presence of either AA or fructose. A further increase in carnosine only partially decreased concentrations of two markers of lipid oxidation but was not efficient in significantly reducing the overall oxidation level of the environment compared to the control. In the model of a burger meal combo with beverage-simulating solutions, significantly reduced oxidation and glycation were observed in the carnosine-enriched

groups, irrespective of the presence of AA or fructose. When comparing these two models, intrinsic and enriched levels of carnosine in the first burger meal (including a supplemental level of AA) were lower than the level of carnosine in the control and enhancement groups in the model of a burger meal combo. On this basis, the overall pro-oxidant effect of carnosine observed at relatively low levels (in the first burger meal) and the antioxidant effect obtained at relatively high levels (in the burger meal combo) are close to a hormesis phenomenon, which usually describes a bi- or tri-phasic response to increasing exposure to a substance, generally characterized by a low-dose stimulation and a high-dose inhibition and graphically represented by an inverted U-shape or by a J- or U-shape dose-response curve. Dietary components such as essential vitamins and minerals have been reported to act as hormetic nutrients in vivo. A hormesis effect of carnosine has also been observed in vivo and studied at the cellular level and is attributed to its capacity to affect redox-sensitive protective genes called vitagenes. In the present study, however, the potential hormesis-like effect of carnosine was in the in vitro gastrointestinal tract, representing an extracellular occurrence that depends solely on the chemical properties of carnosine, whose performance in turn would depend on the redox potential of the environment. In these two burger-based meal models, the dual (pro- or antioxidant) effect of carnosine observed was likely concentration-related. Intermediate levels of carnosine were capable of sustaining the ferric-ferrous cycle through the ferric-reducing capability of carnosine, leading to increased free radical production, while higher levels of carnosine, such as in the model of a burger meal combo, were likely sufficient to reduce the overall oxidation through further enhanced ferric-reducing capability together with radical scavenging and other abilities. Nevertheless, the potential

dose-response curve of dietary carnosine observed herein, which might represent a hormesis effect of the molecule during digestion, warrants further validation and more studies.

Although differences in the oxidative environment and the concentration of carnosine may lead to different final results in studies of the effect of carnosine, the series of studies conducted in the current thesis generally confirmed the benefits of higher levels of carnosine containing in meat throughout a whole meal and emphasized the need for muscle carnosine enrichment in some types of meat, such as pork. In addition, the benefits of carnosine and health potential of carnosine-enriched meat could also be underlined by the capacity of carnosine to inhibit the formation of nitrosamine. Specifically, different levels of carnosine, which covered the range of possible carnosine levels found in meat and digests, reduced the formation of different types of nitrosamines. Given that carcinogenic nitrosamines are associated with health concerns of processed meat products, this ability of carnosine indicates the potential of dietary carnosine to lower the risks associated with the consumption of processed meat products, subsequently suggesting the need for carnosine-enriched meat in the production of these meat products.

7.3 Proposals for further research

a) Carnosine content in pork and animal feed

Different levels of intrinsic carnosine in meat were observed across studies conducted in this dissertation. Breed, nutrition, and carnosine measurement methods can all lead to

variations in the carnosine content of meat. Given that all pork used was from the same company and the carnosine determinations were made by validated methods, the observed differences in intrinsic carnosine levels in the present work could be related to the feeding practices.

Considering that carnosine-enriched meat is suggested to provide health benefits, adjustments in feeding can be an approach to enrich the carnosine content of meat. However, the popular feeding diets nowadays primarily prioritize sustainability and productivity, paying relatively less attention to others, including carnosine biosynthesis. For instance, the popular low-protein (crude) diet in the swine industry leads to a low level of L-histidine, which is necessary for carnosine biosynthesis. This low level of L-histidine could be attributed to its involvement in multiple physiological roles, such as wound healing and inflammation-related processes. Therefore, there is an opportunity to increase the intrinsic level of carnosine by including more L-histidine in the current animal feeding practices. However, there is still limited information in this regard, and more studies are required.

b) Proposed mechanism of the observed effect of dietary components

A hypothetical but likely explanation was provided for the pro-oxidant interaction between carnosine and ascorbic acid. It was based on the capacity to convert ferric to ferrous ions and on their respective contributions to the Fenton-like reaction in the presence of hydrogen peroxide in the in vitro gastrointestinal tract. This ferric-reducing capacity of carnosine has been documented, but the exact mechanism is not well

understood. For instance, whether carnosine, like AA, directly reduces ferric ions to ferrous ions and is oxidized itself in the process or acts through a different mechanism, such as binding to ferric ions and then releasing ferrous ions after intramolecular electron transfer, remains unclear.

In the healthy meal model containing AA and other antioxidants, the pro-oxidative potential of carnosine could be observed in the formation of MDA (a lipid oxidation product) at an intermediate level of carnosine. This occurred without any changes in hydrogen peroxide levels or ferric-reducing capacity. These observations in the healthy meal study suggest that, in addition to the hypothesis that ferric-reducing capacity contributes to the increase in oxidation, other mechanisms could be involved. For example, the pro-oxidative effect of phenolic compounds can also be related to the generation of phenoxyl radicals and superoxide anion through autoxidation, which is unrelated to ferric-reducing ability. Similarly, little information is available on the observed effects of fructose. Fructose, as a reducing sugar, would be expected to contribute only to oxidation and glycation but instead showed a reducing potential against lipid oxidation. To date, the influence of fructose reported in vitro and in vivo is debatable, with no detailed mechanisms available. Future research on these aspects is recommended.

Nitrites also have an antioxidant capacity that can be associated with radical-related reactions, which suggests the possibility that nitrites and carnosine can synergistically reduce oxidation and impart additional benefits during the digestion of meals. On the

other hand, carnosine's role in suppressing the nitrosamine formation induced by nitrites would decrease the availability of carnosine for its antioxidant, anti-glycation, and other beneficial functions. To evaluate the effect of carnosine in the combined presence of nitrites and to study the application of dietary carnosine and even better utilization of nitrites in practical cases, additional detailed investigations would need to be aimed at (i) detailed mechanistic understanding of the suppression of nitrosamine formation by carnosine, (ii) identifying the priority of various reactions in which carnosine can participate, and (iii) direct assessments in meat products and systems closer to daily diets, including digestion processes.

Although only a few significant differences in protein oxidation were found during digestion of meal systems in the present thesis, there is still some information on free thiols requiring further research. The level of free thiols is a widely used marker for protein oxidation because of their susceptibility to modification during the oxidation process. However, detailed mechanisms of how antioxidants, such as carnosine, directly exert their protective effects on free thiols are not fully elucidated. Furthermore, when more dietary components are included in a diet, more interactions may need to be considered. For instance, the peroxynitrite generated from nitrites can attack sulfhydryl groups and is reported to oxidize cysteine thiol groups. Overall, protein oxidation and potential antioxidant mechanisms to protect protein during digestion may need further research.

c) Potential effect of cooking methods and the manufacturing processes of meat products

In the current dissertation, the meal models studied thus far were exclusively based on fresh meat cooked in a water bath, in which cooking intensity is controlled by only two parameters, namely, time and temperature. However, apart from the fat content in meat and cooking intensity considered in the thesis, other sources of variability related to various cooking methods and manufacturing processes of meat products may bring about many changes. For instance, frying processes can introduce oils into the system, thereby contributing to lipid oxidation in a manner that depends on the lipid profile of the frying oil. At the same time, the cooking process can also bring antioxidants into the system, for example, antioxidants from spices during the seasoning step, such as curcumin from turmeric and lutein from basil. In addition, the processing of certain meat products can provide antioxidants, such as phenolic compounds generated by wood smoking. These antioxidants can be transferred to the meat products and later synergistically contribute to the decrease in oxidation with other health-beneficial compounds. Simultaneously, the cooking and manufacturing processes also promote various reactions, including oxidation, nitrosation, and Maillard reactions, and generate more hazardous molecules, such as ROS, nitrosating agents, α, β-aldehydes, and heterocyclic amines, which may consume some of the beneficial compounds. Overall, the potential conflicting effects of these food ingredients and process-generated compounds involved in cooking and manufacturing need to be considered and included in further studies.

d) Potential differences between in vitro and in vivo models

Although in vitro models can be valuable alternatives to in vivo ones, particularly for studies on lipid oxidation, aspects not covered by the in vitro studies, such as iron absorption, may have relevance for the effect of dietary carnosine. Iron is an important meat-related nutritional compound, and some meat components, such as cysteine and histidine-containing peptides, can facilitate iron absorption. On this basis, carnosine, an inherent muscle histidine-containing dipeptide, may also be associated with iron absorption. Because iron is a vital catalyzing factor for these reactions, the changes in iron absorption could affect lipid and protein oxidation and nitrosamine formation during digestion. Unfortunately, the current dissertation only includes the potential bioaccessibility of carnosine during in vitro digestion of different meals. Further investigations are needed to assess the absorption of iron under different carnosine treatments and potential changes in related reactions, such as iron-induced lipid oxidation. Additionally, bioaccessibility and absorption related to other beneficial food ingredients are also worthy of more attention. Overall, information on these aspects of carnosine and other health-beneficial components could contribute to providing better diet guidance on meat consumption as part of a daily diet.

7.4 Conclusion

The overall health impact of dietary carnosine together with other functional molecules is not simply additive. Concentrations and redox potentials of all bioactive compounds can contribute to the final health outcome. Therefore, properties of dietary carnosine and other health-beneficial compounds found in the diet require assessment under different

environmental conditions so that the expected benefits of carnosine and other beneficial compounds can be achieved.

This thesis is the first-ever report of the nitrosamine-decreasing ability of carnosine, the first-ever report of the beneficial and bioactive effects of dietary carnosine imparted during digestion of different meals, and the first-ever report of the different interactive effects that carnosine can have in the presence of other dietary redox components. Overall, the functional role of carnosine as an exclusive muscle food constituent during digestion of different meal models was demonstrated and shown to be dependent on its concentrations and the interplay with other dietary components. By elucidating mechanisms and conditions leading to consistent beneficial properties of carnosine, further studies on dietary carnosine could contribute to mitigating health concerns associated with meat consumption and even providing additional health benefits to meat-containing healthy meals.