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CICLO XXXV

THE OTHER FACE OF NUTRITION: DIETARY COMPOUNDS AT THE CROSSROAD BETWEEN RISK AND SAFETY

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"If you want something you have never had,

you must be willing to do something you have never done"

Thomas Jefferson

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Abstract

È noto che un'alimentazione sana ed equilibrata è un fattore fondamentale per prevenire le malattie croniche legate all'invecchiamento. La dieta mediterranea, caratterizzata da un alto consumo di verdure, cereali e legumi e povera di grassi saturi e zuccheri, viene spesso consigliata per ridurre il rischio di patologie cardiovascolari e metaboliche. Quando i prodotti alimentari vengono preparati o consumati, spesso vengono sottoposti a trattamenti termici che provocano reazioni chimiche tra i componenti alimentari, modificandone le proprietà e riducendo la presenza di microrganismi dannosi. Tuttavia, tali trattamenti possono portare alla formazione di composti indesiderati con un impatto negativo sulla salute umana e un ridotto valore nutrizionale. Tra questi composti troviamo l'acrilamide, l'idrossimetilfurfurale, i composti α-dicarbonilici come il metilgliossale (MGO) e i prodotti di glicazione avanzata (AGEs). Diversi studi hanno infatti collegato l'accumulo di questi composti a diverse malattie croniche come diabete, aterosclerosi, cancro e malattie neurodegenerative, ma ulteriori ricerche sono necessarie per comprendere gli effetti di questi composti sulla salute, nonché i loro livelli negli alimenti e il loro potenziale rischio. Sulla base di queste premesse, per il mio progetto di dottorato ho portato avanti due linee di ricerca differenti ma complementari. In primo luogo, volevamo capire se una dieta ricca di AGEs fosse in grado di indurre alterazioni a livello cerebrale e instaurare un ambiente che predisponesse al deterioramento cerebrale legato all'età. A tal fine, topi anziani sono stati trattati con alti dosaggi di MGO, un composto altamente reattivo e uno dei principali precursori degli AGEs. Questi esperimenti hanno dimostrano che il trattamento orale con MGO (100 mg/kg) per 4 settimane ha causato un deterioramento cognitivo e ha indotto alterazioni biochimiche a livello ippocampale che possono predisporre a condizioni patologiche simili alla malattia di Alzheimer. Questi risultati suggeriscono che un'elevata assunzione di MGO potrebbe favorire la creazione di un ambiente dannoso, riducendo la capacità di risposte adattative necessarie per preservare l'integrità del cervello. In questo quadro, diventa fondamentale valutare l'esatto contenuto di questi composti nocivi negli alimenti per valutarne la sicurezza. Di conseguenza, per la seconda parte del mio progetto di dottorato, mi sono concentrata sull'identificazione e quantificazione di diversi composti derivanti dal processamento alimentare in diverse matrici alimentari. Recentemente si è registrata una tendenza crescente nel consumo e nella richiesta di prodotti a base vegetale, a causa di una trasformazione globale verso un sistema alimentare più

sostenibile. Anche gli alimenti a base vegetale vengono lavorati con diverse tecniche durante la loro produzione, dalla raccolta delle piante fino al confezionamento, eppure la conoscenza di come e in che misura avviene la formazione di prodotti potenzialmente tossici è ancora piuttosto limitata. Pertanto, per lo studio sono state analizzate diverse marche di carne e prodotti lattiero-caseari a base vegetale utilizzando tecniche chimico-analitiche avanzate. È interessante notare che specifici composti potenzialmente dannosi sono stati identificati in diversi alimenti di origine vegetale. Ulteriori ricerche devono essere condotte in questa direzione, al fine di identificare nuove strategie per diminuire la formazione di tali composti negli alimenti a base vegetale, con l'obiettivo finale di stabilire regole che possano essere applicate durante i processi di produzione allo scopo di ottenere alimenti con elevati standard di sicurezza. In sintesi, il mio progetto di dottorato ha voluto porre le basi per un approfondimento sistematico su aspetti dell'alimentazione ancora poco noti il cui studio deve essere approfondito nel contesto di un intervento nutrizionale per la prevenzione delle malattie croniche.

Introduction

"Let thy food be thy medicine and
thy medicine be thy food"

Hippocrates, 460 BC

No existing evidence showed that Hippocrates literally stated the above quotation. Nonetheless, whether his or someone else's, this philosophy has been recently reevaluated in light of the complementarity between nutrition and pharmacology that is nowadays recognized as extremely valuable [1]. From the earliest stages of our life to adulthood and old age, proper food and good nutrition are considered fundamental for many health aspects like physical growth, mental development, performance, productivity, and general well-being [2]. During the last decades, nutrition has also become increasingly popular as a therapeutic strategy to promote longevity and to prevent disease onset [3]. Recent research has emphasized the therapeutic benefits of lifestyle changes and gained valuable understanding of the intricate and changing processes that lead from good health to pathological state. In this frame, a healthy diet and dietary bioactive compounds are considered valuable supports in non-communicable diseases such as cognitive impairment [4], [5], diabetes [6], cardiovascular diseases [7] and cancer[8], among others. But what does make a diet healthy? The definition of what constitutes a healthy diet is constantly changing as new insights on the roles played by essential nutrients and other food components in health and disease are continuously emerging [9]. However, if we want to identify a proper definition, it could be well-summarized with the following words: "a healthy diet is health-promoting and disease-preventing. It provides adequacy without excess of nutrients and health-promoting substances from nutritious foods and avoids the consumption of health-harming substances" [10]. Different approaches have been identified throughout the years to translate the definition of a healthy diet into specific food-based recommendations. One approach consists of the observation and consequent identification of specific dietary patterns associated with a lower incidence of specific diseases. A one-widely famous example is represented by the Mediterranean diet, which healthy nutritional model comprises mostly olive oil, nuts legumes, vegetables, whole grain, fresh and dried fruit, a moderate amount of fish, as well as dairy, meat, and red wine

[11]. This dietary pattern has been the object of many studies as it showed both a remarkable nutritional profile and a sustainable ƒmodel of food production and consumption. However, employing the Mediterranean diet in non-Mediterranean countries represents a challenge. Recently, another valuable approach is to promote a healthy and sustainable dietary model based on the nutritional properties of the Mediterranean Diet but implemented using food products available locally in different world areas. This "Planeterranean" approach takes into account cultural contexts, local foods, dietary customs but also meets the principles and the great demand of a sustainable and circular economy [12].

Based on the opposite principles of the two above-mentioned dietary patterns, Western diet represents the dominant type of dietary pattern in most developed countries. Western diet is characterized by high consumption of high-processed food such as processed and red meat, desserts and sweets, fried foods, high-fat dairy products, refined grains and low consumption of vegetables, fruits, whole grains, and legumes (Figure 1).

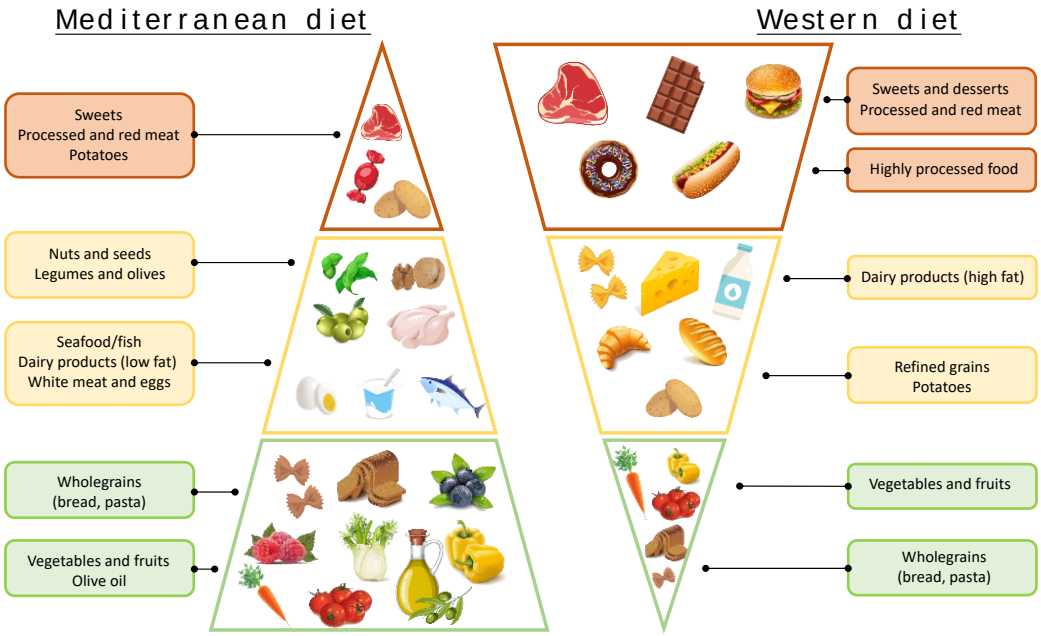

Figure 1. Characteristics of Mediterranean and Western diet. Figure re-designed from Zielinska *et al. 2022*.

Although many aspects of the beneficial or detrimental effects of dietary patterns on health have been widely explored, the effects that processing mechanisms or cooking styles have on food still need to be investigated in more detail. Foods are mixtures of chemical compounds like protein, amino acids, carbohydrates, lipids, vitamins, and minerals usually with water as a solvent. When food products are produced or consumed, they are heated either in households and/or industry. This kind of treatment triggers specific chemical reactions between food components, resulting into desired sensory attributes of food products such as flavour, and colour and also into the reduction of the number of pathogenic microorganisms present in the food. However, on the other side of the coin, unwanted compounds that might affect human health and bring food nutritive losses may be generated as well. In this context, it remains largely unclear what is the effect of these processing compounds on human health as well as their specific content in food and the associated risk and safety.

In this regard, for my PhD project, two different but complementary research lines were investigated:

- ***Chapter 1*** *(in vivo study):* evaluation of the effects of prolonged administration of high dosages of a reactive processing compound called methylglyoxal in aged mice.
- ***Chapter 2*** *(chemical-analytical study)*: identification and quantification of different food processing compounds developed on various food matrices.

Chapter 1

1. Methylglyoxal and its adducts

First recognized in 1913 as a vital component of glucose metabolism in animals, plants, and microorganisms, Methylglyoxal (MGO) is a reactive α-dicarbonyl compound characterized by a yellow colour and a pungent odor [13]. The formation of MGO can occur endogenously as well as in foods through different mechanisms [14]. Via the Maillard reaction, MGO can be transformed into aroma compounds and provide good flavours to food, which are the most important property in consumer acceptability [15]. At the same time, MGO in foods generates other food toxins, especially advanced glycation end products (AGEs), which have been linked to different pathological states [16]. Consequently, restrictions on dietary MGO and MGO-derived AGEs intake could represent an effective strategy to reduce the health risk associated with these compounds [17]. However, a great debate still exists regarding the risk posed by dietary MGO and MGO-derived AGEs. On one side, some scientists support the fact that they do not represent a threat to health. These hypotheses have mostly to do with the stability of these compounds, their availability after food intake and their concentration in food which was claimed to be not harmful [18]. On the other hand, some results have highlighted their potentially detrimental effects, especially in those patients who already suffered from chronic diseases, such as diabetes, renal failure, overweight or obesity [19]–[21].
From this point of view, studying the role played by one of the main toxic precursors of AGEs such MGO has attracted a lot of attention lately and has driven our force to study more in deep this compound as well as its effect in *in vivo* models.

For this reason, in the next sections, I will focus on describing how MGO is formed, how it is catabolized, and its potentially toxic effects. I will also thoroughly analyze the potential role played by MGO as well as by MGO-derived adducts in several chronic diseases. Next, the aim of our *in vivo* study and our findings will be covered.

1.1 MGO production

1.1.1 Exogenous MGO production

Exogenous sources of MGO include cigarette smoke, food, and beverages [22]. MGO was initially found in coffee, with levels ranging from 1.6 to 7.0 mg/L but it can also be found in fermented products like wine (0.1 to 2.9 mg/L), yogurt (0.6 to 1.3 mg/kg), and soy sauce (8.7 mg/kg) [23]–[25]. Comparable levels of MGO were found in various foodstuffs, mainly in sugar-rich and intense heat-treated products such as balsamic vinegar and soy sauce as well as cookies and jams and at very low levels in milk products (<1.6 mg/L) [26], [27]. Interestingly, MGO, identified as the compound responsible for the pronounced antibacterial activity of manuka honey, is present in exceptionally high levels in this specific kind of honey ranging from 40 to 760 mg/kg [28], [29].

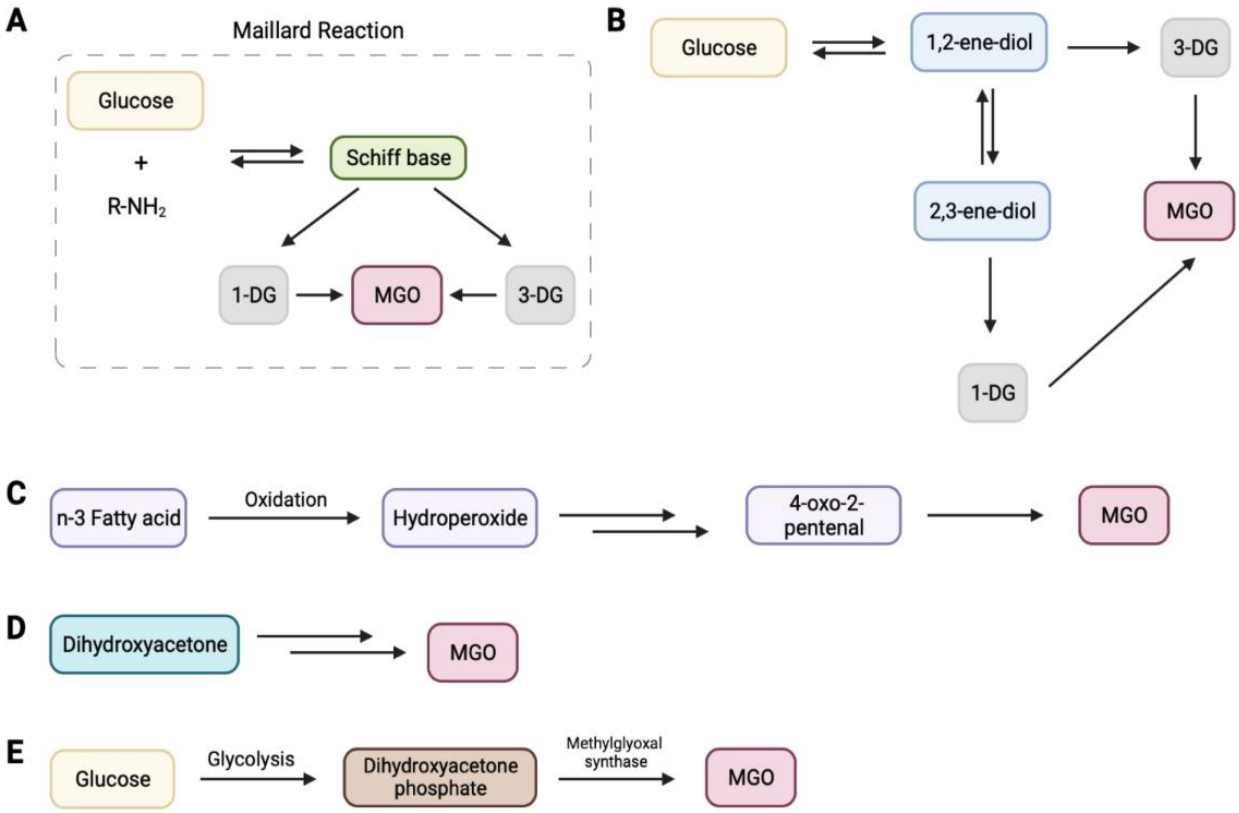

Figure 2. Methylglyoxal formation pathways in foods. (1-DG: 1-deoxyglucosone, 3-DG: 3-deoxyglucosone). Figure adapted from Zheng *et al.* 2021.

In food, the formation of MGO is influenced by processing technology and food formulations and can occur through five main pathways (Figure 2).

The leading pathway is the Maillard Reaction (Figure 2A), which takes place during food storage at room temperature and during heat treatment. The chemical reactions involved in Maillard Reaction will be covered in deep in section 4.2. Briefly, for the formation of MGO, this reaction involves hexoses (i.e. glucose and fructose) reacting with amino acids to produce Schiff bases, which are transformed into Amadori products. These Amadori products then undergo enolization, deamination, and dehydration to form 1- and 3-deoxyglucosones, which can both be converted into MGO [30], [31]. The second pathway is the autoxidation of hexoses (Figure 2B). The isomerization and subsequent fragmentation of sugars by retro-aldol condensation lead to MGO [15], [32]. This process occurs greatly in food with high content of hexoses, such as honey [15].

The third pathway involves the oxidation of unsaturated fatty acids in lipids [31] (Figure 2C) This comprises a chain reaction of unsaturated fatty acids resulting in dihydroperoxide, which is cleaved to form 6-hydroperoxy-2,4-heptadienal and 4-oxo-2-pentenal. 4-oxo-2-pentenal directly generates MGO [33], leading to high MGO levels in foods high in polyunsaturated fatty acids such as fish oils [22]. The fourth pathway is the dehydration of dihydroxyacetone (Figure 2D), a compound specifically present in Manuka honey [28]. Heat treatment leads to high levels of MGO production in commercial Manuka honey products [29]. This MGO pathway formation does not represent a problem in foods such as baked and fried goods because dihydroxyacetone has not been detected in these kinds of products. However, the addition of Manuka honey in such foods may be of concern for the high MGO content that will occur after the heat processing steps. The final route is the conversion of dihydroxyacetone phosphate (Figure 2E) to MGO through microbial metabolism. This intermediate is formed during glycolysis and can be transformed to MGO through the action of methylglyoxal synthase [34]. As a result, MGO can also be found in fermented beverages and foods.

Since MGO formation widely occur in many foods and beverages, its effects on human health have attracted a lot of interest. *In vivo*, the administration of elevated doses of MGO through drinking water was associated with specific pathophysiological changes. For example, Sena *et al.* have pointed out that a prolonged intake of MGO through drinking water induced endothelial dysfunction in normal Wistar rats and exacerbated the endothelial impairment observed in diabetic GK rats by a pathway that involved oxidative stress and decreased NO bioavailability in addition to increased glycation and inflammation [35]. In a study of Hofmann

et al., the consumption of AGE- and MGO-enriched diets caused in diabetic *db/db* mice glucose and insulin intolerance [36]. One criticism that requires clarification is to which extent exogenous MGO may be absorbed into the circulation and exert its effects. The data on this issue are still conflicting [37]. Some results showed that short-chain α-dicarbonyl compounds such as MGO are scavenged during digestion and consequently could not be absorbed [18]. However, in a recent study conducted on a large number of individuals, an increase of MGO was observed in human plasma samples after higher habitual intake of dietary MGO, therefore suggesting its absorption [38]. These contradictory data, together with the observation that MGO showed cytotoxic properties also in *in vitro* experiments [39], [40], highlight the importance of further investigating the metabolic transit, absorption and the effects of dietary MGO on human health.

1.1.2 Endogenous MGO production

MGO can also be produced endogenously as a byproduct of glucose, protein, lipid and ketone metabolisms as well as from lipid peroxidation or ketone bodies' oxidation (Figure 3). Therefore, the exogenous MGO introduced with the diet could potentially contribute to the endogenous pool of MGO [19].

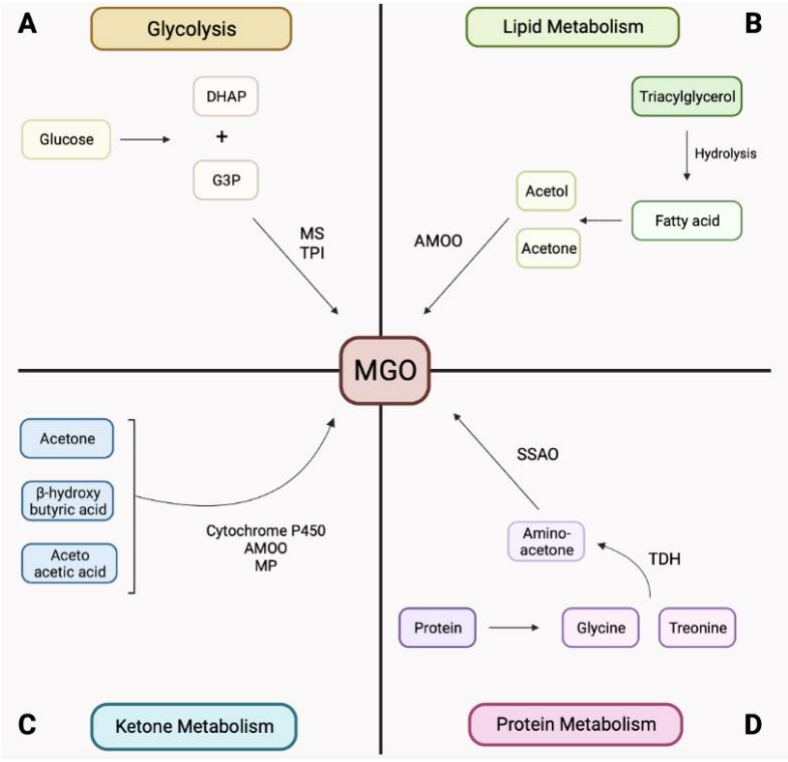

Figure 3. MGO production from metabolic pathways: (A) glycolysis, (B), lipid metabolism, (C) ketone metabolism and (D) protein metabolism. Figure re-design from Lai *et al*. 2022.

Glycolysis is the predominant pathway by which endogenous MGO formation occurs. During glycolysis, MGO can be produced after the degradation of two triosephosphates: dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) [41] (Figure 3A). The reaction could happen both in the presence and absence of enzymes. In the first case, the loss of the α-carbonyl group and phosphate causes the non-enzymatic breakdown of G3P and DHAP and the formation of MGO. Otherwise, MGO synthase (MS) and triose phosphate isomerase (TPI) can catalyze the enzymatic conversion of G3P and DHAP into MGO [42]. MGO can also derive from protein metabolism through the formation of aminoacetone (Figure 3D). During protein metabolism, amino acids such as glycine and threonine are metabolized by threonine dehydrogenase (TDH) into aminoacetone, which could be then converted to MGO through semicarbazide-sensitive amine oxidase (SSAO) [43]. In lipid metabolism, triacylglycerol undergoes hydrolysis to form fatty acids, which in turn are converted first into acetol and acetone and then into MGO through acetol monooxygenase (AMOO) [44] (Figure 3B). MGO can also derive from lipid peroxidation. In lipid peroxidation, oxidants such as free radicals and Reactive Oxygen Species (ROS) form peroxyl radicals and hydroperoxides by attacking unsaturated fatty acids [45]. These compounds can in turn be fragmented and produce different kinds of α-DCs, including MGO. Also, ketones (e.g., acetone, β-hydroxybutyric acid and acetoacetic acid) can be converted to MGO by different enzymes such as cytochrome P450, AMOO and myeloperoxidase [46], [47](Figure 3C). Ketone oxidation represents another route for the formation of MGO, but this mainly occurs in pathological conditions such as ketosis and diabetic ketoacidosis [48].

In the literature, a wide range of plasma concentrations was identified for MGO, going from about 100 nM to 400 μM [49]. This great variability could reflect the sample processing and the different analytical techniques employed for its measurement. For example, during sample processing, MGO formation may occur by degradation of monosaccharides, glycated proteins, glycolytic intermediates or other samples components, as well as by exposing samples to several parameters such as heating, high pH, oxidizing condition and peroxidase activity. Taken together these factors can lead to an overestimation of MGO content in samples. Nowadays, the most-widely used technique for MGO detection is ultra-performance liquid chromatography–tandem mass-spectrometry (UPLC-MS/MS) [50]. The use of this technique along with avoiding the above-mentioned conditions has allowed the estimation of MGO concentrations in both plasma and tissue of healthy adults to be of about 50–150 nM and 1–4 μM, respectively. In case of a pathological state, increased values of MGO can be measured.

For example, in patients with type 2 diabetes, MGO plasma concentrations increase of about 2-fold [51] whereas patients with renal disease showed 3-fold higher MGO plasma levels, which further increase with the progression of the disease [52], [53]. In order to counteract the accumulation of MGO, different detoxifying mechanisms are employed by the cells such as the glyoxalase, aldose reductase and aldehyde dehydrogenase pathways that represent the system for MGO scavenging.

1.2 MGO scavenging

1.2.1 Glyoxalase system

The glyoxalase gene is highly conserved in the tree of life and is found in various living systems, such as humans, plants, yeast, bacteria and fungi. This widespread distribution directly denotes the crucial physiological functions exerted by glyoxalase enzymes in eukaryotic and prokaryotic cells [54].

The glyoxalase pathway represents the main system involved in MGO detoxification, which involves the activities of two enzymes: glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2). The nonenzymatic reaction between MGO and glutathione (GSH) leads to the formation of the methylglyoxal-glutathione hemithioacetal (MGO-GSH), which in turn is recognized by GLO1 and converted into S-D-lactoylglutathione. This intermediate is then converted by GLO2 into D-lactate, with GSH as a byproduct (Figure 4) [55].

The availability of GSH as well as the recognition of the hemithioacetal from GLO1 are two essential and limiting steps for MGO detoxification [56], [57]. Dysfunctions of GLO1, which results in an accumulation of MGO and MGO-derived AGEs, have been correlated with pathological conditions such as diabetes, cardiovascular disease, neurodegenerative disorders and psychiatric disorders [58]. In particular, hyperglycemic episodes and therefore diabetes-related late complications have been frequently associated with a lower GLO1 expression and consequently with the accumulation of MGO [13], [49], [50]. Likewise, Brouwers et al. demonstrated that the overexpression of GLO1 in *in vivo* model of diabetic rats can reduce hyperglycemic-induced levels of carbonyl stress, AGEs levels and oxidative stress [59]. Low expression and activity of GLO1 have also been found in ruptured plaques, leaving cells in atherosclerotic plaques more susceptible to AGEs accumulation [60]. Furthermore, decreased levels of GLO1 have been shown to naturally occur during ageing and during later stages of

Alzheimer's disease, being one the reason why MGO-induced neuronal impairment, apoptosis and AGEs formation in plaques and tangles occur [61]. Recently, consistent results have also pointed out the connection between GLO1 and anxiety-related behaviour. In this regard, MGO was shown to act as a $GABA_A$ receptor agonist and increased GLO1 activity was therefore linked to an increase in anxiety by its ability to detoxify MGO [62].

1.2.2 Oxidation and reduction breakdown

Besides the glyoxalase system, other minor routes are implicated in MGO metabolism, involving both oxidation and reduction reactions (Figure 4). MGO has two functional groups, which can be either oxidized or reduced; these reactions are catalyzed by different dehydrogenase and aldo-keto reductase enzymes, respectively [63]. MGO can be oxidized to pyruvate by two enzymes: i) aldehyde dehydrogenases, a NAD-dependent enzyme, and ii) 2-oxoaldehyde dehydrogenase, a NADP-dependent enzyme. Aldose reductase (AR) is instead involved in catalyzing MGO reduction and consequent breakdown in two different pathways: i) by converting with NADPH the MG-GSH, formed between the nonenzymatic reaction with GSH and MGO, to a lactaldehyde (GSH dependent pathway) and ii) by direct reaction with MGO and NADPH to form acetol (GSH independent pathway). Compounds obtained by either reducing pathways are then converted into propanediol. AR demonstrated to be more efficient in MGO detoxification than aldehyde dehydrogenases because it shows a higher selectivity and preference toward MGO [64]–[66]. In addition, in GLO1 knockout Schwann cells, the inhibition of AR increases intracellular MGO levels suggesting that AR-mediated detoxification of MG represents an important compensatory mechanism if the glyoxalase system is impaired [67]. This can have potential clinical turnouts with regard to those diseases characterized by an impaired glyoxalase system and elevated levels of MGO [68].

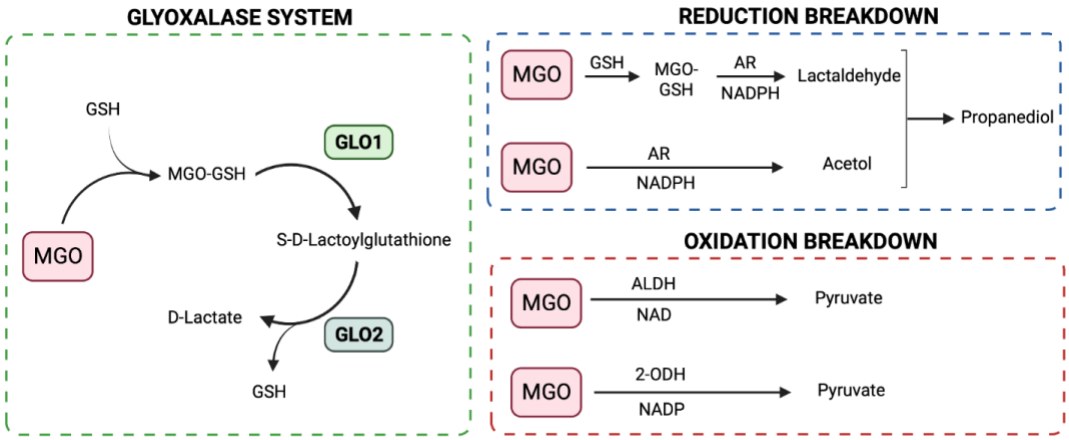

Figure 4. Pathways involved in MGO scavenging.

1.3 MGO and MGO-derived AGEs mechanisms of action

MGO can exert its effects through direct and indirect mechanisms of action. With regards to its direct effects, MGO can induce oxidative stress, inflammation and cell apoptosis in different cell types. Indeed, MGO is a potent inducer of oxidative stress because it can stimulate the formation of superoxide, hydrogen peroxide, and peroxynitrite, and it is also responsible for the decrease of antioxidants and the modulation of their activity [13]. Besides, in hippocampal neural cells, MGO has shown to cause a significant increase in the expression and release of the pro-inflammatory cytokine IL-1β, thus participating in inflammation [69]. Moreover, MGO promotes apoptosis by increasing the Bax/Bcl-2 ratio and activating caspase-9 and caspase-3, that in turn causes the release of cytochrome c from the mitochondria to the cytosol, triggering mitochondrial apoptosis pathways [70]. Besides these direct effects, MGO can act through indirect effects thanks to its ability as a potent glycating agent. Protein and DNA represent the two main targets of MGO, resulting in the formation of MGO-derived AGEs and MGO-derived DNA adducts. This modification leads to important alterations in protein and DNA structure and consequently serious changes in their function. In addition, MGO-derived AGEs, both in their free and protein-bound form, can act as ligands of AGEs receptor, activating several downstream intracellular pathways [45], [71].

1.3.1 Alteration of DNA and protein structure

The high reactivity of MGO towards nucleophiles drives the formation of covalent adducts. The formation of these adducts occurs mainly with DNA and proteins resulting in extended modification of their functions. Adducts formation depends on different factors, including MGO concentration, time of exposure to MGO, intrinsic reactivity of the protein or DNA, and chemical stability of the formed adduct [72]. The most stable MGO-DNA adducts observed in biological matrices are the R- and S-diastereomers of N^2-(1-carboxyethyl)-2'-deoxyguanosine (CEdG), but 1,N^2-(1,2-dihydroxy-2- methyl)ethano-2'- deoxyguanosine (cMG-dG) can also be formed [73]. Such DNA adducts are mutagenic, can induce genomic instability and affect transcription. Besides DNA, MGO can also bind to amino acids resulting in protein adducts with altered structure and function. Different studies demonstrated that modification of albumin by MGO can cause an increase in the release of TNF-α [74] and IL-1β [75] as well as a dramatic impact on anti-oxidant capacity and physicochemical properties of albumin [76]. Collagen can

also be modified by MGO. This modification was linked to: i) an increase in the formation and migration of myofibroblast, which are crucial processes involved in fibrosis development in diabetes [77]; ii) an alteration of integrin binding region which in turn, impairs collagen binding, required for phagocytosis of collagen fibers [78]; iii) an impairment in endothelial cell survival and angiogenesis, likely contributing to vascular dysfunction [79]. Recently, MGO was found to target histones, proteins; Galligan *et al.* demonstrated that MGO glycation of histone H2A led to adduct formation, altered DNA binding, and changes in histone posttranslational modification, chromatin architecture, and gene expression [80]. According to these results, Zheng *et al.* showed that histone glycation disrupts the assembly, stability and compaction of chromatin both *in vitro* and *in cellulo* models [81].

1.3.2 AGEs receptors activation

One of the main mechanisms by which MGO-derived AGEs, free or protein-bound, affect cell function is by binding and activating the receptor for AGEs, called RAGE. RAGE is a member of the immunoglobulin superfamily of cell surface receptors that acts as a pattern recognition receptor. Under physiological conditions, RAGE's expression was found to be low in all tissues but lung, which instead exhibited very high levels of RAGE. In pathophysiological settings - such as diabetes, chronic inflammation, or neurodegenerative disorders – the expression of RAGE increases in various areas, such as in the vasculature, in the hematopoietic compartment, or in the CNS [82]. This multiligand receptor is characterized by three Ig-like domains, one variable (V type) domain and two constant (C type) domains (C1 and C2) in the extracellular part, a transmembrane domain, and a short-cytoplasmic tail [83]. Apart from AGEs, RAGE can interact with multiple ligands having AGEs common motifs such as S100 calcium-binding protein, DNA-binding proteins like high mobility group box-1 (HMGB1), amyloid-β peptide, lipopolysaccharides and phosphatidylserine. The engagement of RAGE by its ligands activates intracellular signaling cascades that lead to the activation of the proinflammatory transcription factor NF-κB. In physiological conditions and in resting cells, NFκB is bound to its protein inhibitor IκBα. When the binding between RAGE and its ligand occurs, IκBα is phosphorylated and degraded. The free NFκB can translocate into the nucleus to increase the expression of target genes, such as cytokines, adhesion molecules, prothrombotic and vasoconstrictive gene products [84]. NF-κB can also induce RAGE expression and the prolonged activation of NF-κB causes an amplification of RAGE activity. Therefore, even if RAGE, even if is expressed at low levels in many tissues, it tends to accumulate in those areas where its ligands are more present

[85]. The binding of ligands to RAGE activates other cellular signaling cascades. For example, the activation of ERK1/2 (p44/p42) MAP kinases was shown to occur in different types of cells, as well as the activation of p38 and SAPK/JNK MAP kinases [86], [87]. Furthermore, also rho-GTPases, phosphatidylinositol-3-kinase, and the JAK/STAT pathway have been implicated in RAGE signaling [88]. In addition, RAGE–ligand interactions may directly induce the generation of ROS via NADPH oxidases [89]. Many of the above-mentioned signaling pathways result in the activation of the downstream effector NF-κB. The multitude of pathways involved in RAGE signaling mirrors the involvement of different RAGE ligands as well as the different cell types that express RAGE, further highlighting the complexity of the RAGE network [90].

RAGE is usually present in two primary forms: as a full-length membrane-bound receptor (flRAGE or mRAGE) and as soluble forms (sRAGE), which lack of the transmembrane domain [91]. Two main mechanisms are involved in the generation of sRAGE. The first one includes the cleavage of total extracellular RAGE by the action of matrix metalloproteinases (MMPs) and A-disintegrin and metalloprotease (ADAM)-10, leading to a RAGE form known as cleaved RAGE (cRAGE). The second one involves alternative mRNA splicing resulting in multiple variants among which endogenous secretory RAGE (esRAGE), is the primary secreted soluble isoform. Soluble forms of RAGE have been generally described as protective forms as they can mitigate the deleterious effects that instead are triggered by the activation of the full-length receptor [92]. Indeed the expression of sRAGE has been found to be decreased in some pathological conditions such as atherosclerosis, coronary artery disease, essential hypertension, rheumatoid arthritis and Alzheimer's disease in individuals without diabetes [93]–[95]. Another receptor involved in counteracting the detrimental effects of AGEs is Advanced Glycation Endproduct Receptor 1 (AGER1). AGER1 can ease AGE turnover by mediating AGEs uptake, degradation and removal and is critically involved in cell survival and ROS regulation [96]. The sequestration and degradation of AGEs by AGER1 prevent AGEs accumulation in the cytoplasm and in the extracellular milieu, blocking the production of ROS as well as new AGEs [96]. The protection offered by AGER1 may result from its long extracellular tail's high-affinity AGE-binding domain that blocks other AGE cell interactions and reduces the likelihood of ROS formation. Data have shown that AGER1 expression levels correlate positively with the levels of other intracellular anti-oxidant mechanisms (SIRT1, NAMPT, SOD2, GSH) and negatively with pro-oxidant pathways (i.e., RAGE, NADPH oxidase, $p66^{shc}$). Thus, considering the important role played by AGER1 in the maintenance of normal AGE homeostasis, reduced AGER1 expression levels may highlight an impairment in host defenses [97].

1.4 MGO- and MGO-derived AGEs in diseases

MGO and MGO-derived AGEs are associated with the pathogenesis of numerous diseases including diabetes, cardiovascular disease, neurodegeneration, and cancer. Here in this section, the relevance of MGO and MGO-derived AGEs in different human diseases will be discussed.

1.4.1 Diabetes mellitus

Over the years, the occurrence of diabetes has globally increased and it is classified as one of the leading causes of high mortality and morbidity rate [98]. Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by insulin deficiency and consequent hyperglycemia [99]. While the prevalence of T1D is circa 10% of all cases of diabetes, type 2 diabetes (T2D) represents the most common type of diabetes, characterized by a defective insulin secretion by pancreatic β-cells and the inability of insulin-sensitive tissue to properly respond to insulin [100]. The discoveries that high glucose concentrations result in MGO formation and that both TD1 and TD2 patients have higher plasma levels of MGO and MGO-derived AGES were the two main driving factors that led to further investigate the role played by MGO in diabetes and diabetes complications. In particular, MGO can contribute to the pathogenesis of diabetes by i) direct effect on β-cells, ii) functional modifications to the insulin molecule and/or iii) interferences in insulin signalling [101]. In particular, it has been shown that adding high concentrations of MGO to β-cells cause an impairment in the release of insulin. This modulation occurs only at high concentrations of glucose while at physiological glucose concentration, MGO induces a depolarization of pancreatic β-cells resulting in the release of Ca^{2+} and a modest and transient secretion of insulin [102]. Moreover, MGO has shown to be able to glycate insulin by attaching the Arg residue in its β-chain, leading to changes in its structure and function. These changes are indeed responsible for altered insulin-mediated glucose uptake, impaired insulin release from pancreatic β-cells and reduced hepatic clearance of insulin from liver cells [103]. In addition, MGO has shown to modify the activity of the insulin-signalling pathway in skeletal muscle. In this regard, Riboulet-Chavey *et al.* demonstrated that short exposure to MGO of skeletal muscle cells restrains glucose uptake through the inhibition of insulin-stimulated phosphorylation of protein kinase B and ERK 1/2. Of note, these effects are independent of ROS production but derived from a direct consequence of MGO binding to these proteins [104].

In diabetes, chronic complications manifest after a long-term exposure to hyperglycemia and can be categorized as microvascular complications, which are restricted to damage to small blood vessels, and macro-vascular complications due to damage to the arteries. Microvascular complications include diabetic kidney disease, diabetic retinopathy, and neuropathy. Both experimental and clinical studies have established that hyperglycemia induced by MGO is involved in the pathogenesis of micro- and macrovascular complications. Focusing on micro-vascular complications, high plasma concentrations of MGO and MGO-derived AGEs have been shown to be early indicators of the progression of important diabetic nephropathy lesions [105]. Higher plasma concentration of MGO in patients with diabetic nephropathy could be due to increased hyperglycemia, impaired clearance consequent to kidney failure and compromised detoxification due to a reduction in GLO1 expression [101], [106]. In the kidney, MGO has been linked to hemodynamic changes (through endothelial dysfunction and hypertension), inflammatory changes (by the activation of RAGE signalling), metabolic changes (via proteasome impairment and oxidative stress) and structural changes (such as glomerular basement thickening, tubular damage and loss of podocytes) which in turn lead to a progression of diabetic kidney diseases [101]. Growing evidences have shown the involvement of MGO also in diabetic retinopathy, one of the most common microvascular complication of diabetes [107], [108]. It has been shown that increased MGO in the eyes of patients with diabetic retinopathy can contribute to the impairment of those mechanisms that are involved in the regulation of retinal blood flow [109]. Moreover, in rats MGO can cause the loss of tight junctions increasing the permeability of the blood-retinal barrier [110]. In vitro, exposure of retinal endothelial cells to high levels of MGO induces the upregulation of vascular endothelial growth factor (VEGF) that may contribute to increased capillary permeability and apoptosis and reduced proliferation of retinal endothelial cells [111]. Even if glycation has been identified as an important player in diabetic neuropathy, only few studies have focused on the specific role played by MGO. In particular, Bierhaus *et al*. have shown that high concentration of MGO can cause metabolic hyperalgesia through MGO glycation of $Na_v1.8$, a voltage-gated sodium channel exclusively expressed in pain-signaling neurons or nociceptors. This modification increases either voltage sensitivity or functional channel availability, leading to the hyperexcitability that is responsible for diabetic hyperalgesia and spontaneous pain [112]. MGO administration can also be used as a valuable approach to investigate the pathways that are involved in diabetic neuropathy. In this regard, Griggs *et al.* showed that intraplantar administration of MGO produces, through the activation of the cation channel transient

receptor potential ankyrin 1 (TRPA1)- calcium-sensitive adenylyl cyclase 1 isoform (AC1) signaling pathway, dose-dependent nociception, hyperalgesia and affective pain, resembling diabetic neuropathy [113].

1.4.2 Cardiovascular diseases

Recent data have shown that patients with heart failure secondary to diabetes exhibited increased MGO modification on actin and myosin in heart muscle when compared to heart failure patients without diabetes. These modifications interfere with processes that are critical for proper cardiac function such as calcium sensitivity and maximal calcium-activated contractile function [114]. In addition, in patients with T1D, higher plasma concentrations of MGO were linked to fatal cardiovascular events while in T2D patients, to a high incidence of atherosclerosis and high blood pressure. These results identify MGO as a possible biomarker for heart disease [115], [116]. In a study conducted on mice, Blackburn et al. revealed that the accumulation of MG-AGEs after myocardial infarction is the cause of adverse cardiac remodelling and functional loss of the heart [117]. Moreover, in spontaneously hypertensive rats, increased aortic MGO, AGEs formation and oxidative stress were associated with an increase in blood pressure, leading to endothelial dysfunction and altered vascular reactivity [118]. These results are in line with the ones observed in patients with diabetes where elevated plasma levels of MGO and MGO-derived AGEs have been associated with microvascular complications, high blood pressure and the presence of atherosclerosis markers and coronary heart disease [119]–[122]. In addition, the role of RAGE and consequently the activation of the AGE/RAGE axis has been investigated in the development of cardiovascular diseases. Increasing results have highlighted that the AGEs/RAGE axis affects the development, severity and progression of coronary artery disease both in patients with or without diabetes [88], [123]. The administration of sRAGE in mice suppressed diabetic atherosclerosis in a glycemia- and lipid-independent manner due to a blockage of AGEs/RAGE axis. These results additionally confirmed the role played by AGEs/RAGE axis in the development of accelerated atherosclerosis in diabetes and identified RAGE as a therapeutic target in diabetic macrovascular disease [124].

1.4.3 Cancer

Contradictory results have been published sustaining both a pro-tumorigenic and an anti-cancer effect of MGO. On one hand, the experimental evidence collected so far suggested that

MGO induce cytotoxicity and impairs the expression of factors involved in cancer invasiveness leading to an overall inhibition of tumor growth [125]–[127]. On the other hand, recent studies have highlighted a supporting role of MGO in tumor growth that can be linked to survival mechanisms and increased migration, invasion and extracellular matrix remodelling processing [128], [129]. This dual role played by MGO can be explained by several factors. First, MGO levels and accumulation play are essential parameters in deciding cancer cell destiny. Indeed, if low levels of MGO are beneficial to cancer by promoting tumor growth as a consequence of apoptosis elusion and activation of survival mechanisms, high MGO levels induce toxic effects on cancer cells. This cellular mechanism is called hormesis, which is a "process in which the exposure to a low dose of a chemical agent, that is damaging at higher doses, induce an adaptive beneficial effect on the cell or organism" [130], [131]. Secondly, cancer cells possess different abilities to face MGO-induced dicarbonyl stress, making their ability to survive tumor-specific [132].

Given the complexity of carcinogenesis, the precise concentration of MGO and MGO-derived AGEs that delineates a pro- or anti-tumor effect is still not clear yet and further studies are needed to clarify the molecular pathways affected by MGO and involved in tumor progression. This will allow the identification and optimization of personalized therapeutical treatment on the basis of different types of cancer.

1.4.4 Neurological diseases

Many studies have shown that MGO and MGO-derived AGEs play a key role in the etiopathogenesis of neurological disorders such as Alzheimer's disease (AD), cerebral atrophy, polyneuropathies, and Parkinson's disease (PD) [133]–[136].

In elderly people, Srikanth *et al.* have demonstrated that greater MGO serum levels are associated with poorer memory and executive function and lower grey matter volume, linking MGO with cerebral atrophy and subsequent neurodegeneration [133]. In addition, less GLO1-expressing neurons were found in older individuals than in younger ones, clarifying the increased MGO concentrations in elderly brains [61].

MGO and MGO-GEs can exert their neurotoxicity through different possible mechanisms. Krautwald and Münch proposed AGEs involvement in the pathogenesis of AD by i) the cross-links of cytoskeletal proteins that induce neuronal dysfunction and death and ii) their accumulation on Aβ deposits, which instead results in chronic activation of micro- and astroglial cells [137]. In addition, MGO has shown to provoke tau hyperphosphorylation by a

double mechanism that includes the enhancement of kinase activities, from one side, and the reduction of phosphatase levels, from the other[138]. In a study, Lissner *et al.* observed in rats that an acute intracerebroventricular administration of high concentrations of MGO affected astroglial functions and both short- and long-term learning and memory [139]. In a consequent study, the group also demonstrated that MGO treatment damaged hippocampal tissue and caused a loss in BBB integrity, further explaining the alteration observed in the animal behaviour [140]. In AD, MGO toxicity is mediated also through the increase of oxidative stress. The activation of several redox signalling pathways leads to apoptosis and cellular dysfunction. In addition, AGEs accumulation further amplifies ROS production by inducing the glycation of important antioxidant enzymes and by providing precursors of oxidative stress [138]. RAGE have shown to act as a cell receptor for β-amyloid (Aβ), which is a central player in the pathology of AD [141]. Multiple studies have identified RAGE as an important cellular cofactor for Aβ-mediated perturbation. The analysis of human brains identified an increased expression of RAGE in neuronal, microglial and endothelial cells in patients with AD compared with age-matched, non-demented controls, supporting its role in the pathogenesis of neuronal dysfunction [142]–[144]. Moreover, the expression levels of RAGE are correlated to the severity of the disease indicated by the clinical score of the amyloid plaque or tangle [143]. Recently, Fang *et al.* demonstrated that a genetic deficiency of RAGE can significantly reduce Aβ accumulations in the brain by reducing the activity of β- and γ-secretase activity, which are two enzymes involved in the cleavage of the Amyloid-β Precursor Protein (APP) [145].

Xie *et al.* have shown that MGO increases dopamine levels in SH-SY5Y neuroblastoma cells, which results in high oxidative stress in neurons, contributing to damage and loss of dopaminergic neurons [146]. MGO was also found to be the precursor of a neurotoxin identified in Parkinson's disease human brain tissue called 1-acetyl-6,7-dihydroxyl-1,2,3,4-tetrahydroisoquinoline (ADTIQ). The direct reaction between MGO and dopamine lead to ADTIQ, which is responsible for hyperglycemia and death of dopaminergic neurons [147], [148]. Moreover, the parkinsonism-associated protein DJ-1 (or PARK7) known to be highly expressed in PD, has been identified as an important anti-glycating protein as it is involved in the detoxification of MGO-glycated protein by acting on early glycation intermediated and releasing repaired proteins [149].

Altogether these results strongly support the evidences that elevated MGO levels in the brain, leading to an increase in MGO-derived AGEs production and consequent RAGE activation, can drive neurological disease onset and progression.

2. MGO: the trojan horse in ageing and Alzheimer's disease

2.1 Ageing and Alzheimer's disease

Ageing is a process characterized by physiological and molecular changes that leads to an increased risk of disease and death [150]. According to the World Health Organization, the number of persons aged 80 years or older is expected to triple between 2020 and 2050 to reach a number of 426 million people [151]. As the elderly population increases, a consequent growth in the financial burden of age-related health disorders will occur, making it urgent to identify preventive and/or therapeutic approaches [152]. Among the different age-related health diseases, neurodegeneration and the associated cognitive decline are particularly relevant as they can greatly influence patients healthspan and quality of life [153]. Ageing represents one of the main risk factors for neurodegeneration. In particular, Alzheimer's Disease (AD) is the most common neurodegenerative disorder and is estimated to affect 131.5 million by 2050 if no effective therapies are available [154]. One of the pathological hallmarks of AD is the accumulation of extracellular senile plaques in the brain, particularly at the level of cerebral cortex and hippocampus. Senile plaques are mainly composed of amyloid- β, a 40-42 amino acid peptide generated by proteolytic cleavages of the amyloid- β protein precursor (APP) [155]. Three proteolytic secretase enzymes are mainly involved in the cleavage of APP: i) α- secretase, ii) β-secretase, among which BACE1 is the major one found in the brain, and iii) γ-secretase, which consists at least of four core components including presenilins (PS1 and PS2), nicastrin, PEN2 and APH1 [156]. APP can be cleaved via two pathways, the nonamyloidogenic or the amyloidogenic one. The non-amyloidogenic pathway involves the cleavage of full-length APP by α-secretase, which releases the sAPPα ectodomain outside the cell membrane and retains an 83 amino acid-C-terminal APP fragment (C83) within the plasma membrane. Then, C83 can be further cleaved by γ-secretase, releasing a small p3 fragment into the extracellular space. The amyloidogenic pathway includes sequential proteolytic cleavage of APP by β-secretase and the γ-secretase complex. After β-cleavage, the sAPPβ ectodomain is released, and a 99 amino acid APP carboxy-terminal fragment (C99) can be further cleaved by γ-secretase at various sites. This may generate amyloid peptides with different chain lengths including Aβ37, 38, 39, 40, 42 and 43 [156]. Among them, Aβ42 and Aβ40 comprise the two

major Aβ species in the brain. Although soluble Aβ40 is much more abundant than soluble Aβ42, Aβ42 exhibits a higher susceptibility for aggregation, due to hydrophobicity within its two terminal residues [157], [158]. Indeed, Aβ42 is the main component of amyloid plaques and is shown to be neurotoxic, identifying it as a key player in initiating plaque formation and AD pathogenesis. Notably, non-amyloidogenic and amyloidogenic pathways have been shown to compete, suggesting that both enhancing non-amyloidogenic pathway and reducing amyloidogenic pathway represent viable strategies to reduce Aβ generation [159] (Figure 5).

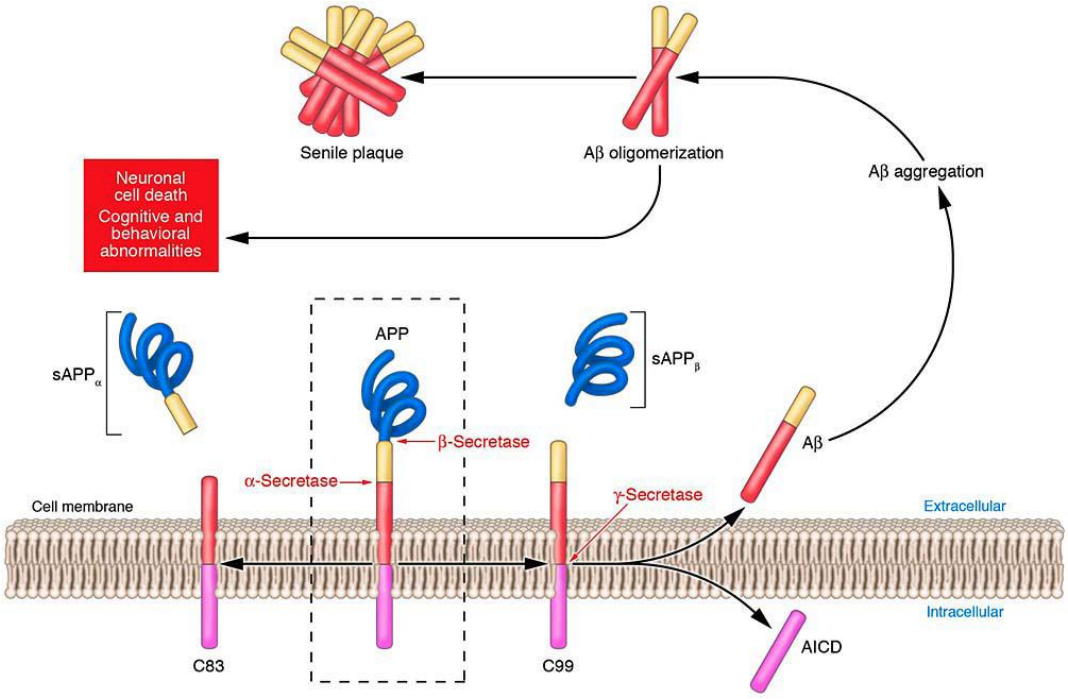

Figure 5. The non-amyloidogenic and amyloidogenic pathways of APP processing.

Another distinctive feature of AD is neurofibrillary tangles which are aggregates of hyperphosphorylated and misfolded tau protein. Under normal physiological conditions, tau promotes the assembly and stability of microtubules and facilitates axonal transport [160]. In AD, abnormal processing of APP is responsible for the overproduction and/or reduced clearance of Aβ, leading to Aβ accumulation in the brain. In turn, the formation of Aβ plaques triggers further development of neurofibrillary tangles and disruption of synaptic connections, ultimately leading to neural cell death and synapse loss [161].

2.2 Our contribution

The world is witnessing a rise in neurological disorders due to the growing elderly population [162]. There is a strong correlation between diet and cognition, and nutrition may offer a cost-effective and safe solution to complement pharmaceutical treatments, prevent cognitive decline and improve the quality of life for aging individuals [5], [163], [164]. The Western diet, characterized by highly processed food and ingredients and cooking techniques that promote the formation of MGO and its derivatives, is currently a major concern [9]. As previously described, MGO has been linked to the development of neurodegenerative diseases by causing cellular damage, protein cross-linking, activation of the AGEs/RAGE pathway, and glycation. Therefore, reducing the intake of foods high in MGO and AGEs could be an effective approach in preventing or delaying dementia in the elderly. However, there are conflicting findings in the literature regarding the relationship between dietary MGO and cognitive impairment. For instance, Zunkel *et al.* discovered that a prolonged intake of MGO through drinking water is not toxic to mice as the detoxification of MGO through renal excretion and other mechanisms effectively prevents its toxicity [165]. On the contrary, Cai et al demonstrated that mice fed with an MGO-supplemented diet developed metabolic syndrome, increased brain amyloid-β42, deposits of AGEs, gliosis, and cognitive deficits [166]. In light of the need to increase awareness about the potential harm posed by MGO in diets, we aimed to gain further understanding of the neurotoxicity of MGO dietary intake. In this regard, we decided to treat aged mice (18-20 months) with a high dosage of MGO or vehicle (VH) once a day by oral gavage for 28 days. Many studies in the literature usually administer MGO through drinking water or supplemented with diet. Although these administration methods are less invasive, they have some limitations and may account for the inconsistent data and conflicting effects of MGO seen over the years. On one side, one challenge with administering MGO through drinking water is the lack of consistent dosing as the amount of MGO actually consumed by the animals can vary, making it difficult to control the dose for each animal. On the other side, supplementing the diet with MGO can result in interactions with other nutrients and compounds in the food, potentially leading to different effects compared to administering MGO orally alone. In turn, our decision to administer MGO through oral gavage was motivated by the need to accurately measure the specific daily amount of MGO given to the mice and to ensure consistent treatment throughout the study. The selected dosage of MGO was 100 mg/kg body weight, based on the findings of Uribarri *et al.* database, which reported MGO levels in various foods [167]. The calculation of the MGO dosage was done to

imitate a high consumption of MGO-rich foods, commonly seen in Western diets. The treatment was administered daily for 28 days, followed by a series of behavioral tests on mice, including rotarod for measuring locomotor activity, open-field for exploring behavior, and Y-maze for testing spatial memory. After the tests, the mice were sacrificed, and their blood and brain areas were collected. The MGO concentration was measured in serum samples after the derivatization with 1,2-diamino-4,5-methylenedoxybenzene (DMB) and using High-Performance Liquid Chromatography coupled with a fluorescent detector. This method was found to be effective for analyzing a large number of samples with low MGO content [168]. In addition, the ability of the brain to scavenge MGO was evaluated by assessing GLO1 activity in different brain areas. Finally, the expression of RAGE, neuroinflammation and redox homeostasis markers as well as AD-related APP processing ones were evaluated by RT-PCR and Western Blot. This study has led to a publication of a manuscript in an international scientific journal:

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Methylglyoxal affects cognitive behaviour and modulates RAGE and Presenilin-1 expression in hippocampus of aged mice

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ABSTRACT

Methylglyoxal (MG), a potent glycotoxin that can be found in the diet, is one of the main precursors of Advanced glycation end products (AGEs). It is well known that modifications in lifestyle such as nutritional interventions can be of great value for preventing brain deterioration. This study aimed to evaluate *in vivo* how an oral MG treatment, that mimics a high MG dietary intake, could affect brain health. From our results, we demonstrated that MG administration affected working memory, and induced neuroinflammation and oxidative stress by modulating the Receptor for Advanced glycation end products (RAGE). The gene and protein expressions of RAGE were increased in the hippocampus of MG mice, an area where the activity of glyoxalase 1, one of the main enzymes involved in MG detoxification, was found reduced. Furthermore, at hippocampus level, MG mice showed increased expression of proinflammatory cytokines and increased activities of NADPH oxidase and catalase. MG administration also increased the gene and protein expressions of Presenilin-1, a subunit of the gamma-secretase protein complex linked to Alzheimer's disease. These findings suggest that high MG oral intake induces alteration directly in the brain and might establish an environment predisposing to AD-like pathological conditions.

1. Introduction

Nutrition is becoming increasingly popular as a therapeutic strategy to promote longevity, health and even prevent disease onset (Kalacheet al., 2019). Healthy dietary patterns, such as the Mediterranean diet, and dietary bioactive compounds are nowadays considered valuable supports for cognitive health (Gómez-Pinilla, 2008; Dominguez and Barbagallo, 2018). Although the correlation between food components or diet pattern and cognition has been widely explored (Serra et al., 2020), the effects of food processing or cooking are less considered yet. Indeed, many foods that are part of modern diets are cooked and exposed to thermal processes that enhance their flavor, palatability, and attractiveness but at the same time are responsible for the formation of dietary glycotoxins, known as Advanced Glycation End-products (AGEs) (Tamanna and Mahmood, 2015; Abate et al., 2017). AGEs derived from the non-enzymatic glycation of proteins, lipids, and nucleic acids with sugars (Chen et al., 2018) and one of their major precursors is Methylglyoxal (MG), a highly reactive dicarbonyl compound and a potent glycating agent (Bellieret al., 2019). Diet is one of the main sources of MG and AGEs but they can also be produced endogenously as part of the normal metabolism (Sergi et al., 2021). When AGEs, both exogenous and endogenous, reach high levels in tissue and bloodstream, they can induce oxidative stress and inflammation contributing to the onset of chronic diseases, such as diabetes, atherosclerosis, and neurodegenerative disease, as well as to aging process (Yamagishi and Matsui, 2018; Singh et al., 2014; Li et al., 2012; Chaudhuriet al., 2019). AGEs mediate their pathological effects by cross-linking with body proteins, changing their structure and function or by binding with cell surface receptors. Among them, the receptor for Advanced Glycation End-products (RAGE), a member of the immunoglobulin superfamily receptor (Hudson and Lippman, 2017; Leerachet al., 2021), plays a pivotal role. AGEs/RAGE axis activates several signalling pathways, including the pro-inflammatory transcription factor NF-κB, NADPH oxidases, mitogen-activated protein kinases (MAPKs), p21ras, extracellular signal-regulated kinase (ERK) p38 and protein kinase C (PKC) (Jandeleit-Dahm et al., 2008).

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In addition to AGEs, RAGE is also capable of binding other ligands, including amyloid-beta (Aβ) (Chaneyet al., 2005; Bongarzone et al., 2017), a key toxic peptide derived from the cleavage of amyloid precursor protein (APP), involved in Alzheimer's disease (AD) pathogenesis.

AD is the most common form of dementia worldwide, characterized by a progressive and irreversible loss of neurons in the cerebral cortex and hippocampus which ultimately leads to impaired daily activities and deterioration of cognitive and functional skills (Petersenet al., 2021). Disease progression involves the misfolding and aggregation of different Aβ proteoforms generated by the pro-amyloidogenic sequential cleavage of β-amyloid precursor protein (APP) by β-secretase and then by γ-secretase (Guo et al., 2020). γ-secretase attracts a lot of attention in AD research because its cleavage determines the Aβ42/Aβ40 ratio, a key factor in AD-related amyloidogenesis (Xu, 2009). Considering that it is now well established that lifestyle and diet can contribute to cognitive deterioration and even to AD development, (Martins et al., 2021; Hu et al., 2013) investigating the effects of dietary glycotoxins might clarify the connection between long-lasting dietary habits and disease exacerbation.

Thus, this study investigated the effects of an oral MG administration, that mimics a high dietary MG intake, in aged mice. In detail, we evaluated whether MG can affect cognitive performances, induce changes directly in specific brain areas, and finally whether MG can induce alterations involved in AD-related pathological mechanisms.

2. Materials and methods

2.1. Animals

Both male and female B6/129PF2 mice (18–20 months, average weight: 35 g) were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). The mice were housed three-to-four per cage in a 12 h light/dark cycle (light phase from 8:00 a.m. to 8:00 p.m.) with food and water available *ad libitum* and were maintained at 50% relative humidity and a 12 h light/dark cycle at 20–22 °C. The mice were *ad libitum* given water and a normal weighted chow (70% carbohydrate, 20% protein, 10% fat, total 3.95 kcal/g; standard diet 4RF21, Mucedola). All experiments were conducted in conformity with the European Communities Council Directive of 1986 (86/609/EEC), approved by the Italian Ministry of Health, and the Animal Care and Use Committee of the University of Brescia.

2.2. Chemicals and treatment

Mice (18–20 months) were gavage fed daily for 4 weeks either with Methylglyoxal (MG) solution (40% in H2O) (Sigma-Aldrich) (10 mice) or vehicle (10 mice). Mice oral dosage of MG was selected as 100 mg/kg according to literature (Di Emidioet al., 2019).

Individual bodyweight and cage food consumption were recorded weekly. At the end of the 4 weeks treatment, mice were placed for acclimatization in the behavioural room for 30 min preceding each test. In the behaviour room, behavioural test area was separated from the operator area by means of a dark sliding door. Rotarod, open field and Y-maze tests were executed as reported below. These behavioural tests were performed during the light phase of the circadian cycle, between 09:30 a.m. and 05:30 p.m. After behavioural tests, mice were sacrificed, and their brain areas (hippocampi and cortices) and blood were collected. Samples were then stored at −80 °C until further analysis.

2.3. Rotarod test

To measure the motor performance, mice were placed one at a time on the rotarod treadmill (Ugo Basile) and a trial of 30 s at a constant speed of 2 rpm was executed. Immediately after, the test was performed at an initial intensity of 2 rpm and a final intensity of 20 rpm that was reached 300 s later. Finally, the time that each rodent was able to stay on the top of the rotarod treadmill has been recorded (Mastinuet al., 2019).

2.4. Open field

Each of MG and VH mice was individually videotaped during a 5-min exploration session in a 40 cm × 40 cm Plexiglas open field activity box. Mice were placed in the center of the arena at the beginning of the test period. Their movement around the arena was recorded by a portable video camera vertically mounted 1.5 m above and remotely controlled by the experimenter. The Plexiglas box was cleaned after each individual test session to prevent subsequent mice from being influenced by odors deposited by previous mice. Locomotor activity was recorded, and total distance travelled and average speed were automatically analyzed with Ethovision XT software (version 13, Noldus, Wageningen, The Netherlands) (Ariaet al., 2020).

2.5. Y-maze

Y-maze paradigm was used to measure the working memory ability of mice. The black Y-maze consisted of three identical arms placed at 120°. Every arm was height 15 cm, width 8 cm and length 30 cm. Mice were placed at the end of one arm and allowed to move freely through the Y-maze during 5 min of test. The number of arm entries was video-recorded, tracked, and analyzed by EthoVision XT software (Noldus). Alternation was defined as triplets of explored arms and counted only if the mouse entered into the three arms of the maze (without revisiting the first arm at the third visit). Max alternation was defined as the number of possible alternations counted by the total number of arms entered minus 2 (Rungratanawanichet al., 2019).

2.6. HPLC methylglyoxal measurement in mice serum

MG levels were determined by high-performance liquid chromatography coupled to a fluorescent detector. MG was derivatized with 1,2-diamino-4,5-methylenedyoxybenzene (DMB) to allow fluorometric detection (Fig. 1A), as previously described by Ogawasara et al. (Ogasawara et al., 2016) with minor modifications. Briefly, DMB solution (7 mM) was prepared with 1 M β-mercaptoethanol and 28 mM sodium dithionite. Serum samples were centrifuged, and the supernatants were diluted 2-fold with MilliQ water. Twenty-five microliters of MG standard (final concentrations: 0,07-4 μM) or previously prepared serum samples were added to 25 μl of DMB solution, and the solution was incubated at 60 °C for 1h. After cooling on ice, samples were resuspended in 25 μl of methanol:acetonitrile:water (35:10:55). After centrifugation, 10 μl of supernatants were collected and injected into a C18 column (KINETEX EVO C18 (Phenomenex, Italy) 5 μm, 250 × 4.6 mm, 100 Å) pre-equilibrated with a mobile phase solution consisted of methanol: acetonitrile:water at a 35:10:55 ratio. Standard curves were generated with concentrations ranging between 0,07 μM and 4 μM (Fig. 1B and C). The flow rate was 0,7 ml/min and run time was 16 min. The fluorescence was monitored using an excitation of 355 nm and an emission of 393 nm. MG concentrations were measured by extrapolating the area from each run using a calibration curve.

2.7. Gene expression

Half hippocampi and cortices derived from MG and VH mice were processed for mRNA analysis. The total mRNA was extracted using the TRI Reagent (Sigma-Aldrich) and 2 μg of total RNA were reverse-transcribed using M-MLV reverse transcriptase (Promega) following the manufacturer's instructions and as reported in (Marzianoet al., 2019). Table 1 shows the murine-specific primers used for qRT-PCR. Amplification and detection were performed with the ViiA7 Real Time PCR Detection System (Applied Biosystems). The reaction mix contained: 6 μL of SYBR Green Master Mix (BIO-RAD Laboratories), 6 pmol

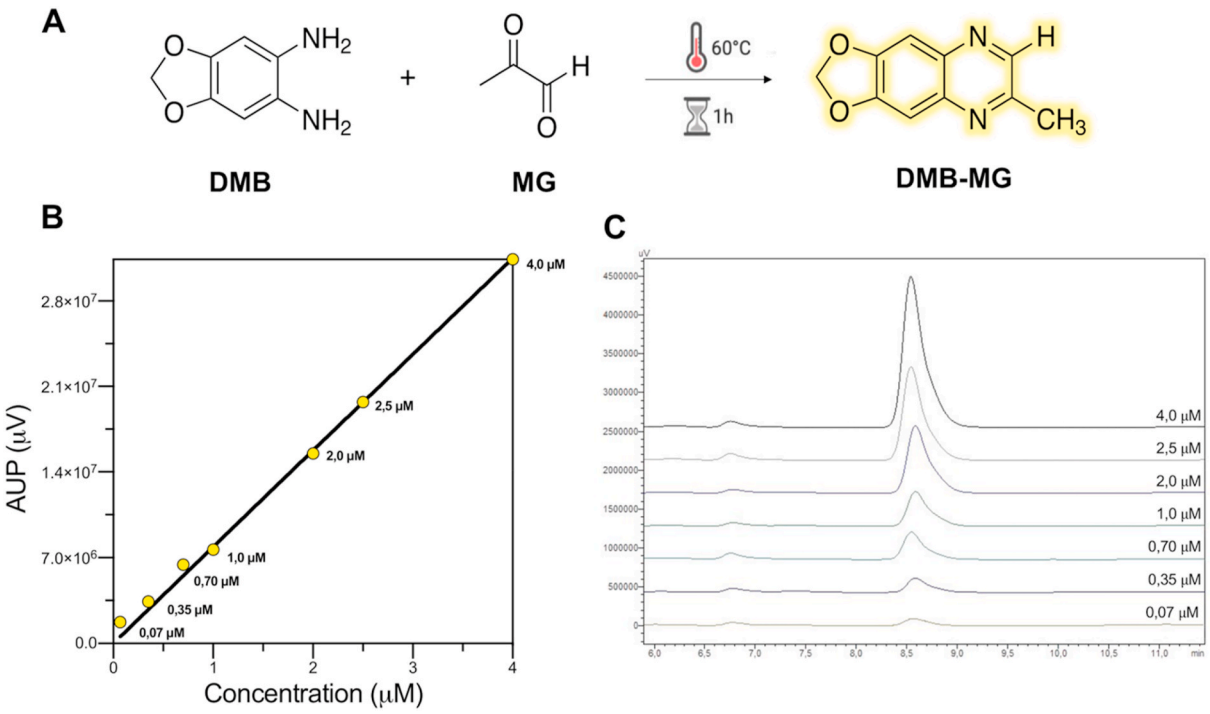

Fig. 1. (A) Derivatization reaction of MG with 1,2-diamino-4,5-methylenedyoxybenzene (DMB) to allow the formation of the fluorescence derivative DMB-MG. (B) Standard curve obtained from the analysis of different concentrations of standard MG derivatized with DMB. (C) Chromatograms obtained from the HPLC analysis with fluorescence detection of different concentrations of standard MG derivatized with DMB.

Table 1
Primers used for q-PCR.

GENES	PRIMER SEQUENCES
Advanced glycation end products receptor (RAGE)	f-5′-CTGAACTCACAGCCAGTGTCC-3’; r-5′-CCCTGACTCGGAGTT-3′
Interleukin 1 beta (IL-1β)	f-5′-GCTTCAGGCAGGCAGTATC-3′ r-5′-TAATGGGAACGTCACACACC-3′
Interleukin 6 (**IL-6**)	f-5′-CCTACCCCAATTTCCAATGCT-3′ r-5′-TATTTTCTGACCACAGTGAGGAAT-3′
Ionized Calcium Binding Adapter Protein 1 (**IBA-1**)	f-5′-GGATTTGCAGGGAGGAAAAG-3′; r-5′-TGGGATCATCGAGGAATTG-3′
Glial Fibrillary Acidic Protein (**GFAP**)	f-5′-ATTGCTGGAGGGCGAAGAA-3’; r-5′-CGGATCTGGAGGTTGGAGAA-3′
Amyloid precursor protein (**APP**)	f-5′-TCGCTGACGGAAACCAAGAC-3′; r-5′-GGCTCGACTTCATTTTCGGTAT-3′
Beta-Secretase 1 (**BACE-1**)	f-5′-GTCAGCTTCTATACCCGGAAGG-3’; r-5′-CTTTGGCCTACAGTCTCAGTG-3′
Presenilin 1 (**PS-1**)	f-5′-GGAGACCGACGAGGAATCG-3′; r- 5′-GCAAAGTTACTACTGCCCGTG-3′
Actin (**β-act**)	f-5′-AGCCATGTACGTAGCCATCC-3’; r-5′-CTCTCAGCTGTGGTGGTGAA-3′

of each forward and reverse primer, and 2 μL of diluted cDNA. The samples were run in duplicate, and the PCR program was initiated by 10 min at 95 °C before 40 cycles, each one of 1 s at 95 °C and 30 s at 64 °C. The gene expression levels were normalized to β-Actin expression and the data were presented as the fold change in target gene expression. Relative quantification was performed using the comparative Ct method (Panet al., 2014, 2015).

2.8. Protein extraction and Western Blot analysis

The remaining half parts of their hippocampi were processed for total protein extraction to assess enzymatic assay and protein expression by Western Blot (Panet al., 2019; Pan et al., 2017). In details, hippocampi were homogenized in ice-cold buffer (0.32 M Sucrose, 1 M Tris/HCl pH 8, 0.1 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin) and sonicated for 1 min on ice. Then protein samples (20 μg) of hippocampal extracts were electrophoresed in 12% acrylamide gel and electro-blotted onto nitrocellulose membranes (Sigma- Aldrich, Merck KGaA, Darmastadt, Germany). Membranes were blocked for 1 h in 3% w/v Bovine Serum Albumin in TBS-T (0.1 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with primary antibodies. Primary antibodies were anti-GAPDH (1:2500, Sigma- Aldrich), anti-AGER (1:300, Sigma-Aldrich), anti-PS1(1:1000, StressMarq, Biosciences). IR Dye near-infrared dyes-conjugated secondary antibodies (LI-COR, Lincoln, Nebraska USA) were used. The immunodetection was performed using a dual-mode western imaging system Odyssey FC (LI-COR Lincoln, Nebraska USA). Quantification was performed using Image Studio Software (LI-COR, Lincoln, Nebraska USA) and the results were normalized over the GAPDH signal.

2.9. Measurement of glyoxalase 1 activity

The activity of glyoxalase 1 (GLO1) was measured as previously described (Pan et al., 2017; Hansenet al., 2017; Mannervik et al., 1981) with some modifications. In a 96-well microplate, the reaction mixture (200 μL/well), containing 50 mM of sodium-phosphate buffer (pH 6.6), 2 mM of MG, and 2 mM of reduced glutathione (GSH), was preincubated for 20 min at 37 °C. Then mice hippocampi or cortices protein extracts were added to the mixture. The reaction was monitored spectrophotometrically by following the formation of S-D-lactoylglutathione at 240 nm. A unit (U) of glyoxalase I activity corresponds to the amount of enzyme that catalyzes the formation of 1 μmol of S-(D)-lactoylglutathione per minute. The activity of GLO-1 was then calculated as units per milligram of protein (U/mg).

2.10. Antioxidant enzyme activities assays

Superoxide dismutase (SOD) activity was measured following the

inhibition of epinephrine oxidation. Hippocampi protein extracts were analyzed, and the reaction was monitored measuring the decrease of absorbance at 480 nm.

Catalase (CAT) activity was measured by monitoring the decomposition of H_2O_2 in hippocampi's protein extracts. The enzymatic reaction was stopped by the addition of ammonium molybdate and measured at 405 nm. The results were extrapolated by a standard curve.

NADPH oxidase (Nicotinamide adenine dinucleotide phosphate oxidase, NOX) activity was measured exploiting the capability of NOX to convert molecular oxygen to superoxide through the oxidation of NADPH to NADP plus H^+. NADPH has an absorbance spectrum at 340 nm. The reduction in absorbance at this length is proportional to the decrease in NADPH through its consumption by NOX. Data were expressed in μM NADP/μg protein/min and was calculated using the equation C = A/(ExL), where C is the concentration of NADP reduced by NOX, A is the absorbance (the gradient of the initial rate of reaction), E is the extinction coefficient of NADPH (6.22 $mM^{-1}cm^{-1}$) and L is the pathway length (1 cm).

2.11. Statistical analysis

Two-way Repeated Measures ANOVA tests with the Bonferroni post-test were used to determine the significance of the pharmacological effect on body weight during the chronic treatment (Panet al., 2020). Student *t*-test with the Bonferroni post-test was instead adopted to determine the statistical difference between MG and VH groups. Data are presented as the means ± S.E.M. (standard error mean). All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego CA, USA), and the statistical significance level was set at $p < 0.05$.

3. Results

3.1. Chronic administration of methylglyoxal affects working memory and increases MG serum level in aged mice

Mice orally treated with 100 mg/kg MG for 4 weeks did not show significant modifications in body weight (data not shown), while a defect in working memory was observed. In particular, Y-maze test showed a significant decrease in the Max Alternation index in MG-mice compared with VH mice (Fig. 2D; VH-mice: 10.33 ± 1,481, MG-mice 6.250 ± 0.8814, $p < 0,05$). No differences were observed in their locomotor activity and exploratory behaviour as shown in Fig. 2B and C. After behavioural tests, mice were sacrificed, serum was withdrawn for MG measurement and hippocampus and cortex were collected for molecular analysis. To further confirm the efficacy of the treatment, MG serum levels were measured by HPLC after derivatization with 1,2-diamino-4,5-methylenedyoxybenzene (DMB) as previously described (see methods section). Increased MG levels were found in MG-treated mice compared to VH mice (Fig. 3A and B, VH-mice: 0.9788 ± 0.149 and MG-mice: 1.593 ± 0.238, $p < 0.05$).

3.2. MG is less degraded by GLO1 and modulates RAGE expression selectively in the hippocampus both at gene and protein levels

AGEs receptor (RAGE) mRNA gene expression was found remarkably increased in MG treated mice, but this selectively occurred only at hippocampus level (Fig. 4A, VH-mice: 1.784 ± 0.558, MG-mice: 4.376 ± 0.545, $p < 0,01$). No differences have been found considering mRNA levels of RAGE in the cortex (Fig. 4B). The significant increase of RAGE in hippocampi of MG-treated mice was further confirmed at protein levels (Fig. 4C, VH-mice: 0.6519 ± 0.0346, MG-mice: 0.7825 ± 0.0193, $p < 0,05$; Fig. 4D). In addition, glyoxalase 1 (GLO1) activity, one of the enzymes involved in MG detoxification, was found reduced at hippocampal level (Fig. 5A, VH-mice: 0.137 ± 0.0119, MG-mice: 0.0926 ± 0.0134, $p < 0,05$), but not in cortex (Fig. 5B).

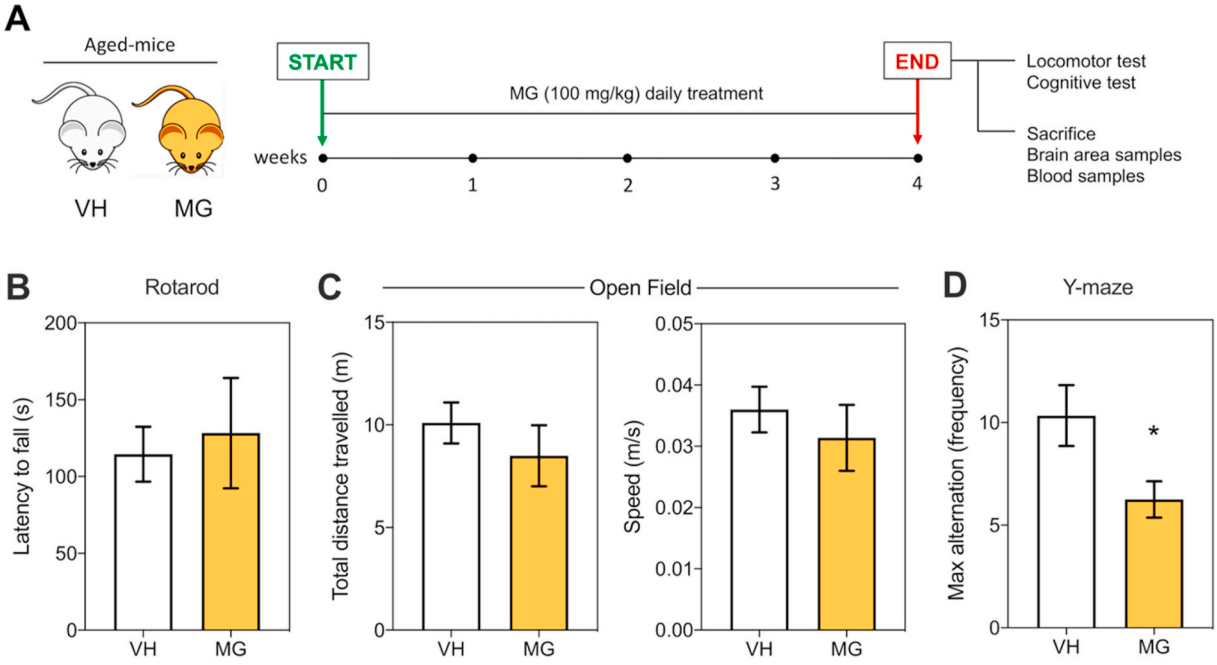

Fig. 2. (A) **Schematic representation of the experimental design.** Twenty aged B6/129PF2 mice (18–20 months) were divided into two groups: (1) MG-mice received 100 mg/kg of MG and (2) control group (VH) received normal saline by oral gavage once a day for 4 weeks. (B–D) **Behavioural battery tests of MG and VH mice.** Rotarod (B), Open Field (C) and Y-maze (D) tests. Data are shown as mean ± standard error of the mean (SEM). For Y-maze: *p < 0.05 vs. control. Student t Tests followed by Bonferroni were used to test statistical significance.

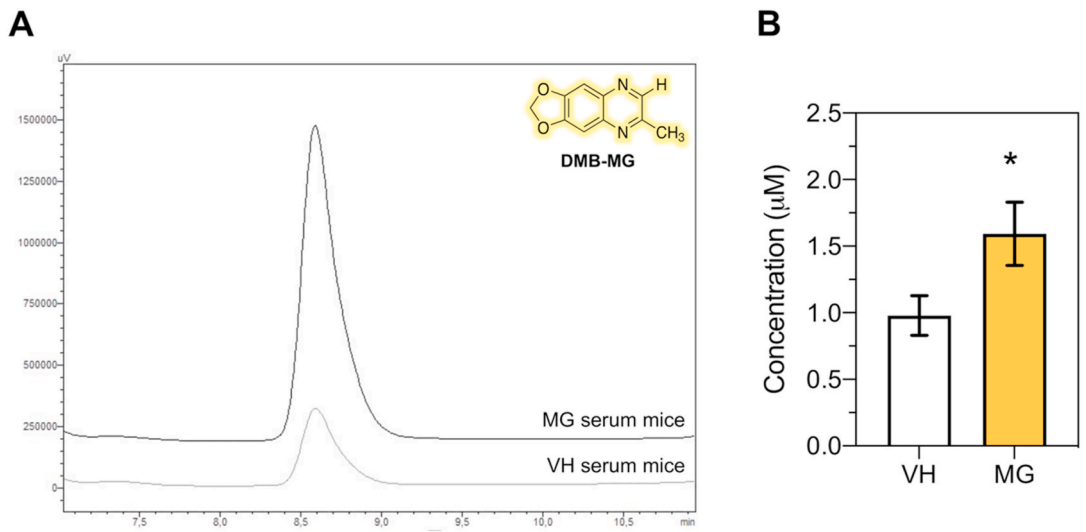

Fig. 3. (A) **Chromatograms obtained from the HPLC analysis with fluorescence detection of MG and VH serum mice derivatized with DMB.** (B) Graphic representation of DMB-MG concentrations in MG and VH serum samples expressed in μM. Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance. *p < 0,05 vs. control VH.

3.3. MG treatment induces low-grade pro-inflammatory effects in hippocampus

As the main MG-related alterations have been exclusively found in the hippocampus, but not in cortex, the further experiments were focused on this brain area.

To evaluate the downstream effect of RAGE activation, we tested the gene expression of pro-inflammatory markers as IL-1β and IL-6. MG treatment increased the mRNA gene expression of IL-1β and IL-6 when compared to the control group (Fig. 6A, VH-mice: 1.063 ± 0.134, MG-mice: 1.781 ± 0.284, $p < 0.05$; Fig. 6B, VH-mice: 1.018 ± 0.0728, MG-mice: 2.579 ± 0.756, $p < 0.05$, respectively). To test if astrocyte and microglia were involved in MG-induced neuroinflammation, the gene expression of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (IBA-1) gene expressions were also assessed. No differences were observed for neither IBA-1 nor for GFAP (Fig. 6C and D). Also the mRNA gene expression of inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were not significantly different in MG and VH mice (data not shown).

3.4. MG treatment induces alteration in enzyme activities involved in redox homeostasis regulation in the hippocampus

AGEs/RAGE activation pathway is known to enhance oxidative stress through ROS production via NADPH oxidase (NOX). Therefore, to test if MG treatment induces increased production of ROS, NOX activity has been evaluated in hippocampi of MG and VH mice. NOX is a multisubunit enzyme that acts as a key producer of ROS in many cells and tissues. NOX activity was statistically enhanced in MG mice compared to VH mice (Fig. 7A, VH-mice: 0.944 ± 0.135, MG-mice: 2.069 ± 0.476, $p < 0.05$). We also evaluate the antioxidant enzyme cascade involved in the detoxification of ROS. In particular, we tested the activity of Superoxide dismutase (SOD) and Catalase (CAT) involved in the dismutation of superoxide and neutralization of hydrogen peroxide, respectively. CAT activity (Fig. 7C, VH-mice: 37.65 ± 2.741, MG-mice: 48.36 ± 3.791, $p < 0.05$), but not SOD activity (Fig. 7B) was increased in the hippocampi of MG mice when compared to VH mice.

3.5. MG modulates hippocampal gene and protein expression of Presenilin-1 (PS1)

Besides being the receptor of AGEs, RAGE is linked to AD pathology since it mediates the binding and uptake of beta-amyloid (Aβ). For this purpose, we tested whether MG/RAGE axis can influence APP-amyloidogenic process by modulating the expression of β-secretase (BACE-1) and γ-secretase (PS-1), as well as APP itself at hippocampal level. MG treatment did not alter mRNA APP levels (Fig. 8A). On the other hand, MG significantly increased both mRNA gene expression (Fig. 8C, VH-mice: 1.219 ± 0.2751, MG-mice: 2.590 ± 0.552, $p < 0.05$) and protein levels (Fig. 8D, VH-mice: 0.487 ± 0.0517, MG-mice: 0.644 ± 0.219, $p < 0.05$; Fig. 8E) of Presenilin-1 (PS1), the catalytic subunit of γ-secretase complex. As BACE1 cleavage of APP is a prerequisite for γ-secretase pro-amyloidogenic cleavage, we also investigated BACE-1 expression. An increasing trend of BACE-1 (Fig. 8B) mRNA gene expression was found in hippocampi of MG mice compared to VH mice.

4. Discussion

In this study, we evaluated behavioural and biochemical parameters to explore the hypothesis that a diet highly enriched with Methylglyoxal (MG) can contribute to cognitive decline and predispose to Alzheimer's disease-like pathology in aged mice. Our hypothesis is based on several data reported in literature that highlight the tight link between diet and cognition (Scarmeas et al., 2018; Klimova et al., 2020; Reichelt et al., 2017).

MG is a potent protein-glycating agent and a major precursor of advanced glycation end-products (AGEs) (Majtan, 2011). When MG irreversibly reacts with the amino groups of the side chain of arginine, lysine and cysteine residues (Zhao et al., 2019), several glycoxidation processes occur which, besides inducing AGEs formation, also generate oxidative damage to proteins (Butterfield and Halliwell, 2019). Humans are mainly exposed to two different sources of AGEs: endogenous AGEs that are physiologically produced in the body and exogenous AGEs that are ingested with foods in the diet. Among different dietary patterns, the Western diet is generally characterized by a high dietary intake of AGEs, whose formation depends on food composition, high-temperature processing, or cooking methods (Abate et al., 2017).

Since controversial findings related to the close association between dietary glycotoxins and cognitive impairment can be found in literature (Caiet al., 2014; Zunkel et al., 2020), this study aimed to disclose this issue by treating aged mice with MG. One of the goals of this study was to get new insights into the potential neurotoxicity of MG dietary intake as more awareness needs to be raised on this threat hidden in diet.

Our data demonstrated that aged mice orally treated with 100 mg/kg

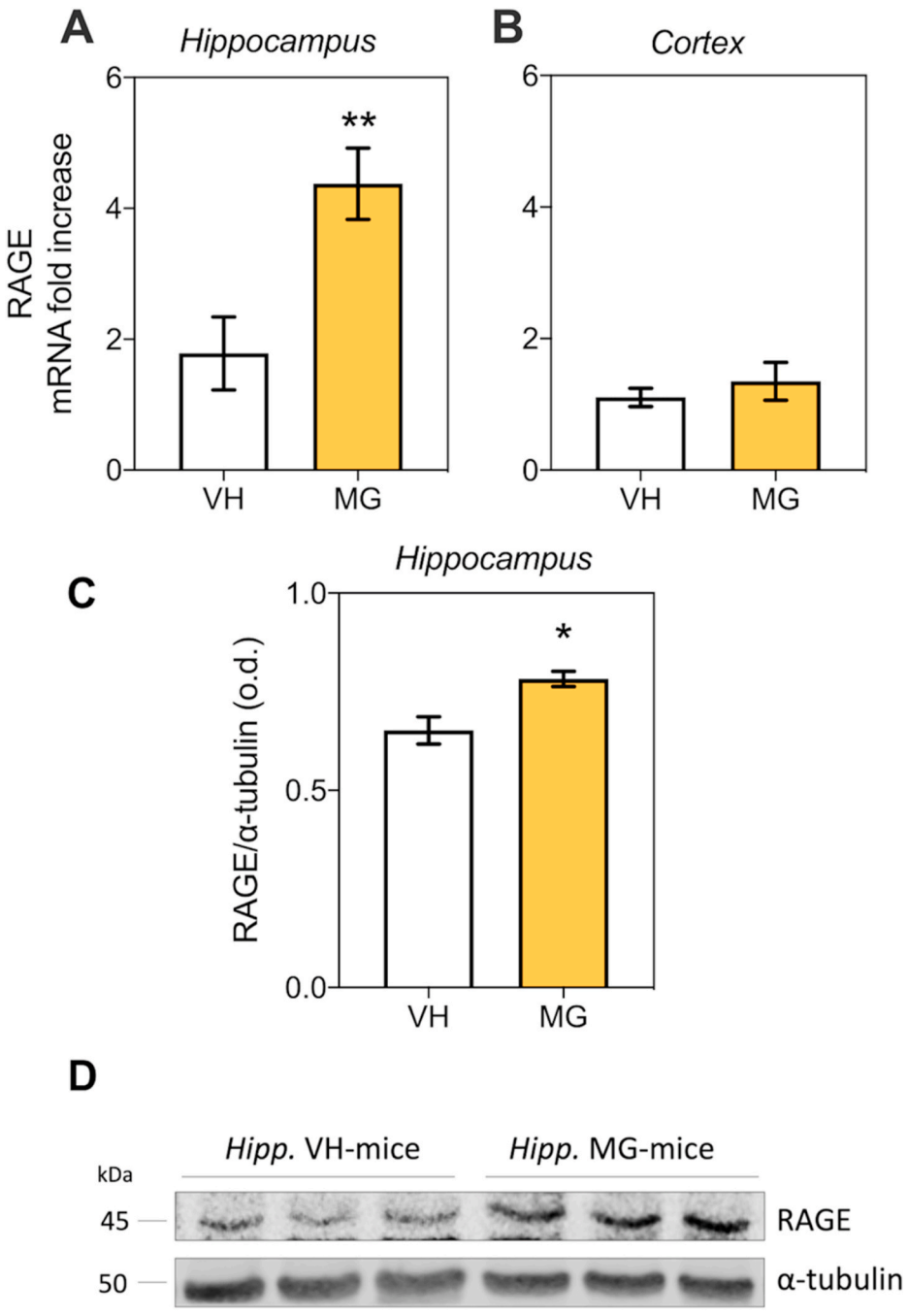

Fig. 4. (A,B) **Effects of MG on RAGE gene expression in two brain areas**: RAGE gene expression in Hippocampus (Hipp-A) and Cortex (Cx-B) of MG and VH mice. Data are expressed as the fold change of the target gene normalized to the internal standard control gene (β-actin). Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance $^{**}p < 0.01$ vs. control (C,D) **Effects of MG on RAGE protein expression in hippocampus:** (C) Western Blot quantitative analysis of hippocampal RAGE normalized over the α-tubulin signal; (D) Representative WB of RAGE protein expression in Hippocampi derived from MG and VH mice. Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance $^{*}p < 0.05$ vs. control.

MG over a prolonged period (4 weeks) developed cognitive impairment. Y-maze test highlighted a defect in working memory in MG-mice compared to the control counterpart, as a significant reduction in the Max Alternation index was found. MG-treated mice also showed higher MG levels in serum than age-matched VH- mice, suggesting and confirming that MG is adsorbed at intestinal level and circulated in the bloodstream. Contradicting results can be found in literature considering the increased MG serum after administration of MG (Zunkel et al., 2020). These discrepancies can be due to the different techniques used for serum MG-detection. In this manuscript, MG was assayed by its derivatization with DMB followed by high-performance liquid chromatography with fluorescence detection. This is a reliable method for analyzing a large number of samples with a low MG content (Ogasawara et al., 2016). The relationship we found between high oral MG intake and cognitive impairment in MG-treated mice is also supported by data in humans that investigated the association between high serum MG levels and cognitive decline (Caiet al., 2014; Srikanthet al., 2013).

Detoxification of MG is known to occur mainly through the conversion of MG to D-Lactate (D-LAC) by two enzymes: glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2). GLO1 is involved in the conversion of a methylglyoxal-glutathione hemithioacetal, formed by the condensation of MG with GSH, into S-(D)-lactoylglutathione (SLG). Then GLO2 hydrolyze SLG in D-LAC by releasing of GSH (de Bari et al., 2019). In vitro, an overexpression of GLO1 prevents MG accumulation (Shinoharaet al., 1998); conversely, GLO1 inhibition increases MG accumulation and decreases cell viability (Kuhlaet al., 2006).

Interestingly, we found that in MG-treated mice GLO1 enzyme activity was found reduced in hippocampus, but not in cortex. Hence, we suggest that the reduced hippocampal activity of GLO1 failed to limit brain MG's cytotoxic effects in a brain-area specific manner,

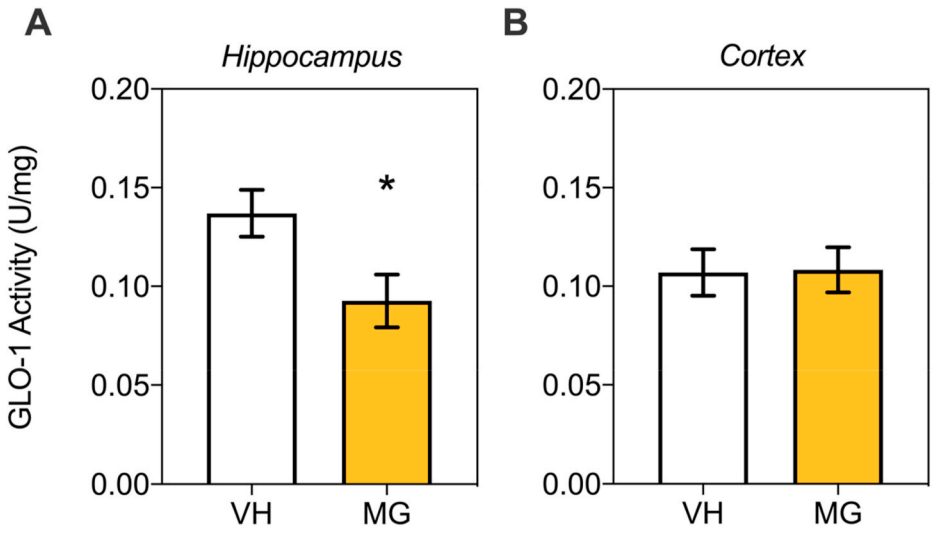

Fig. 5. Effects of MG on GLO1 activity in two brain areas: GLO1 activity in Hippocampus (A) and Cortex (B) of MG and VH mice. Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance * $p < 0.05$ vs. control.

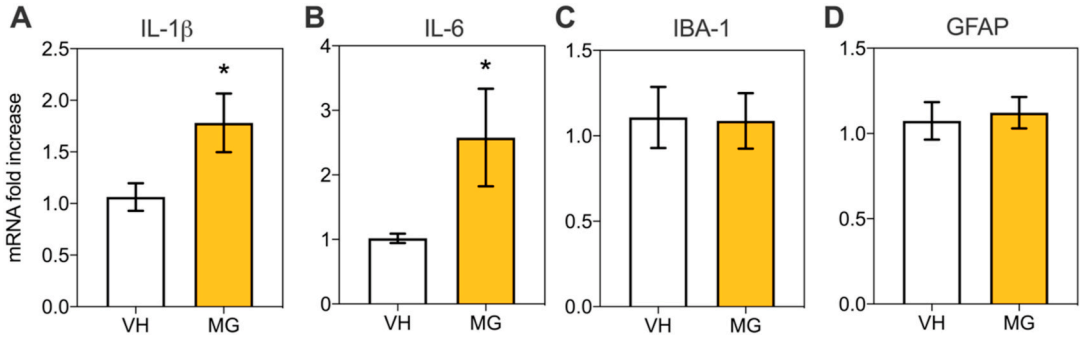

Fig. 6. Effects of MG on inflammation evaluating the gene expression of pro-inflammatory cytokines and the activation of microglia and astrocyte. Hippocampal gene expression of IL-1β (A), IL-6 (B), IBA-1 (C) and GFAP (D) in MG and VH mice. Data are expressed as the fold change of the target gene normalized to the internal standard control gene (β-actin). Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance. *p < 0.05 vs. control.

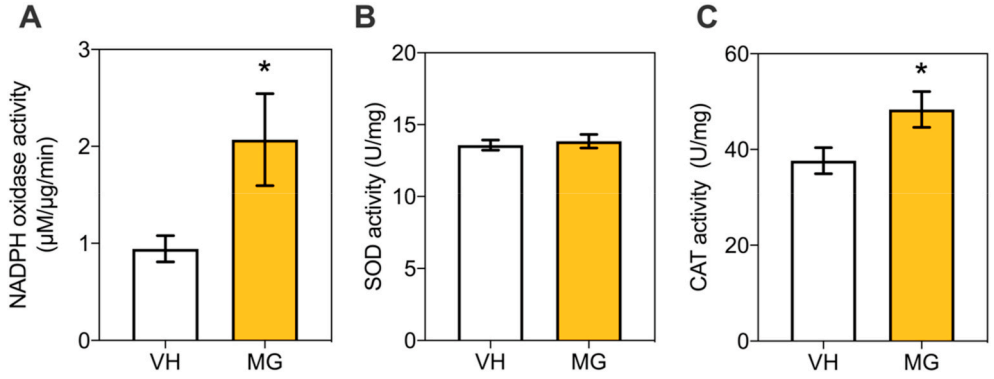

Fig. 7. Effects of MG on redox homeostasis evaluating the activity of pro-oxidant and antioxidant enzymes. Graphic representation of the enzymatic activities of NADPH oxidase (A), SOD (B) and CAT (C) in hippocampi from MG and VH mice. Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance. *$p < 0.05$ vs. control.

contributing to the cognitive defect. Furthermore, the increased expression of RAGE always found in the hippocampus could be the result of MG accumulation favored in turn by the reduced GLO1 in this area. Similarly, Cai and colleagues (Caiet al., 2014) observed an increased protein expression of RAGE in whole-brain extracts derived from aged mice fed up to 18 months of age on an MG-supplemented (MG+) diet. These MG + mice also confirmed cognitive impairment and increased MG serum levels, and in addition exhibited increased levels of MG-lipid and MG-proteins in the brain, further supporting that high MG levels can act dangerously as brain glycotoxins.

AGEs/RAGE axis can trigger multiple intracellular signaling pathways including the activation of transcription factor nuclear factor-κB

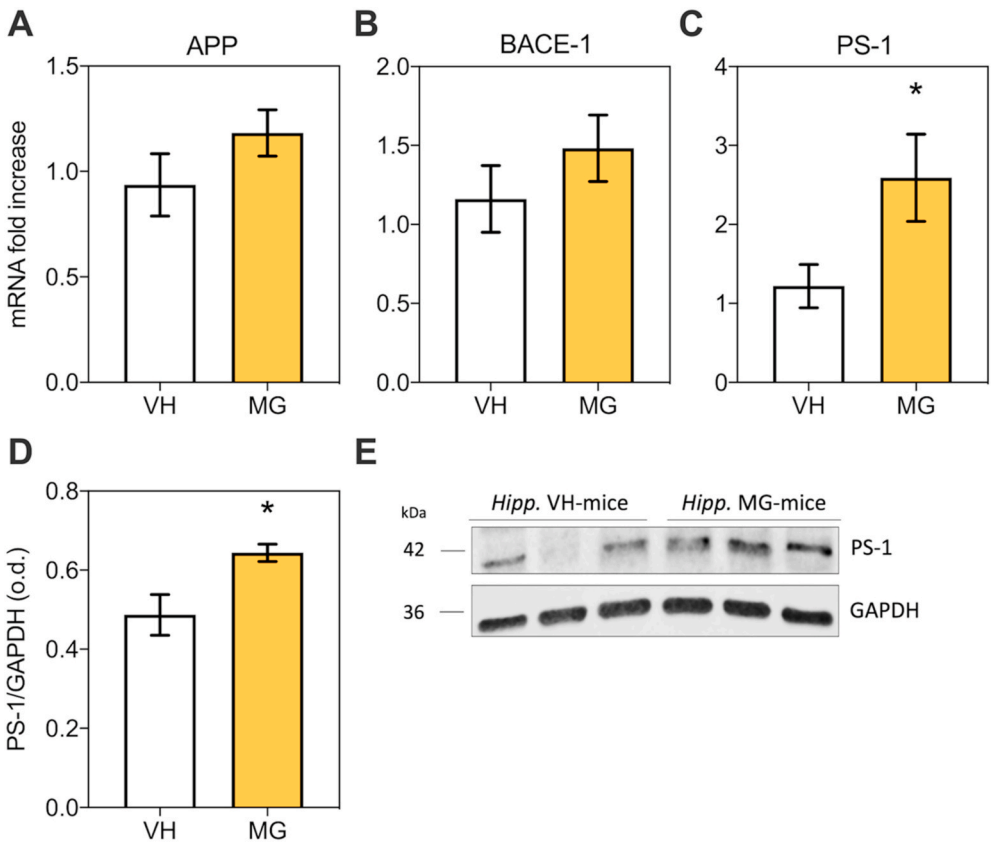

Fig. 8. Effects of MG in the modulation of genes involved in the APP amyloidogenic pathway.(A–C) gene expression of APP(A), BACE1(B) and PS1(C) in Hippocampus of MG and VH mice. Data are expressed as the fold change of the target genes normalized to the internal standard control gene (β-actin). Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance. (D) Representative Western blot analysis of PS-1 from hippocampi of mice treated with MG and VH. (E) Quantitative analysis of PS-1 normalized over the GAPDH optical signal. Data are shown as the mean ± SEM. Student t Tests followed by Bonferroni were used to test statistical significance. $*p < 0.05$ vs. control.

(NF-κB) that in turn can promote the production of pro-inflammatory cytokines such as IL-1β and IL-6, and inducing an inflammatory response (Juranek et al., 2015; Ramasamy et al., 2005). Here we confirmed MG is able to increase gene expression of IL-1β and IL-6 in the hippocampus of MG-mice. In contrast, astrocytic- and microglial-specific markers such as GFAP and IBA-1 were not found modulated by MG treatment, suggesting that microglia and astrocyte activation were not involved in MG-induced neuroinflammation. In line with this data also Hansen et al. (Hansenet al., 2016) showed that MG i.c.v. administration did not induce gliosis.

The AGEs/RAGE axis, when activated, is also able to trigger the generation of reactive oxygen species (ROS) via NADPH oxidase (NOX) (Piraset al., 2016). NOX catalyzes the one-electron reduction of dioxygen (O_2) to produce superoxide ($O_2^{\bullet-}$). The NOX family consists of at least seven different isoforms among which NOX2 is the specific isoform mainly found at hippocampal level (Tarafdar and Pula, 2018). In our experimental model NOX activity was increased after MG prolonged treatment, supporting the fact that an activation of RAGE pathway led to ROS formation. Consequently, increased ROS generation leads to the activation of the antioxidant defense system. For example, enzymes such as SOD catalyzes the dismutation of superoxide in hydrogen peroxide, which in turn is decomposed in water and oxygen from CAT (Abate et al., 2020). In our animal model, CAT activity was found increased in hippocampi of MG-mice, while SOD activity did not change if compared with VH-mice. Although SOD accelerates the dismutase of superoxide anion into hydrogen peroxide, this reaction can also evolve spontaneously when two superoxide molecules rapidly dismutase to hydrogen peroxide and molecular oxygen (Wang et al., 2018; Laitonjam, 2012). The increased enzyme activity of CAT is therefore perceived as an attempt to counteract the oxidative stress occurring in the hippocampus. Since it is well established that RAGE can bind Aβ and that this receptor is found increased in both AD mice models and AD human brains (Choiet al., 2014), we also investigated possible MG-induced alterations of APP metabolism. APP is a type-I transmembrane protein that, as already mentioned, undergoes sequential proteolytic processing. In particular, the APP pro-amyloidogenic pathway involves two secretases: β-secretase (BACE1) and γ-secretase (PS1) (Guo et al., 2020). In this study, MG treatment did not affect APP gene expression, as it was found unaltered in the hippocampus of MG mice. Interestingly, PS1 was significantly enhanced both at gene and protein levels, while BACE-1, showed only an increasing trend in gene expression. To our knowledge, this is the first time in literature that a direct link between MG treatment and PS-1 has been observed in mice brains. These results suggest that a high dietary MG intake could modulate APP processing in the brain, with a potential effect also on the Aβ42/Aβ40 ratio, further supporting that reduced glycotoxin intake should be favored for AD prevention. Further studies will be needed to better clarify whether the increased expression of PS-1 in the hippocampus of MG-mice is directly linked to MG/RAGE axis activation.

From the data collected, we demonstrated that a diet enriched with MG can induce cognitive impairment by selectively affecting GLO1 activity and increasing the gene and protein expression of AGEs receptor (RAGE) and of γ-secretase (PS-1) in the hippocampus. MG is also able to activate the RAGE intracellular signaling, leading to neuroinflammation and redox-homeostasis unbalance. Since in humans, dietary or smoking-derived MG content is a modifiable lifestyle factor (Uribarriet al., 2010), choosing a diet with a low AGE content and a proper lifestyle may promote healthy aging and even prevent AD pathology.

CRediT authorship contribution statement

M. Pucci: Conceptualization, Investigation, Formal analysis, Writing – original draft. **F. Aria:** Investigation, Writing – review & editing. **M. Premoli:** Investigation, Writing – review & editing. **G. Maccarinelli:** Investigation, Writing – review & editing. **A. Mastinu:** Investigation, Writing – review & editing. **S. Bonini:** Formal analysis, Writing – review

& editing. **M. Memo:** Writing – review & editing, Funding acquisition. **D. Uberti:** Conceptualization, Supervision, Funding acquisition, and, Writing – original draft. **G. Abate:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abate, G., Marziano, M., Rungratanawanich, W., Memo, M., Uberti, D., 2017. Nutrition and AGE-ing: focusing on Alzheimer's disease. Oxid. Med. Cell. Longev. 2017.
Abate, M. Vezzoli, Sandri, M., Rungratanawanich, W., Memo, M., Uberti, D., 2020. Mitochondria and cellular redox state on the route from ageing to Alzheimer's disease. Mech. Ageing Dev. 192, 111385. August.
Aria, F., et al., 2020. Brain structural and functional alterations in mice prenatally exposed to LPS are only partially rescued by anti-inflammatory treatment. Brain Sci. 10 (9), 1–21.
Bellier, J., et al., 2019. Methylglyoxal, a potent inducer of AGEs, connects between diabetes and cancer. Diabetes Res. Clin. Pract. 148, 200–211.
Bongarzone, S., Savickas, V., Luzi, F., Gee, A.D., 2017. Targeting the receptor for advanced glycation endproducts (RAGE): a medicinal chemistry perspective. J. Med. Chem. 60 (17), 7213–7232.
Butterfield, A., Halliwell, B., 2019. Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. Nat. Rev. Neurosci. 20 (3), 148–160.
Cai, W., et al., 2014. Oral glycotoxins are a modifiable cause of dementia and the metabolic syndrome in mice and humans. Proc. Natl. Acad. Sci. U. S. A 111 (13), 4940–4945.
Chaney, M.O., et al., 2005. RAGE and amyloid beta interactions: atomic force microscopy and molecular modeling. Biochim. Biophys. Acta - Mol. Basis Dis. 1741 (1–2), 199–205.
Chaudhuri, J., et al., 2019. The role of advanced glycation end products in aging and metabolic diseases: bridging association and causality. Cell Metabol. 28 (3), 337–352.
Chen, J.H., Lin, X., Bu, C., Zhang, X., 2018. Role of advanced glycation end products in mobility and considerations in possible dietary and nutritional intervention strategies. Nutr. Metab. 15 (1), 1–18.
Choi, B.R., et al., 2014. Increased expression of the receptor for advanced glycation end products in neurons and astrocytes in a triple transgenic mouse model of Alzheimer's disease. Exp. Mol. Med. 46 (2), 1–10.
de Bari, L., Atlante, A., Armeni, T., Kalapos, M.P., 2019. Synthesis and metabolism of methylglyoxal, S-D-lactoylglutathione and D-lactate in cancer and Alzheimer's disease. Exploring the crossroad of eternal youth and premature aging. Ageing Res. Rev. 53, 100915. October 2018.
Di Emidio, G., et al., 2019. SIRT1 participates in the response to methylglyoxal-dependent glycative stress in mouse oocytes and ovary. Biochim. Biophys. Acta - Mol. Basis Dis. 1865 (6), 1389–1401.
Dominguez, L.J., Barbagallo, M., 2018. Nutritional prevention of cognitive decline and dementia. Acta Biomed. 89 (2), 276–290.
the Effects of Gómez-Pinilla, F Gómez-Pinilla, F., 2008. The effects of nutrients on brain function, july Nat. Rev. Neurosci. 9, 568–578. https://doi.org/10.1038/nrn2421. BrainNutrients on Brain Function. Nat. Rev. Neurosci., vol. 9, no. july, pp. 568–578, 2008.
Guo, T., Zhang, D., Zeng, Y., Huang, T.Y., Xu, H., Zhao, Y., 2020. Molecular and cellular mechanisms underlying the pathogenesis of Alzheimer's disease. Mol. Neurodegener. 15 (1), 1–37.
Hansen, et al., 2016. Methylglyoxal can mediate behavioral and neurochemical alterations in rat brain. Physiol. Behav. 164, 93–101.
Hansen, F., et al., 2017. Methylglyoxal induces changes in the glyoxalase system and impairs glutamate uptake activity in primary astrocytes. Oxid. Med. Cell. Longev. 2017.
Hu, N., Yu, J., Tan, L., Wang, Y., Sun, L., Tan, L., 2013. Nutrition and the Risk of Alzheimer ' S Disease, 2013.
Hudson, I., Lippman, M.E., 2017. Targeting RAGE signaling in inflammatory disease. October Annu. Rev. Med. 69, 349–364, 2018.
Jandeleit-Dahm, K., Watson, A., Soro-Paavonen, A., 2008. The AGE/RAGE axis in diabetes- accelerated atherosclerosis. Clin. Exp. Pharmacol. Physiol. 35 (3), 329–334.
Juranek, J.K., Daffu, G.K., Wojtkiewicz, J., Lacomis, D., Kofler, J., Schmidt, A.M., 2015. Receptor for advanced glycation end products and its inflammatory ligands are upregulated in amyotrophic lateral sclerosis. Front. Cell. Neurosci. 9, 485.
Kalache, et al., 2019. Nutrition interventions for healthy ageing across the lifespan: a conference report. Eur. J. Nutr. 58 (s1), 1–11.
Klimova, B., Dziuba, S., Cierniak-Emerych, A., 2020. The effect of healthy diet on cognitive performance among healthy seniors – a mini review. Front. Hum. Neurosci. 14 (August), 1–9.
Kuhla, B., et al., 2006. Age-dependent changes of glyoxalase I expression in human brain. Neurobiol. Aging 27 (6), 815–822.
Laitonjam, W.S., 2012. Natural Antioxidants (NAO) of Plants Acting as Scavengers of Free Radicals, vol. 37. Elsevier.
Leerach, N., et al., 2021. Glycation reaction and the role of the receptor for advanced glycation end-products in immunity and social behavior. Glycoconj. J. 38 (3), 303–310.
Li, J., Liu, D., Sun, L., Lu, Y., Zhang, Z., 2012. Advanced glycation end products and neurodegenerative diseases: mechanisms and perspective. J. Neurol. Sci. 317 (1–2), 1–5.
Majtan, J., 2011. Methylglyoxal - a potential risk factor of manuka honey in healing of diabetic ulcers. Evid. base Compl. Alternative Med. 5–9, 2011.
Mannervik, Aronsson, A.C., Marmstål, E., Tibbelin, G., 1981. Glyoxalase I (rat liver). Methods Enzymol. 77.
L. B. Martins, A. L. Malheiros Silveira, and A. L. Teixeira, "The link between nutrition and Alzheimer's disease: from prevention to treatment.," Neurodegener. Dis. Manag., vol. 11, no. 2, pp. 155–166, Apr. 2021.
Marziano, M., et al., 2019. Monitoring Caco-2 to enterocyte-like cells differentiation by means of electric impedance analysis on printed sensors. Biochim. Biophys. Acta Gen. Subj. 1863 (5), 893–902.
Mastinu, et al., 2019. Gamma-oryzanol prevents LPS-induced brain inflammation and cognitive impairment in adult mice. Nutrients 11 (4), 1–13.
Ogasawara, Y., Tanaka, R., Koike, S., Horiuchi, Y., Miyashita, M., Arai, M., 2016. Determination of methylglyoxal in human blood plasma using fluorescence high performance liquid chromatography after derivatization with 1,2-diamino-4,5-methylenedioxybenzene. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1029 (1030), 102–105.
Pan, Bo, et al., 2017. TFEB activation protects against cardiac proteotoxicity via increasing autophagic flux. J. Mol. Cell. Cardiol. 113, 51–62.
Pan, B., et al., 2014. c-Abl tyrosine kinase mediates neurotoxic prion peptide-induced neuronal apoptosis via regulating mitochondrial homeostasis. Mol. Neurobiol. 49, 1102–1116.
Pan, Y., et al., 2015. STI571 protects neuronal cells from neurotoxic prion protein fragment-induced apoptosis. Neuropharmacology 93, 191–198.
Pan, B., et al., 2019. Highly dynamic changes in the activity and regulation of macroautophagy in hearts subjected to increased proteotoxic stress. Front. Physiol. 26 (10), 758.
Pan, B., et al., 2020. The calcineurin-TFEB-p62 pathway mediates the activation of cardiac macroautophagy by proteasomal malfunction. Circ. Res. 31 (4), 502–518.
Petersen, R.C., et al., 2021. NIA-AA Alzheimer's disease framework: clinical characterization of stages. Ann. Neurol. 89 (6), 1145–1156.
Piras, S., et al., 2016. RAGE expression and ROS generation in neurons: differentiation versus damage. Oxid. Med. Cell. Longev. Figure 1, 2016.
Ramasamy, R., Vannucci, S.J., Du Yan, S.S., Herold, K., Yan, S.F., Schmidt, A.M., 2005. Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. Glycobiology 15 (7).
Reichelt, C., Westbrook, R.F., Morris, M.J., 2017. Editorial: impact of diet on learning, memory and cognition. Front. Behav. Neurosci. 11 (May), 10–11.
Rungratanawanich, W., et al., 2019. Γ-Oryzanol improves cognitive function and modulates hippocampal proteome in mice. Nutrients 11 (4), 1–15.
Scarmeas, N., Anastasiou, C.A., Yannakoulia, M., 2018. Nutrition and prevention of cognitive impairment. Lancet Neurol. 17 (11), 1006–1015.
Sergi, D., Boulestin, H., Campbell, F.M., Williams, L.M., 2021. The role of dietary advanced glycation end products in metabolic dysfunction. Mol. Nutr. Food Res. 65 (1), 1–11.
Serra, M.C., Dondero, K.R., Larkins, D., Burns, A., Addison, O., 2020. Healthy lifestyle and cognition: interaction between diet and physical activity. Curr. Nutr. Rep. 9 (2), 64–74.
Shinohara, M., et al., 1998. Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. J. Clin. Invest. 101 (5), 1142–1147.
Singh, V.P., Bali, A., Singh, N., Jaggi, A.S., 2014. Advanced glycation end products and diabetic complications. KOREAN J. PHYSIOL. PHARMACOL. 18 (1), 1–14.
Srikanth, V., et al., 2013. Methylglyoxal, cognitive function and cerebral atrophy in older people. Journals Gerontol. - Ser. A Biol. Sci. Med. Sci. 68 (1), 68–73.
Tamanna, N., Mahmood, N., 2015. Food processing and maillard reaction products: effect on human health and nutrition. Int. J. Food Sci. 2015.
Tarafdar, Pula, G., 2018. The role of NADPH oxidases and oxidative stress in neurodegenerative disorders. Int. J. Mol. Sci. 19 (12).
Uribarri, J., et al., 2010. AGE's in Foods and practical ways to reduce them. J. Am. Diet Assoc. 110 (6), 911–916.
Wang, Y., Branicky, R., Noë, A., Hekimi, S., 2018. Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. 217 (6), 1915–1928.
Xu, X., 2009. γ-Secretase catalyzes sequential cleavages of the AβPP transmembrane domain. J. Alzheim. Dis. 16 (2), 211–224.

Yamagishi, S., Matsui, T., 2018. Role of hyperglycemia-induced advanced glycation end product (AGE) accumulation in atherosclerosis. Ann. Vasc. Dis. 11 (3), 253–258.
Zhao, Y., Wang, P., Sang, S., 2019. Dietary genistein inhibits methylglyoxal-induced advanced glycation end product formation in mice fed a high-fat diet. J. Nutr. 149 (5), 776–787.
Zunkel, K., Simm, A., Bartling, B., 2020. Long-term intake of the reactive metabolite methylglyoxal is not toxic in mice. Food Chem. Toxicol. 141, 111333. January.

2.2.1 Limitations of the study

The reasons to perform the administration by oral gavage were covered above. However, MGO within the food may interact with other nutrients and compounds leading to the formation of AGEs and other modifications that in turn can influence the absorption of food components and lead to the observation of peculiar effects. Providing an MGO-supplemented diet could better mirrors the effects of a daily high intake of MGO with food. In this regard, this type of administration route may be considered in our future studies.

Furthermore, we hypothesized that the changes observed in the hippocampus were caused by a GLO1 deficiency and the resulting increase in RAGE expression was a result of an accumulation of MGO and MGO-derived AGEs. To validate this hypothesis, we should have measured the levels of MGO and glycated proteins in the hippocampus.

Moreover, another limitation of the study is that no data about MGO hepatic metabolism as well as its liver toxicity were not collected yet. The liver plays a key role in the metabolism of drugs, toxins, and other exogenous compounds, as well as in the metabolism of endogenous substances. Consequently, the ability of the liver to metabolize these substances can have a significant impact on their pharmacokinetics and pharmacodynamics. Several serum biomarkers can be used to assess hepatic metabolism, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). These biomarkers are released into the bloodstream when liver cells are damaged or inflamed, and their levels can be measured using standard assays. Real-time PCR and Western Blot will also be used to measure the mRNA and the protein expression of specific CYP450 enzymes in the liver of mice, respectively.

3. Ongoing and future studies

3.1 Is MGO the link between gut and brain?

MGO and MGO-derived AGEs has been found to increase inflammation in the gut [169], which in turn can cause the release of proinflammatory cytokines that negatively impact the intestinal barrier. This is seen through disruptions of the cytoskeleton and changes in the expression and position of proteins involved in the maintenance of the barrier integrity such as claudin-1 (cldn1), occludin (ocln), and zonula occludens (ZO)-1 [170], [171]. Furthermore, chronic inflammation can affect the plasticity and the function of highly specialized secretory epithelial cells known as Paneth Cells (PCs), which support the stem cell niche and mucosal immunity through the release of growth factors and antimicrobial peptides. If the function of these cells is affected, the development of chronic diseases such as type-1 diabetes, Crohn's disease, and multiple sclerosis may occur [172]. In this regard, the aim of our follow-up study of this project was to investigate the possibility that a high intake of MGO could cause changes in the intestine, potentially contributing to the previously observed cognitive effects through the connection between the gut and the brain. In this frame, a thorough examination of the impacts of MGO on the gut is necessary.

3.1.1 Methods

From the same mice administered either with 100 mg/kg methylglyoxal (MGO) solution or vehicle by oral gavage daily for 4 weeks, small intestinal tissue samples were collected from each group and stored at -80°C until analysis. The intestinal tissues were processed to prepare histological samples for microscopic examination. The morphology of the intestinal tissues was assessed using paraffin slices. Briefly, small intestines derived from MG and VH mice were fixed in formaldehyde, sectioned at 2 µm, and observed using hematoxylin and eosin staining. After conventional hematoxylin and eosin (H&E) staining, the samples were processed for immunohistochemical evaluation. Briefly, sections were deparaffinized with Xilolo 100% and rehydrated with Alcohol 100%. After 20 min of incubation with 0.3% H_2O_2 in methanol, antigen retrieval was incubated using 1.0 mM EDTA buffer (pH 8.0). Then, the sections were washed in Tris-HCl (pH 7.4) and incubated, at room temperature for 1h, with rabbit anti-lysozyme (Cell

Marque) to characterize Paneth cells. Then, the slides were washed in Tris-HCl (pH 7.4) and incubated at room temperature for 15 min with post primary and for 10 min with polymer (Kit BOND polymer refine detection, Leica Biosystem). The immunostaining was performed to carried out DAB-system.

Intestinal tissues derived from MGO and VH mice were processed for mRNA analysis. Total intestinal RNA was isolated using TRI reagent (Sigma-Aldrich). Two μg of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega). RT-qPCR was conducted to analyze pro-inflammatory cytokines (IL-1β, IL-6, IL-17A, IL-22) and specific receptor (RAGE). The murine-specific primers were shown in Table 1. Amplification and detection were performed with the ViiA7 Real-Time PCR Detection System (Applied Biosystems). The reaction mix contained 6 μL of SYBR Green Master Mix (BIO-RAD Laboratories), 6 pmol of each forward and reverse primer, and 2 μL of diluted cDNA. The samples were run in duplicate, and the PCR program was initiated by 10 min at 95 °C before 40 cycles, each one of 1 s at 95 °C and 30 s at 64 °C. The gene expression levels were normalized to β-Actin expression and the data were presented as the fold change in target gene expression. Relative quantification was performed using the comparative Ct method.

Table 1. Primers used for RT-PCR

Genes	**Primers sequences**
B- Actin (β-Act)	f-5'-AGC CAT GTA CGT AGC CAT CC-3’ r-5'-CTC TCA GCT GTG GTG GTG AA-3'
Interleukin-1β (IL-1β)	f-5’-GCTTCAGGCAGGCAGTATC-3’ r-5’-TAATGGGAACGTCACACACC-3’
Interleukin-6 (IL-6)	f-5’-CCTACCCCAATTTCCAATGCT-3’ r-5’-TATTTTCTGACCACAGTGAGGAAT-3’
Interleukin-17A (IL-17A)	f-5'-TCC AGA AGG CCC TCA GAC TA-3’ r-5'-TTC ATT GCG GTG GAG AGT C-3'
Interleukin-22 (IL-22)	f-5'-TCCAACTTCCAGCAGCCATA-3’ r-5'-TTCCAGGGTGAAGTTGAGCA-3'
Receptor for AGEs (RAGE)	f-5'-CTGAACTCACAGCCAGTGTCCC-3’ r-5'-CCCTGACTCGGAGTT-3'

3.1.2 Preliminary results

- **MGO treatment induces pro-inflammatory effects in the small intestine independent from RAGE activation**

In order to assess intestinal inflammation, the gene expression of cytokines that play a role in the maintenance of intestinal balance, such as IL-17A and IL-22, and pro-inflammatory processes, like IL-6 and IL-1β, were assessed. Our results showed that MGO treatment led to a statistically significant increase in the gene expression of IL-17A, IL-22 and IL-6 compared to the control group, as shown in Figures 6A-C (p<0.05).

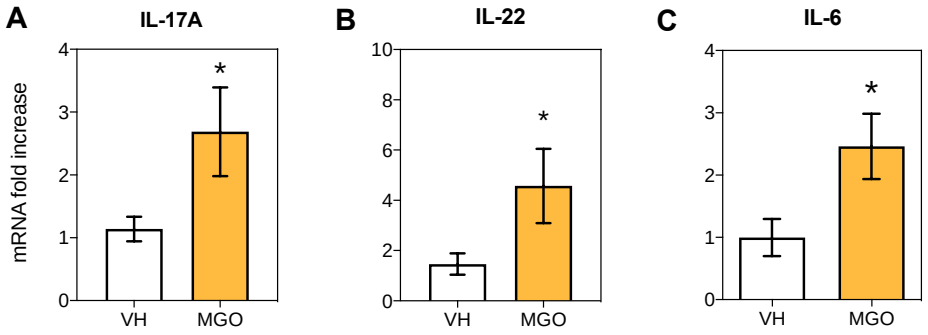

Figure 6. Intestinal gene expression of cytokines important in the maintenance of intestinal balance (IL-17A (A) and IL-22 (B)) and of pro-inflammatory cytokines (IL-6 (C)) in MGO and VH mice.

No differences were found in IL-1β mRNA expression between the two experimental groups (Figure 7A). To determine if RAGE receptors may play a role in the effects of MGO on the gut, we measured RAGE mRNA expression in the small intestine of MGO and control mice. In accordance with the result on IL-1β, no significant differences in RAGE mRNA levels were found between the MGO and control groups (Figure 7B), indicating that MGO effects on the gut may not be related to RAGE activation.

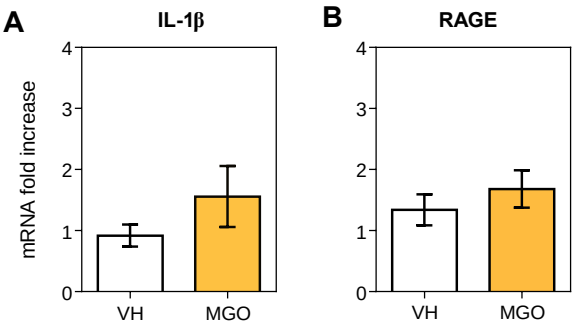

Figure 7. Intestinal gene expression of IL-1β (A) and RAGE (B) in MGO and VH mice.

- **MGO treatment increased the number of PCs**

Immunohistochemical analysis of histological sections of the small intestine revealed morphological changes in the small intestine microenvironment of mice treated with MGO, as shown in Figure 8. With E&O staining, we observed that a chronic administration of MGO led to morphological changes in the base of the intestinal villi, with a greater number of PCs compared to the control group. These changes were confirmed not only with E&O staining but also with the Lysozyme staining. Lysozyme (LYZ) is an antimicrobial peptide that can be released by PCs. As shown in Figure 8, an increase in LYZ in MG-treated mice than VH was observed.

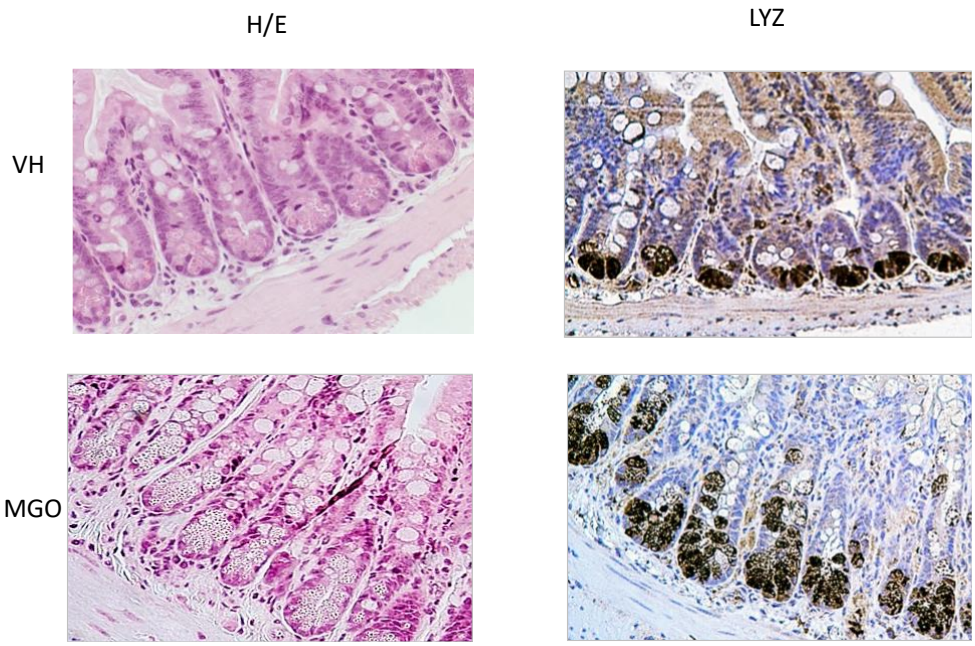

Figure 8. Morphological characterization of Paneth cells in small intestine of VH and MG treated mice.

3.1.3 Preliminary conclusions

In our study, we found through immunohistochemical analysis that long-term consumption of high levels of MGO affected the structure of the intestinal epithelium, leading to an increase in the proliferative capacity of PCs (Figure 8). This finding is consistent with previous studies that have shown a correlation between an increase in the proliferative capacity of PCs and the regenerative response of the intestinal stem cell niche to inflammatory damage [172].

By examining various key pathophysiological elements, we found that chronic administration of MGO can lead to pro-inflammatory conditions (Figure 6). The increase in IL-6 (Figure 6C) is likely caused by the activation of CD4+ T cells [173], [174] and is known to result in the production of IL-17A and IL-22 (Figures 6A-B). Notably, IL-22 plays a crucial role as both a pro- and anti-inflammatory cytokine, promoting and maintaining the integrity of the mucosal barrier [173]. Based on these preliminary results, the data suggests that a diet high in MGO can lead to intestinal inflammation independently of RAGE (Figure 7) and alteration of PCs, causing an imbalance in intestinal homeostasis.

3.1.4 Future experiments

- Further studies are required to confirm the potential link between MGO treatment and the onset of inflammation, as well as to gain a deeper understanding of the specific immune cells and mechanisms involved. In future experiments, we will utilize immunohistochemical analysis to identify and characterize T-lymphocyte populations, particularly CD4+ cells which have been linked to the production of IL-17 and IL-22.
- Membrane permeability plays a crucial role in inflammatory conditions, and the integrity of the intestinal barrier is regulated by tight junctions (TJs) such as ZO-1, cldn1, and occludin (ocln). Disruptions or alterations in TJ structure can disrupt the mucosal immune system and lead to the development of intestinal and systemic diseases. Therefore, in order to assess the integrity of the intestinal barrier, we will investigate the expression and localization of ZO-1, ocln and cldn1 by immunohistochemical analysis.

- Antimicrobial peptides (AMPs) are small peptides (>5 kDa) that are produced and released by PCs in response to pro-inflammatory cytokines, Gram-positive and Gram-negative bacteria, and bacterial products. They can bind to bacterial membranes, causing lysis of their components or forming pores that destroy the integrity of the membrane, which results in the release of bacterial cytoplasmic contents [175], [176]. In this regard, the study of the antimicrobial activity of gut protein extracts of MGO and VH mice will be performed. If the results showed that PCs lost their secretory expression signature, we will study the Wnt and Notch pathways involved in the differentiation and maturation processes of PCs.
- *In vivo* studies have shown that a decrease in microbiome diversity can lead to an imbalance in the gut microbial community [177]. In particular, diets high in AGEs can not only affect microbiota composition and contribute to the development of inflammatory bowel disease [177], [178], but also disrupt glucose homeostasis, leading to insulin resistance and an increased risk of metabolic syndrome [179]. To gain a deeper understanding of the connection between the gut and MGO, an examination of the composition of gut microbiota will be assessed.
- Finally, another aspect we would like to investigate is the impact of MGO on serotonin (5-HT) production in the gut. 5-HT is a neurotransmitter that plays a crucial role in the central nervous system, including the regulation of brain development, sleep, mood, appetite, and temperature [180]. Studies have shown that mice treated with MGO have increased levels of 5-HT in their blood, suggesting that MGO may stimulate 5-HT release by causing an influx of calcium in enterochromaffin cells [178], which can lead to headache and depression-like behavior. The literature suggests that these studies have great potential, as they can help us to understand the impact of diet and lifestyle on the progression of diseases related to gut inflammation and their connection to the central nervous system.

Chapter 2

4. Food processing compounds: friends or foes?

The industrial processing of food often includes heat treatment in alkaline or acidic solutions, that are exploited to: (i) develop pleasing colors and desirable flavors, (ii) improve or preserve texture; (iii) destroy micro-organisms, enzymes, toxins, and proteolytic inhibitors; (iv) prepare protein hydrolysates and concentrates and (v) enhance storage properties and solubility of proteins [181]. Nevertheless, these treatments can also lead to chemical changes that are responsible of undesirable alterations in food. In particular, food protein may be modified to a great extent during processing by different reactions such as, protein oxidation, amino acid crosslinks formation and Maillard reaction [182]. For the aim of the thesis, I will focus mainly on amino acid cross-links formation and Maillard Reaction. The next sections will describe the main compounds that are formed during food processing, focusing on the chemical processes responsible for their formation, the concentration in different kinds of food in which they have been mainly detected, and the data about their toxicity and safety for human health will also be introduced.

4.1 Amino acid cross-links

Protein cross-linking is one modification that occurs in food matrices during heat treatment. This protein modification does not depend on the presence of sugars [183] and happens with a two-step process: i) β-elimination of serine or cysteine takes place resulting in a dehydroalanine derivative (DHA), and ii) this derivative cross-links with either lysine or cysteine residues, forming lysinoalanine (LAL) and lanthionine (LAN), respectively [184]. These compounds–and in particular LAL–represent reliable markers of alkaline and heat treatment of foods [185]. Therefore, unprocessed or native food almost totally lacks LAL and LAN, whereas significant amounts of amino acid cross-links are found in processed food [185]–[187]. In particular, LAL has been detected in different kinds of dairy products [188], [189] while the identification of LAN was conducted to a lesser extent. Milk and dairy products are characterized by high concentrations of amino acid cross-links. The high levels of such

compounds can be due to: i) the high content of the DHA precursor–serine phosphate–in casein and cysteine in lactalbumin ii) the heat treatment used to preserve milk [190]. Also in infant formulas, high levels of both LAL and LAN have been detected [191]. The presence of these amino acid cross-links in formulas that represent for infants the only source of protein in their early phases of growth and development could be of concern. Recently, different studies have been carried out in order to better understand the chemical mechanisms that lead to the formation of these compounds and how, as commonly happens with protein modifications, this could eventually cause a reduction in the digestibility and nutritional quality of protein in infant formulas [192], [193].

In the last few years, the availability of data regarding LAN and LAL content in food is increasing. Today, no general regulatory limits on LAL contents in foods exist, with the exception of potato protein hydrolysate, for which a limit of 500 mg/kg (protein-bound) and 10 mg/kg (free) of LAL has been set in the European Union (EC 2002) [194].

4.2 The Maillard Reaction

The Maillard reaction (MR) was first reported in 1912 by the french chemist Louis-Camille Maillard, who described that upon gently heating sugars and amino acids in water, a yellow-brown colour developed. This phenomenon takes place as a consequence of the reaction between the carbonyl group of reducing sugars and the amino group of amino acids. MR is influenced by physical parameters, such as heating, hydration or pH, and leads to the formation of compounds not naturally present in foods that are referred to as Maillard reaction products (MRPs). The chemistry of MR has been considered extremely complex until 1953 when John Hodge published a consolidated scheme summarizing the reaction pathway. MR is divided into three principal steps: initial, intermediate and final (Figure 9). The initial reaction between sugars and amino acids, peptides or proteins leads to the formation of Schiff's base compounds, which undergo cyclization and in turn form reversible N-substitute glycosylamines. Rearrangements of this intermediate lead to early Maillard reaction products known as Amadori (if the initial sugar was an aldose) or Heyns products (if the sugar was a ketose). The intermediary stage starts with the decomposition of Amadori or Heyns products through several routes. At acidic pH, 1,2-enolization occurs producing furfural or hydroxymethylfurfural (HMF), while at alkaline pH, 2,3-enolization takes place creating reductones and other fission products like glyoxal, methylglyoxal and diacetyl. These α-

dicarbonyl compounds can then react with amino acids through Strecker degradation to form aldehydes and aminoketones or with nucleophic amino acid to produce advanced glycation end-products (AGEs). In the final stage of the reaction, additional polymerization reactions occur, causing the formation of complex compounds that can in turn form melanoidins. These brown pigments are usually identified as the final products of the MR [182], [195], [196]. In the next sections, I will describe in more details some of the main intermediates of the MR.

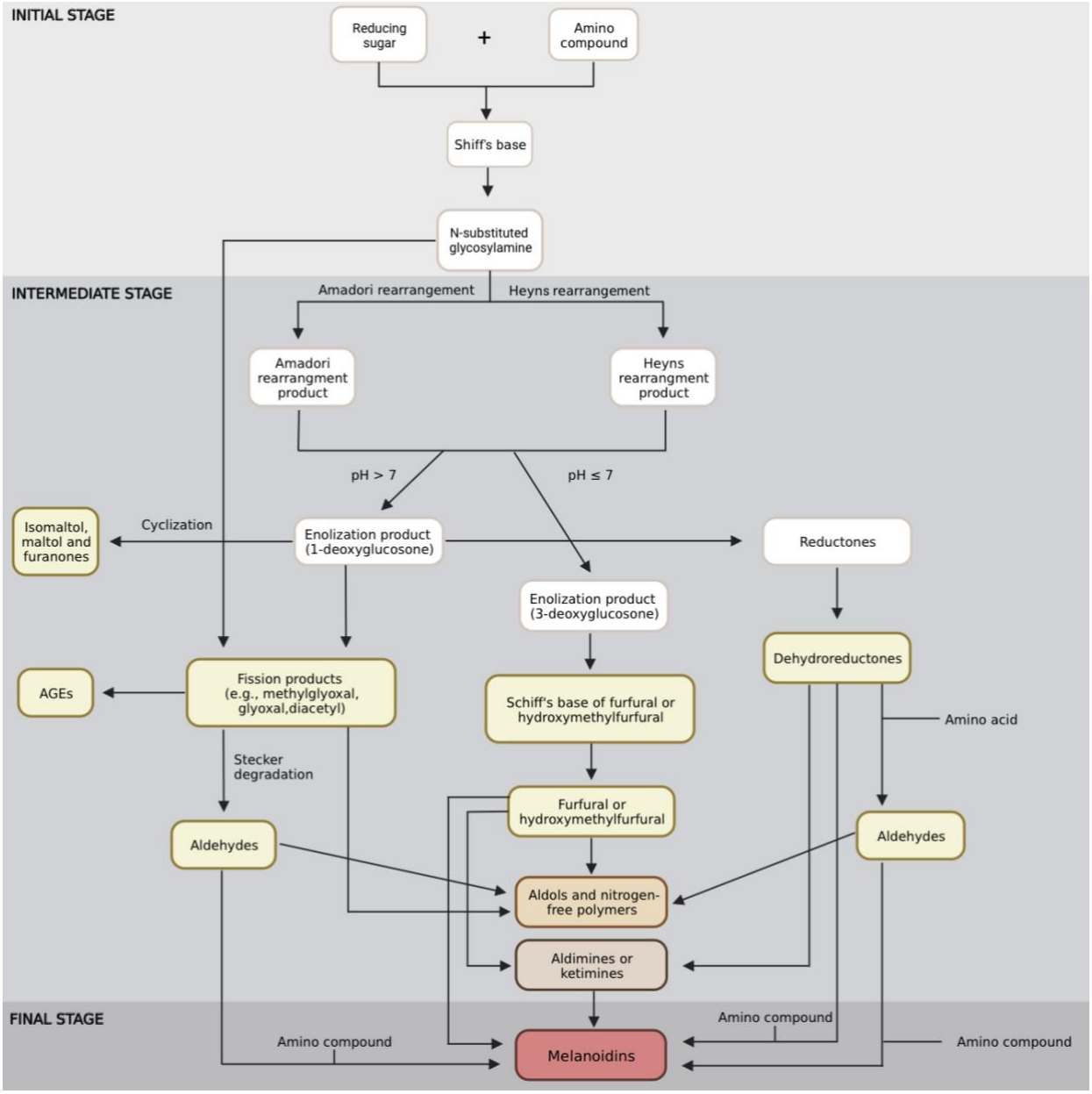

Figure 9. Schematic representation of the Maillard reaction. Re-adapted from Feng et al 2022.

4.2.1 Furosine

Furosine (also called ε-N-(2 furoyl- methyl)-L-lysine) is an amino acid derivative formed during acid hydrolysis of fructosyl-lysine, lactulosyl-lysine and maltulosyl-lysine, which are Amadori compounds produced by the reaction of ε-amino groups of lysine with glucose, lactose and maltose, respectively [197]. Furosine is broadly found in a variety of foods including cereals [198], honey [199], dairy [200] and bakery [195] products and its levels are tightly linked with the extent of a food processing and storage conditions and consequently to the extent of MR. Furosine is also a well-known marker of the nutritional value of heat-treated food, which always mirrors the degree of protein loss and food freshness [201], [202]. Particularly, the highest furosine content has been detected in infant formulas (471.9 mg/100 g to 639.5 mg/100 g) and honey (10.0 g/kg) [201], [203]. Currently, there are not many studies focusing on the toxicity of furosine. Data on kidney HEK-293T and liver HepG-2 cell lines showed that furosine acts as a strong genotoxic agent by inducing significant DNA damage and cell death, even at low concentrations [204]. A recent study also reported that in *in vivo* models high doses of furosine can cause detrimental effects on kidney and liver functions [205]. The same research group confirmed that furosine and one of its metabolic mediators can cause liver damage by triggering necroptosis [206]. Additional data should be collected in order to better evaluate the risks posed by furosine in food, with the final goal to define guidelines to control its formation during food processing and storage.

4.2.2 Acrylamide

Acrylamide is a chemical that naturally forms in starchy food products during everyday high-temperature cooking and in industrial processing at specific conditions. Many chemical mechanisms can lead to the formation of acrylamide in foodstuffs, but the main pathway involved is linked to MR. In particular, acrylamide is produced after the deamination and decarboxylation of asparagine [207]. As a consequence, cooked foods that are rich in free asparagine, like potatoes and cereals, represent a high source of acrylamide [208]. Other factors that influence the reaction are (i) the type and concentration of sugars and amino acids, (ii) temperature, and (iii) time. The highest acrylamide content is formed through roasting, frying, and baking methods. Acrylamide usually forms when elevated temperatures (above 120°C) are employed and low moisture conditions are present. A positive correlation has been found between acrylamide levels and heating time during the baking of biscuits at 200°C and in potato chips that were fried at more than 120°C [209], [210]. The highest level of

acrylamide was found in fried potato products. For instance, the average level of acrylamide in potato crisps was found to be 628 μg/kg, which is higher to the average concentration typically registered in biscuits (317 μg/kg), bread (136 μg/kg) and coffee (253 μg/kg) [211]. Studies in rodent models have highlighted that acrylamide exposure increases the risk of several types of cancer [212]–[215]. Because of this, studies reporting the presence of acrylamide in a wide range of fried and oven-cooked food have raised concerns worldwide [207]. Once introduced in the human body, acrylamide is converted to a compound called glycinamide, which causes mutations in and DNA damage. Despite the association between acrylamide and cancer being described in studies conducted on animal models, a large number of epidemiologic studies in humans have found no consistent evidence that dietary acrylamide exposure is associated with the risk of cancer [216], [217]. One reason for these contradicting evidences may be due to the difficulty in determining the personal acrylamide intake based on the reported diet. However, in 2015, the European Food Safety Authority (EFSA) issued a scientific opinion following a thorough risk assessment of the public health risks of acrylamide in food, in which experts conclude that this compound potentially increases the risk of developing cancer for consumers in all age groups [218]. Recently, EFSA also confirmed the genotoxicity of acrylamide mediated by the formation of glycinamide in addition to a potential contribution of non-genotoxic effects towards acrylamide carcinogenicity [219]. As a guide for food production, the European Union (EU) has set benchmark levels for an array of foodstuffs. The highest benchmark levels are set for instant coffee and gingerbread at 850 μg/kg and 800 μg/kg, respectively, and the lowest levels for food products such as baby food and wheat bread at 40 μg/kg and 50 μg/kg, respectively [220]. Additional epidemiologic studies in which acrylamide adducts or its metabolite levels are serially measured in the same individuals over time are necessary to further investigate the link between dietary acrylamide intake and increased cancer risks. Moreover, the pathways involved in acrylamide formation during the cooking process should be better evaluated and more analysis of food in terms of their acrylamide content should be done. Altogether, these results will allow more accurate and comprehensive estimations of dietary exposure to this compound.

4.2.3 α -dicarbonyl compounds

α-dicarbonyl compounds (α-DCs) are key intermediates formed by the degradation of Amadori compounds. α-DCs include C6-dicarbonyls such as 3-deoxyglucosone (3-DG) and 1-deoxyglucosone, and short-chain α-dicarbonyls (C2-, C3-, and C4-) like methylglyoxal (MGO),

glyoxal (GO), and diacetyl. To date, 3-DG seems to be quantitatively the most important of the C6-dicarbonyls detected, followed by 3-deoxygalacotosone (3-DGal), which is 3-DG C-4 epimer and can be found mostly in liquid food samples [27], [221]. High concentrations of 3-DG were detected in food such as fruit products [222], beer and malt [223], among others [26]. α-DCs are very reactive, especially MGO and GO. Due to their fast reaction with other compounds [224], short-chain α-dicarbonyls are usually detected in food in lower concentrations than C6-dicarbonyls [27], [222]. Measurable amounts of GO and MGO were identified in commercial cookies, bread, alcoholic beverages, coffee, soy sauce, vinegar, and dairy products [26], [225]–[229], and, especially MGO, in higher amounts in food such as honey and roasted sesame seeds [230], [231].

The recent interest in the effects of α-DCs on human health is due to their widespread occurrence in many foods and beverages. The effects of MGO as been fully covered above (Section 1.4). Regarding GO, *in vitro* studies have demonstrated that GO is genotoxic and mutagenic. It has been shown that GO binds to DNA and creates adducts [232], [233], causes mutations [234], [235], leads to chromosomal abnormalities [236], results in sister chromatid exchanges [237], and generates DNA single strand breaks [238], [239]. *In vivo*, Svendsen *et al.* demonstrated that after exposing the carcinogen-susceptible Min mouse to different concentrations of GO via drinking water, GO increased the average tumour size in the small intestine in both male and female mice model [240]. The exposure of diacetyl has been shown to cause severe damage to lung epithelial tissue, leading to a condition called bronchiolitis obliterans or "popcorn lung", which is a common and often fatal occupational hazard for workers in popcorn factories [241]. Recently, Xie *et al.* demonstrated that the administration of diacetyl not only reversed cognitive impairment but also impacts the aggregation of $A\beta_{1-42}$ by promoting fibrillization and significantly reducing the level of harmful soluble Aβ oligomers in the mouse brain [242]. 3-DG has been reported to be associated with diabetes and other age-related human diseases [243], [244]. The levels of 3-DG in the plasma of diabetic patients are approximately two times higher than usual and it plays a role in the progression of diabetic complications [245]–[247]. In addition, 3-DG possesses certain biological properties, such as suppressing enzyme activity during glucose metabolism and cell growth, triggering apoptosis, and deactivating glutathione reductase [248]–[250].

In addition to exerting their effects alone, several different effects have also been observed from α-DCs derivatives. α-DCs indeed can further degrade to form various compounds, including furfural and 5-hydroxymethylfurfural (HMF) or alternatively–as

previously mentioned–they can further react with nucleophilic amino acid residues, leading to the formation of AGEs [182].

4.2.4 HMF and furfural

HMF and furfural are furanic compounds produced in an intermediate stage of the MR during food thermal processing. Several factors influence the generation of HMF and furfural. Among them, we can find the concentration and type of sugars as well as low pH, low water activity and high baking temperatures [251]–[253]. Presence of HMF and furfural in food is not only due to its formation during MR but these compounds are also listed as flavouring substances in the EFSA database. HMF and furfural, but mostly HMF, have been widely used as indicators of the severity of heat treatment or length of storage in several foods. Besides, HMF has been detected in a wide range of various heat-processed foods, such as processed fruit products [222], bakery products [254], UHT milk [224], malt and beer [223], and coffee [255].

In the last few years, many studies have evaluated the safety of HMF [256], [257]. Some reports have shown that HMF at high concentrations is cytotoxic, and can cause irritation to eyes, upper respiratory tract and skin [258], [259]. In order to evaluate acute, subacute, and chronic toxicity of HMF, a toxicological study by the National Toxicology Program was piloted treating rats and mice for different intervals of time: 3 weeks, 3 months, and 2 years, respectively. From this evaluation, HMF was suggested to contribute to liver carcinogenesis in female mice and its administration was also associated with increased incidences of lesions of the olfactory and respiratory nose epithelium, regardless of the gender and the species (both in rats and mice) [260]. Moreover, after both *in vitro* and *in vivo* studies, scientists ascribed to 5-sulphooxymethylfurfural, the metabolite of HMF, a mutagenic and genotoxic activity [261], [262]. Despite these results being reported for rodent models, still no conclusive research conducted on humans confirms the toxicity of HMF. In this regard, EFSA stated no restriction on the use of HMF as a food flavouring [263]. The only product with guidelines concerning HMF is honey, whose levels are set by the Codex Alimentarius Commission (CAC) to be maximum 40 mg/kg (80 mg/kg in honey from tropical regions) [264]. Also, furfural has raised some concerns regarding its safety because short-term studies conducted on in *in vivo* models have shown hepatotoxic effects [265]. In 2011, for furfural and furfural diethyl acetal, EFSA established an accepted daily intake of 0.5 mg/kg body weight/day [266].

4.2.5 AGEs

AGEs are a heterogeneous group of compounds derived from the reaction between α-DCs and nucleophilic amino acid residues, such as the amine group of lysine the guanidine group of arginine, and the thiol group of cysteine [267].

N-ε- (carboxymethyl)lysine (CML) is one of the major AGEs and for this reason typically used as their marker. CML is formed in the reaction between GO and lysine [268] but can also derive by the Hodge pathway which includes the oxidation of Amadori products [269]. N-ε-(carboxyethyl)lysine (CEL) is instead formed by a reaction between MGO and lysine. Both CML and CEL have been identified in various foods and beverages, such as dairy, meat, cereal and bakery products, and nuts [184], [270]–[273]. Apart from CML and CEL, also MGO- and GO-lysine dimers (MOLD and GOLD) are part of lysine-derived AGEs. They are cross-links formed between two lysine residues and have been detected only at minor levels in foods [224], [274]. MG- and GO-derived hydroimidazolones are instead arginine-derived AGEs, which exist as three different isomers (MG-H1, MG-H2, and MG-H3 and GO-H1, GO-H2, and GO-H3) [275]–[277]. MG-H isomers have been detected in a wide range of food products, including UHT milk, dried pasta, malt and beer, meat, potato products, bakery products, and nuts [37], [184], [223], [224], [270], [271], [278]. However, arginine-derived AGEs as well as cysteine-derived AGEs are not well characterized in foods [279].

In foods, AGEs can exist as protein- and non-protein-bound. Usually, foods contain AGEs which are formed on amino acid residues of proteins, e.g., beer [270] and UHT milk [224], but some products, like soy sauce, contain higher concentrations of free AGEs compared to protein-bound AGEs [280].

Many of the effects of MGO-derived AGEs have been already described in the above sections. Among AGEs, CML represents the most well-characterized one. Its role has been studied through the years identifying this AGEs as an active player in inflammation and oxidative stress processes [281]. In general, the full understanding of the interaction between dietary AGEs and biological effects, the significance of the AGE-RAGE axis, and the role of AGEs in common physiological processes throughout life is still lacking. Consequently, advances at the molecular level in comprehending the physiological and pathological processes associated with the progression of AGE-related diseases has become crucial.

5. Foods in plant-based diet: concerning regarding their nutritional value and safety

The definition of a plant-based diet typically encompasses a diet that includes foods originating from plants (such as fruits, vegetables, nuts, seeds, legumes, and whole grains), but may also include small amounts of animal-based foods like dairy, eggs, meat, and fish. Plant-based diets are often associated with higher dietary quality and a reduced risk for chronic metabolic diseases commonly linked to the consumption of animal-based foods [282]–[285]. However, while some consumers are reducing their consumption of animal-based foods, a growing number of consumers still seek distinct sensory experiences, such as flavor, texture, mouthfeel, and satiety, that are traditionally associated with animal-based products. This has led to the development of innovative plant-based alternatives designed to replicate these experiences and meet the evolving demands of consumers [286]. From a public health perspective, there has been limited research on these new emerging products and many different aspects still need to be investigated.

From a nutritional point of view, van Vliet *et al*. caution against categorizing plant-based alternatives as nutritionally equivalent to animal-based products. Based on their metabolomics study, they concluded that the beef and the corresponding plant-based alternative are more likely to be complementary rather than interchangeable in terms of the nutrients they provide [287]. In addition, it should be noted that some plant-based beverages may not be adequate substitutes for animal-derived dairy products due to their limited nutrient diversity [288]–[290]. This discrepancy is particularly important for vulnerable populations, such as the growing trend of plant-based formula and nutrition products for infants and toddlers. Additionally, essential minerals like iron, zinc, magnesium, and calcium may not be as easily absorbed in some of the plant-based ingredients used in alternatives [291], [292]. Food processing may also lead to the loss of certain nutrients and phytochemicals found in plant-based foods. Additionally, some plant-based meat alternatives contain higher salt levels, which can negatively impact cardiovascular health over time [293]. Further research is needed to fully understand the nutritional impact of these products.

In addition, the safety of plant-based foods is another important factor that still needs to be strongly evaluated. The safety of plant-based alternatives is influenced by various factors, including the soil in which the source plants are grown, the methods of agriculture and

harvesting and the storage and processing methods used to obtain protein isolates. The higher diversity of ingredients used in plant-based products in comparison to animal-based products can also increase the potential risks associated with these products, making food safety a challenging issue to address for plant-based alternatives [294]. In this regard, another important aspect that needs to be considered when evaluating the safety of plant-based alternative foods is the formation of MRPs. Plant-based alternative foods are often subjected to more processing than their animal-based counterparts in order to achieve desired sensory experiences. They may also have different content and sources of protein, which can influence the formation of MRPs. Overall, as the demand for plant-based alternatives continues to grow, it is important for researchers, manufacturers, and consumers to consider not only the potential nutritional benefits but also the potential risks associated with these products. More research is needed to fully understand the nutritional and health impacts of these emerging products.

In this regard, by combining pharmacological and chemical analytical approaches, a more comprehensive understanding of MRPs occurrence in plant-based alternatives and their potential health effects can be provided. With the integration of these two approaches, researchers can identify specific MRPs that are most likely to contribute to adverse health outcomes and develop strategies to mitigate their formation during food production and preparation. Moreover, these multidisciplinary studies can help to identify potential therapeutic targets for the treatment of diseases associated with MRPs.

6. Our contribution

From our previous results, we demonstrated that a high MGO intake might favor in the brain the establishment of a damaged environment, reducing the ability of adaptive responses needed to preserve brain integrity. As diet represents one of the main exogenous sources of MGO and other food-processing compounds, it becomes critical to assess the content of these harmful compounds in food in order to evaluate their safety. MRPs formation can occur greatly in animal-derived foods that are high in fat, protein, and sugar, such as meat or milk. These foods are prone to MRPs formation as they can be subjected to cooking or thermal treatments to allow their consumption and ensure an extended shelf life [295], [296]. Numerous studies have been already undertaken over the years to assess the safety of animal-derived foods in terms of their content of food-processing compounds [297]. Recently, there

has been an increasing trend in the consumption and demand of plant-based alternative products due to a global transformation towards a more sustainable food system. However, plant-based foods are also processed by various techniques during their production from harvesting of the plants until packaging, yet very little is known so far about the formation of MRPs in these kinds of products [298]. For example, a study recently showed that glycation of plant proteins takes place in plants due to their photosynthetic metabolism and their proteome is continually subjected to glycation, resulting in early glycation adducts and AGEs [299]. Moreover, plant proteins have different amino acid profiles compared to animal proteins [300], therefore the susceptibility to MRPs formation can differ. Considering these differences between plant- and animal-based foods, proper identification and quantification of MRPs in plant-based foods are indispensable. Consequently, for the second part of my PhD project, I focused on the identification and quantification of different food-processing compounds in plant-based foods. This part of the project was carried out in collaboration with the University of Copenhagen at the Department of Food Science and under the supervision of Marianne Nissen Lund and Halise Gül Akillioglu, who have extensive expertise in the analysis and study of food protein modifications and MRPs formation [182], [184], [301]. The project was divided in two parts:

- ***Part 1- Investigation of Maillard Reaction in Plant-based Milk Alternatives***: different plant-based drinks purchased from local Danish supermarket were used for the study and analyzed in comparison with different kinds of cow's milks. The overall goal was to understand whether thermal processing techniques that occur during the production and packaging of these plant-based drinks can in turn modify their MRPs profile.
- ***Part 2- Evaluation of Maillard Reaction Products in different kinds of traditional and plant-based meat products:*** various types of meat-alternatives products were analyzed in order to investigate the risk posed by plant-based alternatives compared to traditional meats, in regard to the dietary exposure of MRPs.

In the context of nutritional intervention for the prevention of chronic diseases, the novelty of this research approach is to highlight aspects of food that are still little considered. Therefore, this part of my project represented a starting point in the study of Maillard reaction in plant-based food alternatives. Additional research needs to be carried out in this direction in order to identify new strategies to decrease the formation of these compounds in plant-based food with the final goal to set up rules that can be applied during production, manipulation, and thermal processes to obtain plant-based foods with high safety standards.

6.1 Part 1- Investigation of Maillard Reaction in Plant-based Milk Alternatives

6.1.1 Introduction

Milk has an exceptional and balanced composition of nutrients including proteins, amino acids, lipids, vitamins and minerals recognizing it as an essential part of human nutrition [302], [303]. Apart from being a wholesome complete food, the consumption of bovine milk has been linked to a wide range of physiological functionalities including anti-inflammatory [304], anti-oxidant [305], anti-adipogenic [306] and anti-osteoporosis [307] effects. Nevertheless, in the last decade, a great concern has grown around dairy production as it has a negative impact on the environment, contributing substantially to both climate change and water pollution [308]. In this frame, a global transformation towards sustainable food systems is crucial and plant-based dairy alternatives have attracted a lot of attention for their potential role in this green transition [309]. Besides, medical reasons such as lactose intolerance, milk allergies, and cholesterol issues and also lifestyle choices like a vegetarian/vegan diet or concerns about ethical use of animals have influenced consumers toward a growing demand for dairy alternatives [310], [311]. The popularity of plant-based drinks is also due to the presence of different functional components that have been associated with a beneficial role on human health [312]. For example, isoflavones within soy milk have shown to have protective effects against cardiovascular disease and osteoporosis [313], while β-glucans found in oat milk have demonstrated to reduce blood glucose levels and to exert hypocholesterolemic effect by reducing total and LDL cholesterol [314], [315]. Additionally, powerful antioxidant properties and a protective role against free-radical reactions have been associated with α-tocopherol found in almond milk [316]. Other functional components such as β-sitosterol and γ-oryzanol within rice milk are able to lower cholesterol and hypertension and can exert anti-diabetic, anti-inflammatory and anti-oxidant effects [317].

As the consumption of plant-based milk alternatives has increased over the last few years, many questions about these products have been raised, ranging from their actual definition to nutritional content, manufactural issues, health advantages, and safety [318]. Firstly, plant-based milk alternatives can be defined as water-soluble extracts derived from different kinds of plant material like cereals (e.g. oat, rice, corn, spelt), pseudo-cereals (e.g. quinoa, teff, amaranth), legumes (e.g. soy, peanut, lupin), nuts (e.g. almond, coconut, hazelnut, pistachio,

walnut) and seeds (e.g. sesame, flax, sunflower)[312]. Secondly, in order to meet consumer needs, these products should resemble the technical, nutritional and organoleptic properties of cow's milk. As the preparation of plant-based milk alternatives includes the disruption of plant material, colloidal systems characterized by dispersed particles different in size and composition are formed, making it difficult to obtain a stable food product. Moreover, in contrast with the bland but specific flavor of animal-based milk, the flavors of milk alternatives stem from the specific aroma of the plant raw material and from the processing and storage conditions, resulting typically in undesirable off-flavors. Additionally, it is widely recognized that animal and plant-based drinks differ in terms of nutritional composition. Indeed, most of plant-based milks are characterized by a lower protein content compared to cow's milk. In order to overcome these drawbacks, many of the processing steps necessary for plant-based milk production have been standardized throughout the years to mitigate the undesirable organoleptic characteristics and unbalanced nutrition of plant-based drinks [319]–[321]. For example, novel technologies such as ultrasound, pulsed electric fields, ohmic heating, and high and ultra-high-pressure homogenization have been applied to enhance stability [322]. On the other hand, the fortification of plant-based milk alternatives with micronutrients usually found in animal-based milks such as calcium, iodine, vitamin B12 and riboflavin [312], and the use of mixed-culture fermentation, hold great potential for improving the nutritional quality and the sensory profile of plant materials [319].

To ensure the safety and the quality of these products, the application of heat treatment is a crucial step in plant-based milk processing and represents the most-conventional methodology used to improve the shelf-life and to preserve the product from microbial contamination. During heating, the flavor and color of plant-based drinks can change to a great extent due to the Maillard reaction [323]. The Maillard reaction is an inevitable reaction that takes place between the carbonyl group of reducing sugars and the amino group of amino acids, peptides, or proteins, resulting in the formation of Maillard reaction products (MRPs). The Maillard reaction is a cascade of reactions comprising three principal stages: early, advanced and late. The initial reaction between sugars and amino acids, peptides or proteins leads to the formation of a Schiff base. Rearrangements of this intermediate lead to early MRPs known as Amadori (or Heyns, depending on the sugar) products. Furosine represents one marker of the early-stage Maillard reaction, formed from Amadori products during acid hydrolysis. Further rearrangements of Amadori products that include dehydration, elimination and condensations reactions typically occur during advanced Maillard reaction steps and result

in the formation of α-dicarbonyl compounds, furans, Strecker aldehydes and advanced glycation end products (AGEs), such as N-Ɛ-(carboxymethyl)lysine (CML), N-Ɛ-(carboxyethyl)lysine (CEL), methylglyoxal-derived hydroimidazolone isomers (MG-Hs), glyoxal-derived hydroimidazolone isomers (GO-Hs), methylglyoxal lysine dimer (MOLD) and glyoxal lysine dimer (GOLD). Other reactive Maillard reaction intermediates include furfural, 5-hydroxymethylfurfural (HMF), and acrylamide. Finally, additional polymerization reactions cause the formation of complex compounds that in turn can form melanoidins, brown pigments formed in the final stage of the Maillard reaction [182], [195], [196].

Protein cross-linking is another modification that occurs in food matrices during heat treatment. This protein modification does not depend on the presence of sugars [183] and happens by a two-step process: firstly β-elimination of serine or cysteine takes place resulting in a dehydroalanine derivative, secondly, this derivative cross-links with lysine or cysteine residues, forming lysinoalanine (LAL) and lanthionine (LAN), respectively [184].

MRPs and protein cross-links have been the subject of increasing attention as these compounds may form in high concentrations in foods that are rich in protein. Indeed these modifications can cause in turn a reduction in the nutritional value of food and also, some proteins are lost or become non-digestible [324]. Numerous studies have already been undertaken over the years to assess the safety of animal-derived foods in terms of their content of MRPs [296]. On the contrary, very little is known about the formation of MRPs in plant-based food [183] and to our knowledge, no other studies have been conducted in this direction on plant-based milk alternatives. Recently it has been shown that glycation of plant proteins takes place in plants due to their photosynthetic metabolism and their proteome is continually subjected to glycation forming early glycation adducts and AGEs [299]. Moreover, plant proteins have different amino acid profiles and generally a higher content of carbohydrates compared to animal-based proteins, therefore the susceptibility of formation of MRPs differs from animal-based foods [300], [325]. Thus, in the present study, several plant-based milk alternatives currently available in Danish supermarkets were analyzed for their potential to contribute to dietary MRPs. A comprehensive evaluation has been performed by state-of-the-art methods in order to understand the role of different types of plant-based drinks on dietary exposure to AGEs, α-dicarbonyl compounds, HMF, furfural and acrylamide.

6.1.2. Materials and Methods

6.1.1.1 Chemicals and consumables

The following standards and internal standards were purchased from Iris Biotech GmbH (Marktredwitz, Germany), with net weight values given in brackets; CEL (89.6%), CML (95.5%), GOLD TFA salt (94.1%), MG-H3 TFA salt, GO-H3 TFA salt (48.6%), furosine HCl (72.7%), LAL HCl salt (mixture of two diastereoisomers, 62.7%), MG-H1-d3 (90.5%), CEL-d4 (78.3%), MOLD-15N2 acetate salt (88.9%), furosine-d4 HCl (52.8%), and CML-d4 (94.4%). In addition, MOLD acetate salt (≥96%), GO-H1-13C2 (≥97%) and GOLD-15N2 acetate salt (≥96%) were also obtained from Iris Biotech GmbH; no net weight values were available from the supplier for these standards, so chromatographic purities are given in brackets. Glyoxal solution (40% in water), methylglyoxal solution (40% in water), dimethylglyoxal (≥97 %), 2-keto-D-glucose (D-glucosone, ≥98 %), 6- aminocaproic acid (≥ 99%) and amino acid standard for protein hydrolysate were purchased from Sigma Aldrich (Copenhagen, Denmark). 2-(2',3',4' -trihydroxybutyl) quinoxaline (quinoxaline form of 3-deoxyglucosone) was obtained from Biosynth Carbosynth (UK). Orthophenilene diamine (OPD, 98 %), diethylenetriaminepentaacetic acid (DETAPAC, ≥99 %), 5-(hydroxymethyl) furfural (HMF) (99.5 %), furfural (≥99 %), sodium dihydrogen phosphate monohydrate (≥99%), sodium phosphate dibasic anhydrous (≥99%), sodium tetraborate decahydrate (99,5%), sodium azide (≥99.99 %), O-phthaldialdehyde (OPA) (≥99%), 3-mercapto-propionic acid (MPA) (≥99%), acrylamide (≥99%), acrylamide-d3 standard solution (500 mg/L) were purchased from Sigma-Aldrich Chemie GmBh (Steinheim, Germany). Disodium hydrogen phosphate anhydrous (>99%) and formic acid (≥99 %) were obtained from VWR Chemicals (Denmark). Carrez I (potassium hexacyanoferrate (II) trihydrate) and dextrin 15 from maize starch were purchased from Fluka Biochemika (Switzerland) whereas Carrez II (Zinc sulphate heptahydrate), 1-octanol anhydrous (≥99%), maltose monohydrate, β-lactose, D-(+)-galactose, D-(+)glucose, D-(-)-fructose, sucrose, maltotriose were obtained from Sigma-Aldrich (Darmstadt, Germany). LC–MS grade acetonitrile, ammonium formate, methanol (99.995%) and HPLC grade acetonitrile, methanol, sulfuric acid were purchased from Sigma Aldrich (Copenhagen, Denmark). Nylon filter membranes (0.20 µm pore size, 47 mm diameter) were obtained from Phenomenex (Aschaffenburg, Germany). Oasis MCX 1 cc Vac Cartridges were purchased from Waters (Taastrup, Denmark). Milli-Q water was produced from a Millipore purification system (Millipore Corporation, Billerica, MA).

6.1.1.2 Food products

For this study, two UHT treated cow's milk (one whole milk and one semi-skimmed milk) and ten plant-based milk alternatives (six brands of oat, one soy, one rice, one almond and 1 mix (composed of soy, rice, almond and oat) drinks) were purchased from a local supermarket in Copenhagen. The six oat drinks will be referred to in the text on the basis of their fat content: 0.5, 1.5, 1.8, 1.9, 3 and 3.5%. Some of the drinks used in our study were from the same brand that can be divided in four "brand groups": 1) whole and semi-skimmed milk, 2) mix, soy, almond, rice and oat 1.9%, 3) oat 0.5%, oat 1.5% and oat 3.0% and 4) oat 1.8% and oat 3.5%. Table 2 summarizes their nutritional values according to the product labels while Table 3 reports the ingredients, and storage condition as well as other additional label information.

Table 2. Nutritional values of UHT milks and plant-based milk alternatives according to products label.

Products	Energy (kcal)	Fat (g)	Carbohydrates (g)	of which sugar (g)	Fiber (g)	Protein (g)	Salt (g)	Calcium (mg)	Vitamin D (μg)	Vitamin B12 (μg)	Riboflavin (mg)
UHT 1.5%	47	1.5	4.9	4.9	/	3.4	0.1	125	/	/	/
UHT 3.5%	64	3.5	4.9	4.9	/	3.4	0.1	125	/	/	/
Soy	37	2.1	0.6	0.6	0.6	3.7	0.09	/	/	/	/
Oat 0.5%	37	0.5	6.7	4.1	0.8	1	0.11	/	/	/	/
Oat 1.5%	46	1.5	6.7	4.1	0.8	1	0.1	120	0.5	0.38	0.21
Oat 1.8%	44	1.8	5.7	0	1	0.7	0.12	120	0.75	0.38	/
Oat 1.9%	52	1.9	8	3.2	1	0.4	0.08	/	/	/	/
Oat 3%	61	3	7.1	3.4	0.8	1.1	0.1	120	0.5	0.38	0.21
Oat 3.5%	59	3.5	5.7	0	1	0.7	0.12	120	0.75	0.38	/
Almond	21	0.9	2.7	2.4	0.3	0.4	0.09	/	/	/	/
Mix	46	2	3.4	2.3	0.6	3.4	0.19	120	1.5	0.38	0.21
Rice	54	1.1	10	6.5	/	0.1	0.09	/	/	/	/

Table 3. Ingredients, storage conditions and additional info reported on UHT milks and plant-based milk alternatives.

Products	*Ingredients*	*Storage conditions*	*Additional info*
UHT 1.5%	Semi-skimmed Milk (1,5% fat)	Stored in the fridge	
UHT 3.5%	Whole Milk (3,5% fat)	Stored in the fridge	
Soy drink (unsweetened)	Water, SOY *(10%) *organic ingredients.	Can be stored at normal room temperature until opening (max 25°C). After that, storage in the refrigerator (max 7°C) and shelf life approx. 4-5 days.	• Shake before use
Oat (0,5% fat) drink	Oat base (water, oats 10%), sea salt. *organic Free from milk and soy.	Unopened packaging can be stored at room temperature. Opened packaging can be stored as a refrigerated product at +8°C. After opening durable for at least 5 days.	• Natural sugars from oats • Shake before use
Oat (1,5% fat) drink	Water, oats 10%, rapeseed oil, minerals (calcium carbonate, calcium phosphates), salt, vitamins (D2, riboflavin, B12). Free from milk and soy.	Unopened packaging can be stored at room temperature. Opened packaging can be stored as a refrigerated product at +8°C. After opening durable for at least 5 days.	• Natural sugars from oats • Shake before use
Oat (1,8% fat) drink	Oat base (water, oats (8.7%)), sunflower oil, chicory root fiber, pea protein, calcium carbonate, acidity regulator (potassium phosphates), natural flavourings, sea salt, emulsifier (lecithins (sunflower)), stabiliser (gellan gum), vitamins (B12, D2). Free from milk. Naturally lactose-free	Keep in a cool place (max 7°C) and use within 5 days.	• Source of calcium - no sugar - source of vitamins B12 - natural low content of saturated fat, can be used hot or cold- naturally lactose free • Shake before use
Oat (1.9% fat) drink	Water, OATS* (16%), sunflower oil*, inulin*, sea salt. *organic ingredients. May contain traces of gluten.	Can be stored at normal room temperature until opening (max 25°C). After that, storage in the refrigerator (max 7°C) and shelf life approx. 4-5 days.	• Without added sugar-sweet comes from the oats themselves. • Shake before use
Oat (3% fat) drink	Water, oats 10%, rapeseed oil, acidity regulator (dipotassium phosphate), minerals (calcium carbonate), salt, vitamins (D2, riboflavin, B12). Free from milk and soya.	Unopened packaging can be stored at room temperature. Opened packaging can be stored as a refrigerated product at +8°C. After opening durable for at least 5 days.	• Natural sugars from oats • Shake before use
Oat (3,5% fat) drink	Oat base (water, oats (8.7%)), sunflower oil, chicory root fiber, pea protein, calcium carbonate, acidity regulator (potassium phosphates), natural flavourings, sea salt, emulsifier (lecithins (sunflower)), stabiliser (gellan gum), vitamins (B12, D2). Free from milk. Naturally lactose-free	Keep in a cool place (max 7°C) and use within 5 days.	• Source of calcium - no sugar - source of vitamins B12 - natural low content of saturated fat, can be used hot or cold- naturally lactose free • Shake before use
Almond drink	Water, cane sugar*, roasted ALMOND* (2%), sea salt, stabilizer (guar gum*, gellan gum). *organic. Can contain traces of nuts	Can be stored at normal room temperature until opening. After that, storage in the refrigerator (max 7°C) and shelf life approx. 4-5 days.	• Shake before use
Mix drink	Water, SOY, rice, OATS, calcium carbonate, sea salt, ALMOND, vitamin D, vitamin B2 (riboflavin), vitamin B12, natural flavouring.	Can be stored at normal room temperature until opening (max 25°C). After that, storage in the refrigerator (max 7°C) and shelf life approx. 4-5 days.	• Shake before use
Rice drink	Water, rice* (15%), sunflower oil*, sea salt. *organic.	Can be stored at normal room temperature until opening. After that, storage in the refrigerator (max 7°C) and shelf life approx. 4-5 days.	• Naturally lactose-free. • No added sugar-the sediment comes from the rice itself. • Shake before use

6.1.1.3 Freeze-drying of samples and dry matter (DM) determination

Aliquots of 20 mL of milk and plant-based drinks were added to 50 mL tubes and the weight of each sample was determined. Thereafter they were frozen overnight at -80 °C. Samples were then freeze-dried at −50 °C for 72 h under vacuum conditions (Modulyo, Edwards High Vacuum International, UK). After drying, dried drinks in the tubes were weighed and stored in the freezer (-20°C) for further analysis. The water removal during drying (dry matter) was calculated as % of weight change.

6.1.1.4 Protein content determination

Ten mg of dried samples were dissolved in 2.5 mL of 6 M urea. Afterwards, total protein content was measured using PierceTM BCA Protein Assay Kit by following the manufacturer's instructions with minor modifications. Briefly, samples were diluted two-fold with Milli-Q water and then 25 µL of diluted sample were pipetted into a 96-well plate. Each bovine serum albumin (BSA) standard was diluted two-fold with 6 M urea directly in the plate. Then 200 µL of Working Reagent (50:1, Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 sodium hydroxide): Reagent B (containing 4% cupric sulfate)) was added to each well and the plate was mixed thoroughly on a plate shaker for 30 s. The plate was then covered and incubated at 37 °C for 30 min. After cooling the plate to room temperature, the absorbance at 562 nm was measured and the average absorbance measurement of the black standard replicates was subtracted from the absorbance of all other individual standard and sample replicates. A standard curve was prepared by plotting the blank-corrected absorbance measurement for each BSA standard with its concentration in µg/mL and then used for the determination of the protein concentration of samples.

6.1.1.5 Preparation of hydrolyzed samples

For the determination of furosine, CEL, MGO-Hs, GO-Hs, GOLD, MOLD, LAL, LAN and total amino acid content, samples were subjected to acidic hydrolysis. In microwave glass tubes, dried samples containing 3-5 mg protein were added 3 mL 6 M HCl . Tubes were consequently flushed with nitrogen and tightly sealed. Afterwards, samples were hydrolyzed by microwave heating at 150 °C for 1 min followed by 10 min at 165 °C using a Biotage Initiator+ microwave synthesizer. Aliquots of 1000 µL of each hydrolysate were centrifuged at 10000 rpm for 10 min at room temperature and 500 µL of the resulting supernatant were evaporated to dryness by using a centrifugal vacuum concentrator. The residue was dissolved in 500 µL of Milli-Q water,

mixed vigorously and filtered through 0.22 µm nylon filters. For the determination of CML content, samples were reduced by sodium borohydride as previously described. In microwave glass tubes, dried samples with 3-5 mg protein were added 250 µL of Milli-Q water, 750 µL of sodium borate buffer (0.2 M, pH 9.2) and 500 µL 1 M sodium borohydride in 0.1 M NaOH. Samples were then incubated at room temperature for 4 h. Afterwards, 50 µL of the antifoaming reagent 1-octanol were added to reduce foaming that will occur during the subsequent addition of HCl. Samples were finally hydrolyzed by adding 1.45 mL of HCl (37%) using the same microwave protocol and conditions described above. After evaporation of acid, residues were dissolved in 500 µL of Milli-Q water and filtered through 0.22 µm syringe filters.

6.1.1.6 Analysis of furosine, AGEs and amino acid cross-links

The filtered hydrolyzates were used for analysis for furosine, AGEs (CML, CEL, MGO-Hs, GO-Hs, GOLD, MOLD) and amino acid cross-links (LAL and LAL) after proper dilutions. Analysis was performed according to [184]. Dilutions and addition of internal standards were prepared in order to reach a final sample solvent of acetonitrile: water (50:50, v/v). Five µL of the sample was injected into the Dionex UltiMate 3000 LC system (Thermo Fisher Scientific Inc., Waltham, USA) equipped with a Syncronis HILIC column (100 mm length × 2.1 mm internal diameter × 1.7 µm particle size, Thermo Fisher Scientific Inc., USA) maintained at 40 °C. The UHPLC system was directly interfaced to an OrbiTrap Q Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) operated in positive ionization mode. The chromatographic conditions and mass spectrometry parameters are described elsewhere [184]. The quantification of each compound was based on internal standard calibration by using a stable isotopically labeled internal standard.

6.1.1.7 Analysis of total amino acids

The filtered hydrolyzates were diluted with Milli-Q water. Working standard solutions were prepared by proper dilutions of the amino acid standard stock solution (500 µM) with 0.1 M HCl. Concentration levels of amino acids in the calibration solutions were in the range of 0 to 200 µM. Both samples and working standard solutions were spiked with an internal standard (aminocaproic acid solution, 50 µM) in a ratio of 1:1. Samples and working standard solutions were filtered through a 0.22 µm syringe membrane filter before HPLC analysis. For the analysis, the primary amino acids were derivatized by o-phthaldialdehyde in the presence of 3-mercapto propionic acid as described previously [326], [327]. Briefly, the amino acid

composition was analyzed by an ultra-high-performance liquid chromatography–fluorescence detection (UHPLC-FLD) system (Thermo Ultimate 3000 RS, Thermo Scientific, MA, USA) equipped with an Agilent AdvanceBio AAA column (100 mm length × 3.0 mm internal diameter × 2.7 µm particle size; Agilent Technologies, CA, USA) fitted to a guard cartridge. Mobile phase A consisted of 10 mM Na_2HPO_4 in 10 mM $Na_2B_4O_7$ (pH 8.2) whereas mobile phase B consisted of a mixture of acetonitrile, methanol and water at a ratio of 45:45:10 (v/v/v). A flow rate of 0.620 mL/min was used with the following gradient program: 0–0.35 min, 2% B; 0.35–13.4 min, 57% B; 13.4–13.5 min, 100% B; 13.5–15.7 min, 100% B; 15.7–15.8 min, 2% B; 15.8–18.0 min, 2% B. Fluorescence detection was carried out by using an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The amino acids in the samples were quantified based on the internal standard calibration method using authentic amino acid calibration standards.

6.1.1.8 Preparation of water extract of the samples

Dried samples (0.5 g) were added to 10 mL of Milli-Q water and homogenized using an Ultra-turrax homogenizer (T 25 digital ULTRATURRAX®) for 2 min at 12000 rpm. The homogenates were cooled in the freezer (-20 ℃) for 15 min and then centrifuged for 15 min at 7500 rpm at 0 °C to collect any major matrix components in the bottom pellet and separate the top fat layer. Aliquots of 15 mL of the clear supernatant was retrieved and stored in the fridge (5°C) until further use. This water extract was used for analysis of α-dicarbonyl compounds, HMF, furfural, acrylamide and sugar.

6.1.1.9 HMF and furfural analysis

HMF and furfural analysis was performed as described in [328]. Briefly 1.5 mL of the water extract was added of 50 µL Carrez I (7.5 g potassium hexacyanoferrate (II) trihydrate in 50 mL Milli-Q water) and 50 µL Carrez II (15 g zinc sulphate in 50 mL Milli-Q water) solutions. The tubes were vortexed and centrifuged for 10 min at 15000 rpm at room temperature. The clear supernatant was collected and filtered directly into UHPLC vial through 0.22 µm nylon filters. Ten µL of the sample were injected into a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific Inc., Waltham, USA) coupled to a quaternary pump.

The chromatographic separation was performed using a Zorbax Eclipse XDB-C18 column (150 mm length × 4.6 mm internal diameter × 5 µm particle size; Agilent Technologies, CA, USA) maintained at 40°C with isocratic conditions of 20% acetonitrile and 80% Milli-Q water at a flow rate of 0.500 mL/min. The HMF (retention time (RT): 4.197) and furfural (RT: 6.783)

were detected at 285 nm and 277 nm respectively. External standard calibration curves were constructed in the range of 1 and 1000 µg/mL by using standards of HMF and furfural.

6.1.1.10 α-dicarbonyl compounds analysis

200 µL of water extract were mixed with 1000 µL of ice-cold methanol using a vortex mixer and then incubated at -80°C for 2h. Afterwards, samples were centrifuged at 22000xg for 15 min at 0°C. 500 µL of supernatant were then mixed with 150 µL of phosphate buffer (0.5 M, pH 7.0) and 150 µL of OPD (0.2% w/v in 18.5 mM DETAPAC). Samples were filtered through 0.22 µm nylon filters into HPLC vials and incubated at 37°C for 2h in the dark to allow the derivatization of α-dicarbonyl compounds. For soy drink, the addition of phosphate buffer and OPD caused the appearance of an opalescence that could be due to a high concentration of calcium in the samples. In this regard, after the addition of the buffer and OPD, soy samples were first centrifuged at 22000xg for 15 min at 0°C and then the clear supernatant was filtered and incubated in the same condition of other samples. The quinoxaline derivatives of glucosone, galactosone, 3-deoxyglucosone, 3- deoxygalactosone, glyoxal, methylglyoxal, dimethylglyoxal were determined by LC–MS. Ten µL was injected into the same LC–MS system as described in section 2.6 equipped with an Acquity UPLC BEH Phenyl column (100 mm length × 2.1 internal diameter ×, 1.7 µm particle size; Waters, Taastrup, Denmark) at 55°C. The chromatographic conditions and mass spectrometry parameters are reported elsewhere [328].

Working solutions of glyoxal, methylglyoxal, dimethylglyoxal and glucosone in the range of 1–500 ng/mL were derivatized and analyzed as described above to build the external calibration curve of quinoxaline, 2-methylquinoxaline and 2,3-dimethylquinoxaline, and glucosone quinoxaline forms, respectively. Quantification of 3-DG was based on an external calibration curve prepared with 2-(2', 3', 4' -trihydroxybutyl) quinoxaline in Milli-Q water at a concentration range of 1–500 ng/mL. It was not possible to obtain the standards of galactosone or 3-deoxygalactosone or their quinoxaline forms, therefore, their concentrations were determined based on the standard curves of glucosone and 3-deoxyglucosone, respectively, after confirming their identity with LC-MS.

6.1.1.11 Acrylamide analysis

750 µL of water extract, 750 µL of Formic Acid (20 mM) containing acrylamide d-3 (53.3 ng/mL), 50 µL Carrez I and 50 µL of Carrez II were mixed vigorously using a vortex mixer for at least 1 min. Thereafter samples were centrifuged for 15 min at 15000 rpm at 10°C. After

centrifugation, 1 mL supernatant was filtered through a 0.22 µm nylon syringe filter. The filtrate was then passed through preconditioned MCX cartridges, where the first 8 drops were discarded, and the rest of the extract was collected into HPLC vials. Working concentrations of acrylamide were prepared in the range of 0.5-250 ng/mL and then 75 µL of each standard concentration, 75 µL of Formic Acid (20 mM) containing acrylamide d-3 (53.3 ng/mL) and 10 µL of Milli-Q water were mixed directly in HPLC vials. Five µL of samples were injected in a Vanquish LC system (Thermo Fisher Scientific Inc., Waltham, USA) equipped with an Acquity UPLC® HSS T3 column (100 mm length x 2.1 mm internal diameter x 1.8 µm particle size; Waters, Taastrup, Denmark) at 40°C. Chromatographic separation was performed using isocratic condition (98:2) of mobile phase A consisting of 10 mM formic acid solution and mobile phase B consisting of 10 mM formic acid in acetonitrile for a total run of 10 min and at a flow rate of 0.250 mL/min. The LC system was directly interfaced with a TSQ Quantis triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA) operated in positive heated electrospray ionization mode with a voltage of 3500 V and using the following interface parameters: ion transfer tube as well as the vaporizer temperature was 275 °C, sheath gas (60 arbitrary units), auxiliary gas (5 arb. unit), sweep gas (1 arb. unit).

Data acquisition was performed by selected reaction monitoring (SRM) mode where acrylamide was monitored by the transitions of m/z values 72.08 → 55.04, 44.04 and 27.18 and acrylamide-d3 by m/z values of 75.01 → 58.05, 44.04 and 30.20. m/z 55.04 and m/z 58.05 were used as the quantifier ions for acrylamide and acrylamide-d3, respectively.

6.1.1.12 Analysis of sugars

Sugars were analyzed by mixing 0.5 mL of the water extract with 100 µL of Carrez I and 100 µL Carrez II solutions. The tubes were vortexed vigorously and then centrifuged at 22000xg for 15 min at 22°C. 100 µL of the clear supernatant was collected, added to 900 µL of Milli-Q water and then filtered through 0.22 µm nylon filters directly into HPLC vial. Twenty µL were injected into an HPLC system coupled with a Refractive Index detector (RID) equipped with an Aminex HPX-87H Ion Exclusion Column (300 mm length x 7.8 mm internal diameter x 9 µm particle size) at 30°C. The chromatographic separation was performed in isocratic conditions (100% of 5 mM Sulfuric Acid) at a flow rate of 0.500 mL/min for a total time run of 30 min. External standard calibration curves were constructed in the range of 0.1 and 10 mg/mL by using standards of dextrin-15, maltose, sucrose, lactose, glucose, maltotriose, galactose, fructose and xylose for quantification.

6.1.1.13 Statistical analysis

Two-way Repeated Measures ANOVA tests with Tukey post-test were used to determine the statistical difference between UHT milks and plant-based milk alternatives. Data are presented as the means ± SD (standard deviation). All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, San Diego CA, USA), and the statistical significance level was set at $p<0.05$.

6.1.3 Results and Discussion

6.1.3.1 Amino acid composition

Plant-based food production uses less land, water, and contributes to lower greenhouse gas emissions than animal-based food [329]. However, plant-based proteins generally exhibit a lower nutritional quality than animal-derived proteins, due to the limitation of essential amino acids. Essential amino acids cannot be synthesized within the body and consequently must be supplied through the diet. An adequate intake of essential amino acids is critical for good health. For example, lysine is involved in supporting the growth and repair of tissues, whereas leucine has a key role in promoting muscle protein synthesis [330], [331]. Another example is methionine, which initiates the production of all eukaryotic proteins [332]. In this regard, the total amino acid composition of various plant-based drinks was analyzed in this study. The plant-based milk alternatives clearly contained significantly lower levels of essential amino acids compared to the milk sample (Table 1). However, this is not surprising considering the low protein content in these samples except for soy milk. After the hydrolysis, as expected, UHT milk samples showed the highest concentration of total essential amino acids (UHT 1.5%: 1480 $\pm$ 11 mg/100 mL; UHT 3.5%: 1375 $\pm$ 8 mg/100 mL). Among plant-based drinks, soy (1119 $\pm$ 5 mg/100 mL) and mix (1021 $\pm$ 8 mg/100 mL) drinks showed the closest total essential AA levels to UHT milk samples confirming them as the two plant-based drinks of our study with the most similar protein content to cow's milk. Rice drink was shown to contain the lowest amount of total essential AA (11 $\pm$ 1 mg/100 mL) in agreement with the low protein content of this drink. In line with that, lysine and leucine levels reflect this trend as comparable levels to UHT milk samples were found in soy (Lys: 217.1 $\pm$ 6.3 mg/100 mL; Leu: 285.2 $\pm$ 7.9 mg/100 mL) and mix (Lys: 181.6 $\pm$ 7.5 mg/100 mL; Leu: 249.3 $\pm$ 12.9 mg/100 mL) drinks and significantly low contents in rice (Lys: 1.3 $\pm$ 0.4 mg/100 mL; Leu: 4.2 $\pm$ 1.0 mg/100 mL) drink. In addition,

methionine contents were particularly low in all plant-based drinks when compared with milk but with great variability between plant-based protein drinks. These results are in line with the ones observed by Sousa *et al.* [333].

Table 4. Total Amino Acid (AA) content (mg/100mL) of milk and plant-based milk alternatives. Asx and Glx represent the sum of Asp and Asn. and Glu and Gln, respectively. The amount of Trp and Cys could not be determined as they were destroyed during the hydrolysis step with acid.

		Milks		*Plant-based Milk Alternatives*									
		UHT 1.5%	**UHT 3.5%**	**Soy**	**Oat 0.5%**	**Oat 1.5%**	**Oat 1.8%**	**Oat 1.9%**	**Oat 3.0%**	**Oat 3.5%**	**Almond**	**Mix**	**Rice**
Essential AA	**His**	91.9 ± 6.3	91.4 ± 4.2	88.6 ± 4.8	24.0 ± 2.3	22.7 ± 2.8	14.6 ± 1.3	4.0 ± 1.8	29.8 ± 4.6	14.9 ± 2.1	10.6 ± 1.4	75.8 ± 5.8	1.4 ± 0.9
	Ile	161.9 ± 10.4	163.4 ± 8.6	148.1 ± 3.3	36.4 ± 2.1	34.2 ± 3.2	24.6 ± 1.8	4.3 ± 0.3	44.9 ± 2.2	24.5 ± 1.1	14.4 ± 1.2	126.4 ± 7.0	0.9 ± 0.6
	Leu	351.6 ± 22.7	339.1 ± 14.2	285.2 ± 7.9	83.7 ± 3.6	78.2 ± 7.4	53.3 ± 3.6	14.1 ± 0.4	104.4 ± 2.5	53.8 ± 3.5	32.9 ± 2.1	249.3 ± 12.9	4.2 ± 1.0
	Lys	273.1 ± 20.2	252.8 ± 12.9	217.1 ± 6.3	37.6 ± 1.6	34.8 ± 3.3	36.1 ± 1.8	6.6 ± 0.5	42.2 ± 1.1	36.2 ± 2.3	6.4 ± 0.4	181.6 ± 7.5	1.3 ± 0.4
	Met	83.5 ± 4.9	81.8 ± 5.8	35.9 ± 4.7	13.0 ± 1.8	12.1 ± 1.6	4.2 ± 0.6	<LOD	16.3 ± 0.7	4.3 ± 0.6	1.8 ± 0.3	30.6 ± 2.3	<LOD
	Phe	155.8 ± 9.4	151.5 ± 7.9	176.6 ± 5.9	53.3 ± 2.4	49.5 ± 4.9	31.2 ± 2.1	5.7 ± 0.6	65.3 ± 2.6	30.5 ± 1.2	21.8 ± 1.9	148.6 ± 10.3	0.7 ± 0.6
	Thre	99.3 ± 5.4	98.5 ± 4.6	94.0 ± 4.1	24.0 ± 1.9	22.1 ± 2.3	16.2 ± 1.1	5.7 ± 0.6	27.9 ± 2.0	16.1 ± 0.7	8.7 ± 0.7	78.1 ± 5.1	0.9 ± 0.4
	Val	190.8 ± 12.1	195.9 ± 9.7	145.2 ± 5.4	47.8 ± 3.4	45.0 ± 5.8	28.4 ± 2.4	8.5 ± 0.5	59.8 ± 4.0	28.8 ± 2.3	17.7 ± 2.3	130.9 ± 11.2	2.9 ± 1.5
	tot.	1408 ± 11	1375 ± 8	1191 ± 5	320 ± 2	299 ± 4	209 ± 2	49 ± 1	391 ± 2	209 ± 2	114 ± 1	1021 ± 8	11 ± 1
Non-essential AA	**Ala**	111.6 ± 7.7	107.2 ±5.6	145.2 ± 3.9	46.5 ± 2.0	43.7 ± 3.6	29.8 ± 2.1	12.4 ± 0.2	56.7 ± 1.5	29.5 ± 1.8	20.4 ± 1.3	130.0 ± 5.4	4.2 ± 0.7
	Arg	112.2 ± 6.8	104.4 ± 8.0	279.2 ± 12.3	72.3 ± 2.1	66.1 ± 4.7	48.9 ± 2.6	12.3 ± 0.9	87.1 ± 1.7	48.0 ± 2.5	45.4 ± 3.2	237.9 ± 13.1	4.3 ± 0.9
	Asx	246.1 ± 15.9	263.7 ± 17.6	403.3 ± 23.1	83.7 ± 4.8	77.7 ± 6.2	71.6 ± 6.4	24.0 ± 2.3	101.3 ± 3.3	71.0 ± 1.0	46.0 ± 3.1	342.9 ± 24.3	6.5 ± 2.4
	Glx	781.4 ± 51.1	754.8 ± 43.5	718.1 ± 44.0	249.8 ± 10.4	234.8 ± 19.6	131.9 ± 8.7	62.3 ± 3.6	313.3 ± 8.6	131.6 ± 6.3	125.3 ± 9.1	623.3 ± 37.0	13.0 ± 2.6
	Gly	66.7 ± 4.0	62.4 ± 3.6	152.2 ± 5.9	53.9 ± 2.8	50.7 ± 3.7	34.2 ± 1.9	19.3 ± 0.1	63.8 ± 1.8	33.2 ± 2.8	28.2 ± 1.6	142.7 ± 6.9	4.1 ± 0.3
	Ser	151.4 ± 18.8	137.8 ± 15.2	167.5 ± 21.5	41.9 ± 1.1	39.6 ± 4.7	30.0 ± 1.8	15.2 ± 1.1	47.1 ± 2.1	27.8 ± 2.2	14.4 ± 1.3	125.3 ± 9.5	3.0 ± 0.6
	Tyr	165.3 ± 7.8	160.8 ± 7.1	127.6 ± 4.1	35.2 ± 1.4	33.2 ± 3.0	21.5 ± 1.6	7.3 ± 0.5	44.1 ± 1.7	21.4 ± 1.6	11.5 ± 1.1	104.0 ± 8.2	14 ± 0.6

6.1.3.2 *Maillard reaction products and amino acid cross-links*

Furosine is the acid derivative of Amadori compounds and a marker of early stages of the Maillard reaction. From our results, high concentrations of furosine were found in UHT milk samples (UHT 1.5%: 4270 μg/100mL; UHT 3.5%: 4077 μg/100mL). Furosine content was significantly lower in all tested plant-based drink samples except for mix drink where the difference was minor but still significant (3668 μg/100 mL) (Figure 10A). With the progress of the Maillard reaction, Amadori compounds fragment into α-dicarbonyl compounds or oxidizes to form AGEs. Therefore, the low concentration of furosine in plant-based drinks might indicate that glycation progressed to a higher extent.

CML and CEL are typically the most abundant AGEs formed in foods and they are formed by the reaction between lysine and GO or MGO, respectively. For CML, no statistical differences were observed between mix drinks (298 μg/100 mL) and UHT milk samples (UHT

1.5%: 324 μg/100 mL; UHT 3.5%: 299 μg/100 mL). Significantly lower concentrations of CML were instead found in all other plant-based drinks (Figure 10B). Apart from mix drinks, oat 1.8% and oat 3.5% drinks have the highest concentration of CML in comparison with all other plant-based drinks. Interestingly, these two oat drinks are both of the same brand, and they contain similar levels of CML. This high content of CML could be due to the heat processing used during their production. Interestingly, a significant difference was observed between oat 0.5% (47 μg/100 mL) and oat 3.0% (141 μg/100 mL) drink samples. These two kinds of samples of oat drinks are from the same brand and consequently, we can hypothesize that they are subjected to the same processing conditions. As these two drinks present similar protein and sugar levels, this difference in the content of CML could be due to higher fat content. The non-enzymatic oxidation of polyunsaturated fatty acids can generate peroxide intermediates, which can break down into various oxidative products, including glyoxal, which in turn can contribute to CML formation [334]. The Maillard reaction is a series of intricate reactions, encompassing condensation, degradation, and elimination processes, often happening simultaneously. The formation of CML occurs through two pathways: i) the Hodge pathway which includes the oxidation of the Amadori compound or ii) the Namiki pathway which involves the direct reaction of GO with the Ɛ-amino group of lysine. The variation in CML formation in plant-based drinks may be due to differences in GO concentration in the samples (Figure 11).

Compared to UHT milk samples, CEL was found higher in mix (211 μg/100 mL), soy (186 μg/100 mL), and almond (98 μg/100 mL) drinks, lower in oat 1.5% drink (11 μg/100 mL) and was not detected in rice drink (Figure 10C). Also in this case, the difference in the extent of CEL formation could be due to the difference in MGO concentrations formed in the samples.

Arg-derived AGEs include MGO- and GO-derived hydroimidazolones (MG-Hs and GO-Hs), which exist as three different isomers (H1-H2-H3) but have been quantified as MG-H3-equivalents and GO-H3 equivalents corresponding to the sum of MGO- or GO-isomers as described previously [184].

High concentrations of GO-Hs were found in mix (730 μg/100 mL), oat 3.0% (426 μg/100 mL), oat 1.8% (402 μg/100 mL) and oat 3.5% (393 μg/100 mL) (Figure 10D), similar to what was observed for CML concentrations. In particular, mix and oat 1.8% drinks contain significantly higher levels of CML as well as GO-Hs among the plant-based drinks, which is accompanied by a lower concentrations of GO content (Figure 11A). The results from CML and GO-Hs analysis showed that the formation of AGEs derived from GO and the decline of their precursor GO,

could be due to the advancement of the Maillard Reaction in the mix and oat 1.8% drink samples.

MG-Hs have demonstrated to be another important AGEs formed in food [184], [335]. The content of MG-Hs was found to be higher in mix (694 μg/100 mL), soy (281 μg/mL), almond (406 μg/100 mL), oat 1.8% (263 μg/100 mL) and oat 3.5%, (187 μg/100 mL) drinks as compared to UHT milk samples, while oat 1.5% (50 μg/100 mL) and rice (46 μg/mL) contained significantly lower levels (Figure 10E). Soy, almond and mix drinks showed a significant higher levels of MG-Hs in addition with CEL increase. Taken together, also in this case, the concomitant increase in MGO-derived AGEs and decrease in their precursor MGO, is likely due to the progression of Maillard Reaction in soy, almond, and mix drink samples.

MGO- and GO-Lys dimers (MOLD and GOLD), which are cross-links formed between two Lys residues, were below the limit of detection (data not shown). These results are in agreement with previous studies showing that MOLD and GOLD have only been detected in food at minor levels [184]. Finally, LAN and LAL are cross-linked amino acid products, formed during heat treatment by the reaction of lysine and cysteine with dehydroalanine, respectively. LAN content was found to be higher in all tested drinks in comparison to UHT milk samples except for rice and almond where it was lower (rice: 40 μg/100 mL; almond: 78 μg/100 mL) than the UHT milk (Figure 11F). LAL concentration was detected in higher concentrations in mix (1048 μg/100 mL), soy (1102 μg/100 mL) and oat 3.0% (1067 μg/100 mL) drinks than UHT milk samples whereas was found to be significantly lower in almond (68 μg/100 mL), rice (102 μg/100 mL), oat 1.9% (184 mg/100 mL), oat 0.5% (147 μg/100 mL), and oat 1.5% (215 μg/mL) (Figure 10G). The reason for the LAN and LAL differences could be related to the production techniques. During soy protein production, alkali extraction is a common approach. Alkali processes increase dehydroalanine formation through beta-elimination of serine and cysteine residues, therefore LAL and LAN formation increases with alkaline processing. Therefore, a possible alkaline extraction during the production of protein isolate or protein concentrate might have caused an increased level of LAL and LAN [185], [336], [337]. Proteins from oat may also be extracted under alkaline conditions [338]. Also in this case, it has been observed that the formation of cross-linked amino acid may occur during alkali extraction. For instance, the formation of LAL begins at pH 9, and reaches the maximum at pH 12.5. These changes affect protein and are highly undesired as result in a decrease in product quality and nutritional value [339].

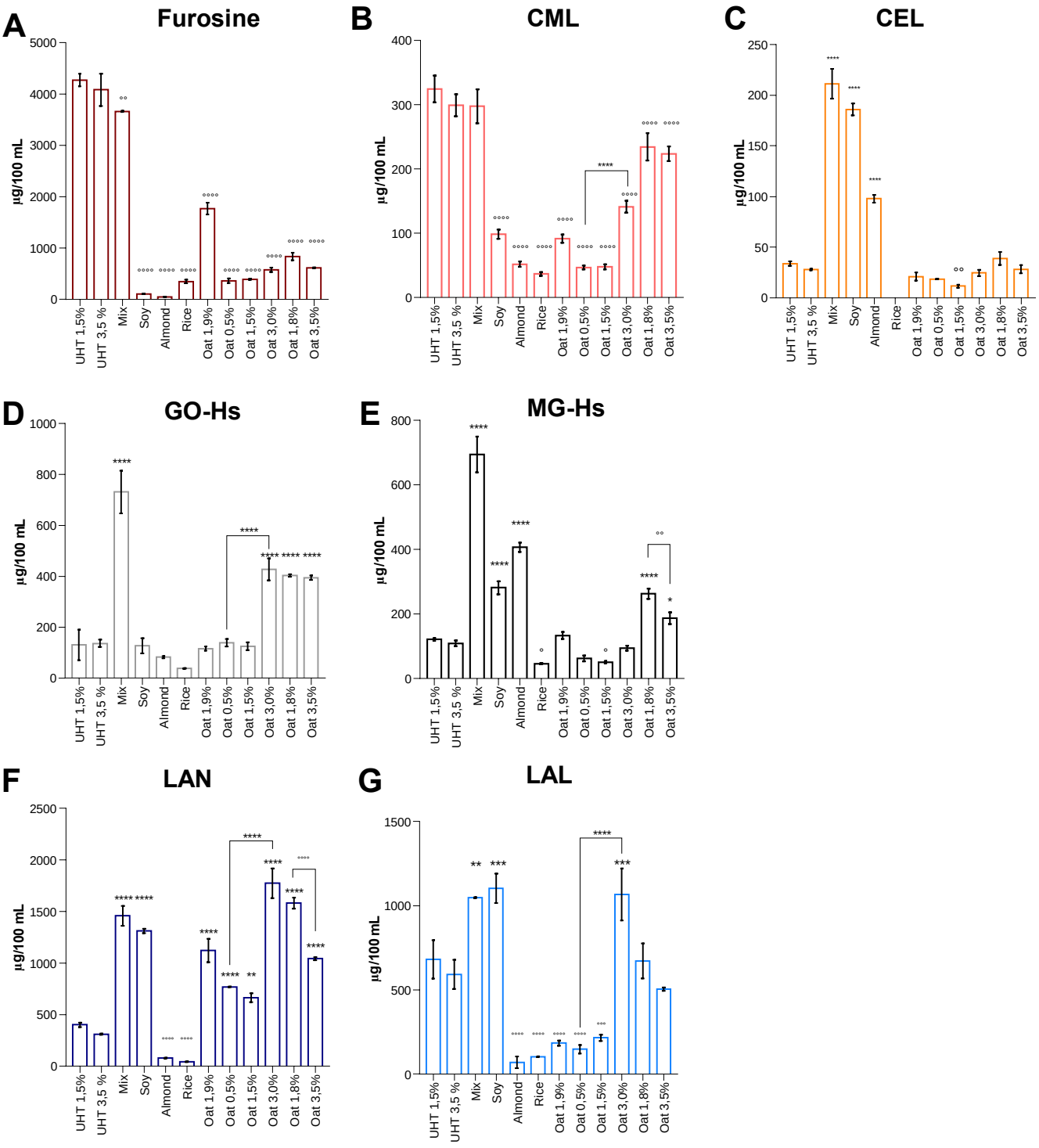

Figure 10. Concentration (µg/100 mL) of furosine (A), AGEs (CML (B). CEL (C). GO-Hs (D). MGO-Hs (E)) and amino acid crosslinks (LAN (F) and LAL (G)) in milks and plant-based drinks. Data are shown as the mean ± SD. One-way ANOVA test with post-hoc Tukey test was used to analyze statistical significance. */°: $p < 0.05$. **/°°: $p<0.01$. ***/°°°: $p<0.001$. ****/°°°°: $p<0.0001$ vs. control (UHT 1.5%). * is used if values are higher vs. control while °if they are lower.

6.1.3.3 α-dicarbonyl compound analysis

α-dicarbonyl compounds can be formed from the degradation of Amadori products through the Maillard reaction pathway or can also be formed directly from the degradation of carbohydrates [31]. The presence of α-dicarbonyl compounds in beverages has been evaluated in different studies. For example, α-dicarbonyl compounds concentrations have been reported for coffee, soft drinks and fermented products such as beer, wine and soy sauce [26]. Recently Maasen *et al.* [14] have identified a food composition database for α-dicarbonyl compounds highlighting that the lowest total concentrations of dicarbonyl compounds in terms of glyoxal, methylglyoxal and 3-deoxyglucosone, were found in tea, dairy and light soft drinks (<10 mg/kg). In the present study, in comparison to UHT milk samples, GO was found higher in mix (147 μg/100 mL), rice (246 μg/100 mL), oat 1.9% (125 μg/100 mL), oat 1.5% (135 μg/100 mL), oat 3.0% (439 μg/100 mL), oat 1.8% (236 μg/100 mL), and oat 3.5% (380 μg/100 mL) drinks (Figure 11A). MGO was instead found higher in mix (100 μg/100 mL), oat 1.9% (131 μg/100 mL), oat 3.0% (98 μg/100mL), oat 1.8% (232 μg/100 mL), and oat 3.5% (324 μg/100 mL) drinks (Figure 11B). Diacetyl was only detected in soy (9 μg/100 mL), oat 1.8% (73 μg/100 mL) and oat 3.5% (57 μg/100mL) drinks (Figure 11C). Another α-dicarbonyl compound, 3-deoxyglucosone, was detected in higher concentrations in mix (4252 μg/100 mL), rice (12169 μg/100 mL), oat 1.9% (27672 μg/100 mL), oat 1.8% (4964 μg/100 mL), and oat 3.5% (5647 μg/100 mL) drinks than UHT milk samples (Figure 11D).

The low concentration of MGO, GO and 3-DG in UHT milk samples as well as in soy milk is confirmed by previous literature [27], [340]. 3-DG is the α-dicarbonyl compound found in the highest concentrations in our samples. In fruit juices, 3-DG was found to be about 6.0 mg/100 mL, while lower amounts were found in soft drinks (340-2800 μg/100 mL)[26]. On the contrary alcoholic beverages are characterized by higher content of α-dicarbonyl compounds because they are subjected to a fermentation step in their manufacturing process. In the plant-based industry, fermentation is often used to enhance the sensory characteristics, nutritional value, texture, and microbial safety of plant-based milk alternatives, making them more desirable and palatable, thus eliminating the need for adding perceived artificial ingredients [319]. High concentrations of 3-DG found in our samples could be due to fermentation to which raw material for plant-based milk alternatives are subjected.

Additionally, a high content of glucosone was detected in mix (859 μg/100 mL), almond (311 μg/100 mL), rice (1037 μg/100 mL), oat 1.9% (947 μg/100 mL), and oat 0.5% (308 μg/100 mL) drinks and not detected in UHT milk samples (Figure 11E). Finally, 1-deoxypentosone (1-

DP) was identified in significantly high concentration in oat 1.9% (412 μg/100 mL), oat 3.0% (346 μg/100 mL), oat 1.8% (364 μg/100 mL), oat 3.5% (461 μg/100 mL), and rice (318 μg/100 mL) drinks than UHT milk samples (Figure 11F).

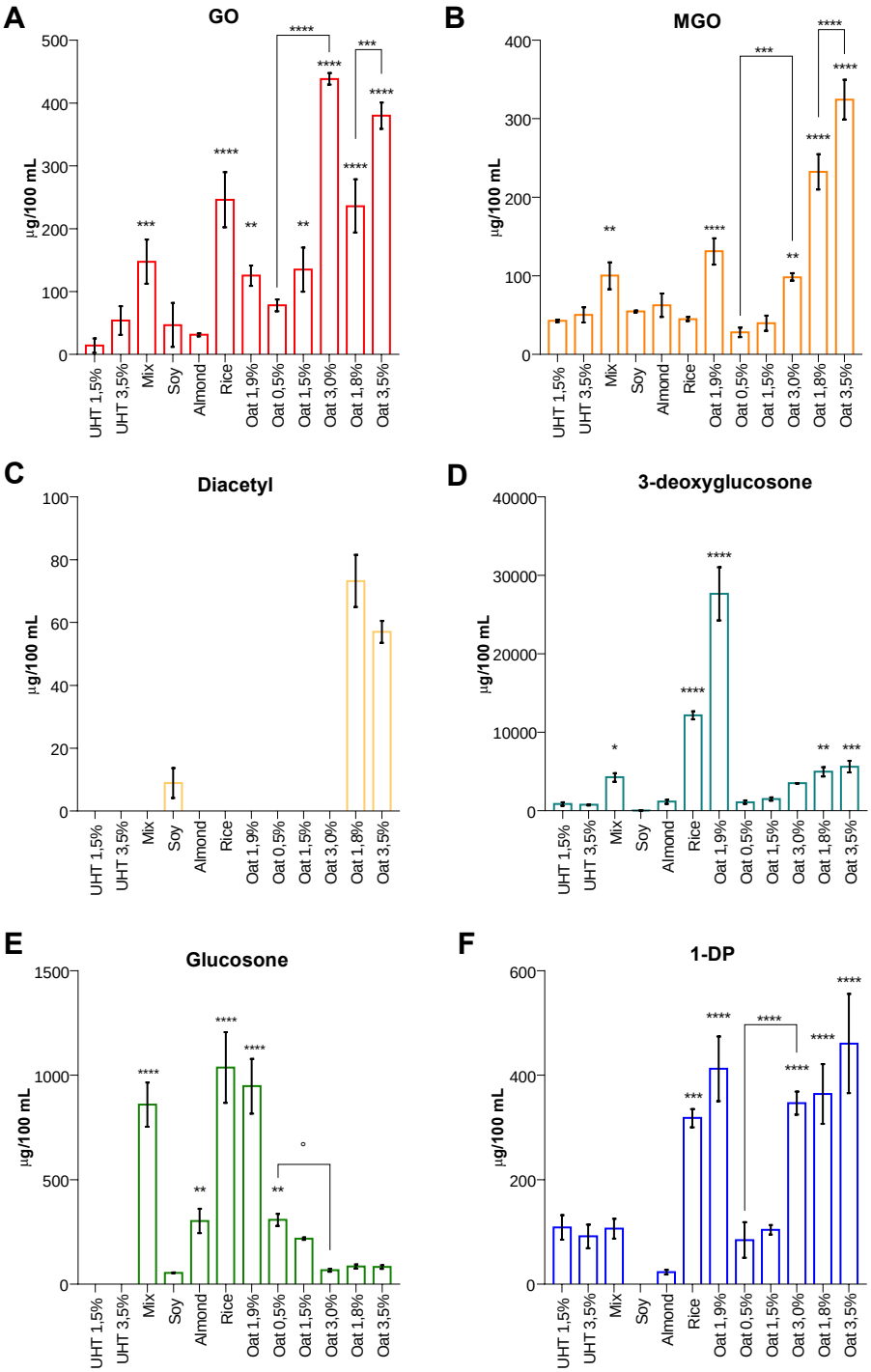

Figure 11. Concentration (μg/100mL) of dicarbonyl compounds (GO (A). MGO (B). diacetyl (C). 3-deoxyglucosone (D). glucosone (E). 1-DP (F)) in milks and plant-based drinks. Data are shown as the mean ± SD. One-way ANOVA test with post-hoc Tukey test was used to analyze statistical significance. */°: p < 0.05. **/°°: p<0.01. ***/°°°: p<0.001. ****/°°°°: p<0.0001 vs. control (UHT 1.5%). * is used if values are higher vs. control while °if they are lower.

6.1.3.4 HMF, furfural and acrylamide analyses

The main mechanisms for the formation of HMF and furfural are either through the Maillard reaction or caramelization via sugar dehydration [211]. HMF is generated from hexose breakdown while furfural is primarily formed from pentose degradation or can also result from the thermal degradation of HMF. HMF and furfural have been identified as indicators of the severity of heat treatment or length of storage in several beverages including fruit juices [341], UHT milk samples [342]–[344], wine and other alcoholic beverages, vinegars and coffee [345], [346]. In the present study, furfural was detected only in almond drinks (1.00 µg/100 mL), whereas HMF was detected in significantly high concentrations in oat 1.9% (28.9 µg/100 ml) and in almond (19.6 µg/100 mL) drinks. Lower pH, low water activity and high temperatures are key factors in HMF and furfural generation [251], [253], [254]. The higher concentration of these two compounds in almond drink samples is likely due to the fact that this kind of plant-based drink was made from roasted almonds. Furans, such as furfural and HMF have been already detected in toasted almonds [347]–[349] as well as other roasted food such as coffee [211]. Besides HMF and furfural, acrylamide is another toxicant that can be found in food subjected to high temperatures like roasting, baking and frying [301]. Acrylamide is formed during the Maillard reaction by the reaction between asparagine and reducing sugars [350], [351] [352]. Acrylamide can be found in varying quantities in a wide range of heat-processed foods such as fried potatoes, baked goods, and roasted food [353]. In our samples, acrylamide was detected mainly in oat 1.8% (0.64 µg/100 mL), oat 1.9% (2.93 µg/100 mL), oat 3.5% (1.08 µg/100 mL) and almond (1.08 µg/100 mL) drinks. The presence of these levels of acrylamide in our samples was unexpected, as the formation of this compound primarily occurs in conditions of intense heat and dryness. Therefore, its formation does not usually occur at elevated levels in food containing a high content of water such as plant-based drinks or milk. The main explanation could be the fact the formation of acrylamide, as well for HMF and furfural, does not occur during the production of plant-based drinks but is likely due to the treatments to which the raw materials used for beverage production are subjected.

6.1.3.5 *Sugars analysis*

The results of sugar analysis are summarized in Table 5 and Figure 12. From the analysis, the sugar content of semi-skimmed (UHT 1.5%) and whole (UHT 3.5%) milk is quite similar (4.46 and 4.40 g/100 mL, respectively). All oat drink samples except for oat 3.5% showed a significantly higher concentration of sugars than UHT milk samples but among plant-based drinks, rice drink sample was the one with the highest sugar content. In soy and almond drink samples significantly low levels of sugars than UHT milk samples were detected whereas mix drink sample does not show any significant differences in sugars content. These data are in accordance with the ones observed by Angelino *et al*. [35] where rice drinks and blended beverages had the highest sugar content, while soy and almond drinks contained the lowest.

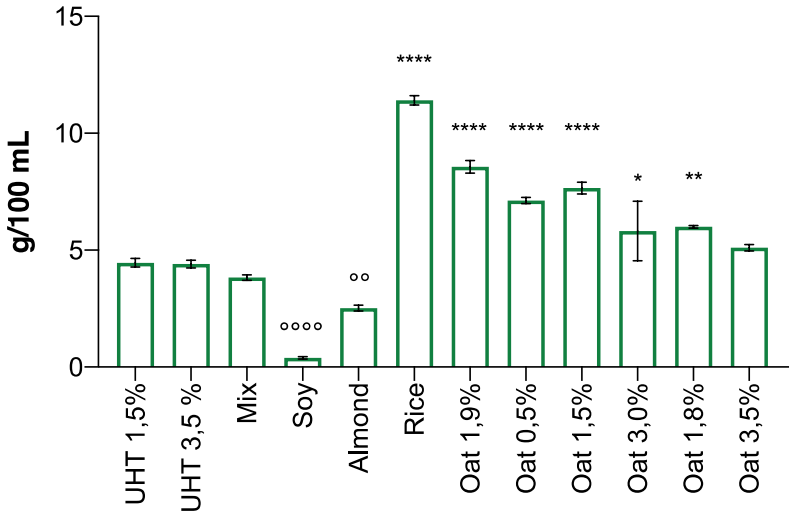

Figure 12. Sugar content in milk and plant-based drinks. Data are shown as the mean ± SD. One-way ANOVA test with post-hoc Tukey test was used to analyze statistical significance. */°: p < 0.05. **/°°: p<0.01. ***/°°°: p<0.001. ****/°°°°: p<0.0001 vs. control (UHT 1.5%). * is used if values are higher vs. control while °if they are lower.

Coming to the type of sugars identified, as expected, only lactose was detected in UHT milk samples, while plant-based drinks were characterized by the presence of dextrose, maltotriose, maltose, and in some cases, glucose (Table 5). These differences can be attributed to the fact that starch is the storage carbohydrate in many plants and can be hydrolyzed into smaller sugars during plant-based beverage production. One significant challenge in the preparation of a stable emulsion during the heat processing of plant-based drinks is the high concentration of starch. When heat is applied, starch begins to gelatinize, and the liquid attains a gel-like consistency with high viscosity and low acceptability. To maintain fluidity and a beverage-like consistency, researchers have explored enzymatic hydrolysis as a solution to

remove starch and prevent gelatinization during thermal treatment [312]. For example, Deswal *et al.* [37] have optimized an enzymatic processing protocol for the production of oat drinks that includes specific conditions and parameters in terms of slurry concentration, enzyme concentration and liquefaction time. Another option besides enzymatic treatment is the natural germination of the plant raw material. This process activates enzymes such as proteases and amylases, which can significantly change the composition and functional characteristics of the plant material [354], resulting in a rise of free sugars and amino acids that can be utilized to produce desirable aromas during fermentation [355]. It has been shown that in oat seeds, germination for 24–144 h reduced starch content from 60% to 21%, while increased free sugars from 5% to 28% [356].

In addition, plant-based drinks can sometimes cause higher blood sugar levels due to differences in their composition and factors affecting insulin secretion and gastric emptying. These higher blood sugar levels can contribute to the development of non-communicable diseases such as type 2 diabetes [357]. Glucose, sucrose and maltose are known to have high-glycemic-index values compared to those of lactose. Jeske *et al.* [358] determined the glycemic index (GI) of several plant-based drinks and cow's milk. They have shown that samples containing glucose such as rice plant-based drink samples have a high GI (>96). In addition, Jeske *et al.* presented the GI of maltose, sucrose, fructose, and lactose to be 105, 61, 19 and 46 respectively, identifying plant-based drinks with higher levels of maltose to have consequently a higher GI. The only exception in this study was represented by oat plant-based drinks because, even if containing mainly maltose, they resulted in a moderate GI of 59. The authors explain this result by the presence of β-glucan in oats, which is known to reduce GI [358], [359]. Furthermore, several plant-based drinks with high content of maltotriose, resulting from high starch contents, can contribute to increasing GI due to hydrolysis into glucose by digestive enzymes [360]. In our study, we did not calculate GI of plant-based milk, however, we can hypothesize that rice would be the plant-based drink with the higher GI due to the presence of high concentrations of both maltose and glucose. In the case of oat drinks, even if having a high concentration of maltotriose, the GI could be still moderate due to the presence of β-glucan.

Table 5. Concentrations of identified sugar in milk and plant-based drinks. Results are expressed as mean in g/100mL.

	Sugar	**Content (g/100 mL)**	**Total Content (g/100 mL)**
UHT 1.5%	Lactose	4.46	4.46
UHT 3.5%	Lactose	4.40	4.40
Soy	Dextrose Maltose	0.23 0.16	0.39
Oat 0.5%	Dextrose Maltotriose Maltose	1.70 4,09 1.33	7.12
Oat 1.5%	Dextrose Maltotriose Maltose	1.56 4.56 1.53	7.65
Oat 1.8%	Dextrose Maltose	5.42 0.58	6.00
Oat 1.9%	Dextrose Maltotriose Maltose Glucose	1.93 3.25 3.23 0.16	8.56
Oat 3.0%	Dextrose Maltotriose Maltose	1.45 2.84 1.54	5.82
Oat 3.5%	Dextrose Maltose	4.43 0.68	5.10
Almond	Dextrose Maltose	0.07 2.48	2.55
Mix	Dextrose Maltotriose Maltose Glucose	0.65 1.12 1.27 0.79	3.83
Rice	Dextrose Maltotriose Maltose Glucose	0.91 5.38 2.35 2.76	11.41

6.1.4 Conclusions

In comparison to semi-skimmed and whole milk, the highest content of AGEs was detected mainly in mix, soy, oat 3.0%, oat 1.8% and oat 3.5% samples. Oat 1.8%, oat 3.5% and oat 1.9% drink samples were found to be higher also in α-dicarbonyl compounds and acrylamide. In addition to oat 1.8% and oat 3.5%, oat 1.9% showed also high concentration of HMF. Finally, almond drink samples showed high concentrations of furfural, HMF and acrylamide. Our results demonstrated that these differences were likely due to plant-based drink compositions, industrial processing techniques as well as heat treatment employed during their production. By conducting this study, we aim to lay the foundation for further research in this direction, with the ultimate objective of producing plant-based foods that meet rigorous safety standards. Our hope is that the findings of this study will prompt further investigations into the impact of food production and manipulation on the levels of MRPs in different plant-based foods, and inspire the development of innovative strategies for reducing their formation. Ultimately, we aspire to ensure that plant-based foods can be produced with the highest standards of safety, in order to meet the growing demand for healthy food options.

6.2 Part 2- Evaluation of MRPs in different kinds of traditional and plant-based meat products

6.2.1 Introduction

Meat represents a precious source of high-quality protein, iron, vitamin B12, B complex vitamins, zinc, selenium, and phosphorus, making it a crucial dietary component [361]. Meat is frequently subjected to various processing methods, such as high-temperature frying, curing, smoking, charcoal grilling, and cooking, to produce a wide range of meat products. While these methods can improve the quality, taste, preservation, and safety of meat products, they can also lead to the formation of Maillard Reaction Products (MRPs) through the Maillard reaction. Maillard reaction is needed for conferring specific color, flavor, and aroma to food [362]. Maillard reaction starts with a condensation reaction between the amino groups on protein, peptides, and amino acids and the carbonyl groups of reducing sugars, resulting in the formation of a Schiff base which rearranges to form Amadori or Heyns products. These molecules can undergo some additional modifications that lead to reactive α-dicarbonyl compounds like methylglyoxal (MGO), glyoxal (GO) and diacetyl able to readily react with additional nucleophiles such as other amines, guanidines, and thiols. Further downstream reactions include the formation of advanced glycation end-products (AGEs), such as N-ε-(carboxymethyl)lysine (CML), N-ε-(carboxyethyl)lysine (CEL), which derive from modification on Lys residues. Arg can also be modified to glyoxal or methylglyoxal-derived hydroimidazolinone isomers (GO/MG-Hs). Acrylamide, hydroxymethylfurfural (HMF) and furfural represent other reactive Maillard reaction intermediates [196].

As meat products are generally high in fat and protein and are exposed to various high-temperature processing and storage conditions, the formation of MRPs can be greatly impacted. Recent studies have revealed high levels of MRPs in many meat products, including sausage, chicken, pork, and beef, and their accumulation through the diet has been linked to various diseases, such as kidney disease, diabetes, and cancer [363]. The significant presence of MRPs in meat products, combined with the significant portion of meat products in daily diets, has raised concerns. Additionally, in recent years, meat production has been recognized as the primary way in which humanity impacts the environment, with excessive use of natural resources and intensive animal farming leading to irreversible environmental changes,

greenhouse gas emissions, concerns about animal welfare, and significant demand for water [364]. As a result, plant-based meat alternatives (PBMAs) have gained popularity among consumers in recent years, attracting substantial financial, media, and scientific research attention [287]. The global plant-based meat market is projected to increase from $5.67 billion in 2022 to $6.69 billion in 2023 at a compound annual growth rate of 17.9% [365]. This rising demand has prompted many companies to launch innovative plant-based meat products designed to mimic the texture and flavor of animal-derived foods [366]. The production of PBMAs typically involves three primary steps: protein isolation and functionalization, formulation, and processing. Firstly, specific plant proteins are extracted from plants and can be subjected to hydrolysis to enhance certain properties such as solubility and cross-linking capacity. Next, plant proteins are combined with i) target ingredients like food adhesives, plant-based fats, and flour to replicate the traditional texture of meat, as well as ii) nutrients to meet or exceed the nutritional profile of meat. Finally, this blend of plant proteins and additional ingredients undergoes protein-reshaping processes (such as stretching, kneading, trimming, pressing, folding, and extrusion) to achieve a fibrous and meat-like consistency. Recently, advanced technologies, such as shear cell technology, mycelium cultivation, 3D printing, and recombinant protein additives, have been utilized to improve the sensory properties of PBMAs [367].

Despite the great success of PBMAs and the technological difficulties in simulating traditional meat properties, various challenges for these kinds of products still need to be addressed in terms for example of food safety and nutrition [368]. Indeed, the specific chemical composition of plant-based meat together with their processing methods make PBMAs more susceptible to Maillard reaction than traditional meat.

In this regard, in this study, we evaluated different categories of traditional meat samples together with their corresponding PBMAs such as minced products (burger patties and schnitzels), muscle-type products (chicken) and emulsion-type products (sausages). These products were subjected to thermal processing according to the product labels. Then the aim of our study was to investigate the occurrence of MRPs and to assess the potential health risks of process compounds such as AGEs, HFM, furfural, acrylamide in traditional meat and PBMAs commonly sold in Denmark. To the best of our knowledge, there is no study yet which investigates these compounds in plant-based foods. The results of this study will provide additional findings regarding the evidence of these compounds in the perspective of real-life risk and safety management.

6.2.2 Materials and Methods

6.2.2.1 Chemicals and consumables

The following standards and internal standards were purchased from Iris Biotech GmbH (Marktredwitz, Germany), with net weight values given in brackets; CEL (89.6%), CML (95.5%), MG-H3 TFA salt, GO-H3 TFA salt (48.6%), furosine HCl (72.7%), MG-H1-d3 (90.5%), CEL-d4 (78.3%), furosine-d4 HCl (52.8%), and CML-d4 (94.4%). In addition, GO-H1-13C2 (≥97%) was also obtained from Iris Biotech GmbH; no net weight values were available from the supplier for these standards, so chromatographic purities are given in brackets. 6- aminocaproic acid (≥ 99%) and amino acid std for protein hydrolysate were purchased from Sigma Aldrich (Copenhagen, Denmark). Orthophenilene diamine (OPD, 98 %), diethylenetriaminepentaacetic acid (DETAPAC, ≥99 %), sodium dihydrogen phosphate monohydrate (≥99%), sodium phosphate dibasic anhydrous (≥99%), sodium tetraborate decahydrate (99,5%), sodium azide (≥99.99 %), 3-mercapto-propionic acid (MPA) (≥99%), acrylamide (≥99%), Acrylamide-d3 standard solution (500 mg/L) were purchased from Sigma-Aldrich Chemie GmBh (Steinheim, Germany). Disodium hydrogen phosphate anhydrous (>99%) and Formic acid (≥99 %) were obtained from VWR Chemicals (Denmark). Carrez I (Potassium hexacyanoferrate (II) trihydrate) was purchased from Fluka Biochemika (Switzerland) whereas Carrez II (Zinc sulphate heptahydrate), 1-octanol anhydrous (≥99%), were obtained from Sigma-Aldrich (Darmstadt, Germany). LC–MS grade acetonitrile, ammonium formate, methanol (99.995%) and HPLC grade acetonitrile, methanol, sulfuric acid were purchased from Sigma Aldrich (Copenhagen, Denmark). Formic acid (≥99%) was obtained from VWR Chemicals (Denmark). Nylon filter membranes (0.20 µm pore size, 47 mm diameter) were obtained from Phenomenex (Aschaffenburg, Germany). Oasis MCX 1 cc Vac Cartridges were purchased from Waters (Taastrup, Denmark). Milli-Q water was produced from a Millipore purification system (Millipore Corporation, Billerica, MA).

6.2.2.2 Food products and thermal processing conditions

Four types of plant-based meat alternative products were included in the study: burger patties, chicken fillets, sausages, and schnitzels. Two Scandinavian plant-based brands (Brand A and B) that are popular in Danish supermarkets were chosen. For each product type, its traditional meat counterpart was used for comparison. The overview of nutritional components and main protein source is shown in Table 6. The list of ingredients for each samples are instead listed in Table 7.

Table 6. Nutritional values and main protein sources of traditional meat and PBMAs samples according to the label.

Products	Brand	Energy kcal/100 g	Fat (g/100g)	Carbohydrates (g/100g)	Fiber (g/100g)	Protein (g/100g)	Salt (g/100g)	Protein source
Burger	A	217	14.00	6.80	1.90	16.00	0.98	pea
Burger	B	197	14.00	2.00	5.00	14.00	0.75	soy
Burger	Traditional	333	30.00	0.50	0.00	16.00	0.88	beef
Chicken	A	216	12.00	7.10	2.70	20.00	1.50	pea
Chicken	B	151	4.70	1.20	5.90	23.00	1.40	soy
Chicken	Traditional	111	1.70	2.00	0.00	22.00	1.80	chicken
Sausage	A	178	11.00	4.30	4.50	12.00	1.50	soy
Sausage	B	115	2.00	5.30	5.90	16.30	1.25	wheat/soy
Sausage	Traditional	278	23.00	3.30	0.00	15.00	1.00	pork/beef
Schnitzel	A	230	13.00	13.00	4.20	13.00	1.00	soy
Schnitzel	B	235	11.70	17.60	5.30	12.30	0.83	soy
Schnitzel	Traditional	176	7.90	7.90	0.00	18.00	1.90	chicken

Table 7. Ingredients of plant-based meat alternatives and traditional meat according to product labels**.**

Product	*Type*	*Ingredients list*
Burger	**Brand A**	Water, pea protein (18%), coconut oil, WHEAT GLUTEN, natural aroma, fermented dextrose, stabilizer (methylcellulose), caramelized sugar, beetroot juice, carrot concentrate, acerola juice, acidity regulator (citric acid), salt.
	Brand B	Rehydrated SOY Protein Concentrate (52.0%) (water, SOY protein concentrate 19,9%) Water, Vegetable Oils (Rapeseed, Coconut), Vinegar spirit, Flavourings, Stabilizer: Methylcellulose, Plant Concentrates (apple, beetroot, carrot, hibiscus), Salt, Malt Extract Powder (from BARLEY). May contain: Egg, Sesame, Celery and Mustard
	Traditional	99 % minced beef, salt and pepper
Chicken	**Brand A**	Water, pea protein (30%), rapeseed oil, natural aroma, fermented dextrose, vegetable broth (salt, dextrose, yeast extract, dried vegetables), onions, garlic, acidity regulator (citric acid). May contain traces of GLUTEN, SOY and CELLERY
	Brand B	Water, SOY / SOY PROTEIN (32%), oil (rapeseed), vinegar, yeast extract, salt, aroma
	Traditional	94% chicken breast fillet, modified tapioca starch, salt, antioxidant (E331), stabilizer (E451, E450), xylose, dextrose.
Sausages	**Brand A**	Water, SOY PROTEIN (17%), rapeseed oil, onion, spices (including paprika, coriander), salt, corn starch, garlic, tomato, apple extract, pea fiber, pea starch, natural aromas, smoke flavor, stabilizing agent (methyl cellulose), couloring (iron oxide)
	Brand B	Water, WHEAT PROTEIN (10.3%), SOY PROTEIN (5.3%), spices (dried onion, dried garlic, black pepper, MUSTARD, paprika, nutmeg), onion, stabilizer (methylcellulose, xanthan gum, guar gum, carrageenan) , starch (WHEAT, potato), potato protein, vegetable oil (rapeseed, sunflower), salt, yeast extract, beetroot powder, flour, hydrolyzed SOY PROTEIN, aroma, vinegar, smoked maltodextrin, dye (lycopene), pea protein.
	Traditional	Pork 63%, beef 20%, water, sea salt, potato starch, cayenne pepper, garlic, onion, paprika, black pepper, nutmeg, larch, parsley, acidity regulator (E331), lamb casing
Schnitzel	**Brand A**	Water, SOY PROTEIN (15%), rapeseed oil, glutenfree rasp (rice flour, chickpea flour, corn starch, salt, dextrose), rice flour, corn starch, onions, salt, spices, pea fiber, pea starch, natural aromas, stabilizer (methylcellulose).
	Brand B	REHYDRATED SOY 34.6% (water, soy 14.4%), water, rasp 16.9% [WHEAT FLOUR / yeast, salt, oil (rapeseed), spices (paprika, turmeric)], vegetable oil (rapeseed, sunflower) in varying proportions, WHEAT FLOUR, corn starch, vinegar, stabilizer (methylcellulose), guar gum, citrus fibers, natural flavors, salt, onion powder, garlic powder, acidity regulator (potassium hydroxide).
	Traditional	Chicken tenderloin, wheat flour, water, rapeseed oil, wheat starch, salt, rice starch, modified potato starch, leavening agents (E450, E500), flavor enhancer (E621), maltodextrin, spices, stabilizer (E331), celery, garlic, yeast extract, lime fiber chili extract, garlic extract, color (E 160c)

6.2.2.3 Thermal Processing Conditions

Thermal processing conditions were based on the home-cooking style. The chosen times and temperatures for these products were based on similar label cooking information and on safety rules for meat cooking procedures where an internal temperature of a minimum of 75°C is required. Products were baked in an industrial oven with a combi-steamer mode at a temperature of 180°C for 12 min, flipped halfway after 6 min. Since schnitzels have cooking time and temperature parameters that differ from the ones reported on burger patties, chicken pieces, and sausages labels, different cooking conditions were used. Schnitzels were baked in the same oven described above at a temperature of 200°C for 16 min, flipped halfway after 8 min. Cooking parameters are summarized in Table 8. After heating, each product was cooled down, finely chopped in a food processor and stored at -80°C until further analysis.

Table 8. Cooking parameters used for plant-based and traditional meat samples.

Process	Time	Temperature	Products
Baking	12 min	180°C	Burger, chicken, sausage
Baking	16 min	200°C	Schnitzel

6.2.2.4 *Dry matter (DM) determination*

To measure the dry matter content, about 2 g of each sample were weighted on aluminum dishes and spread uniformly all over the dishes surface. Dry matter content was determined by the standard gravimetric method (AOAC 950.46) with two separate measurements.

6.2.2.5 Protein content determination

For protein content determination, Dumas assay was used. Dumas assay is based on the combustion of the food sample at temperatures between 900 and 1300°C in an oxygen-rich atmosphere. Samples were used without any pre-treatment. Analyses were run using a standard method with a total analysis time of about 5 minutes and argon as carrier gas. A protein factor of 5.7 was applied to calculate the average protein content.

6.2.2.6 Preparation of hydrolyzed samples

Furosine, CEL, MGO-Hs, GO-Hs, and total amino acid content were identified on hydrolyzed samples. In microwave glass tubes, plant-based and traditional meat samples containing 3-5 mg protein were weighed and added of 3 mL 6M HCl. Tubes were consequently flushed with nitrogen and tightly sealed. Afterwards, the hydrolysis of the samples was performed by microwave heating using a two-step protocol: the first step consists of microwaving the samples at 150°C for 1 min followed by a 10 min at 165°C step using a Biotage Initiator + microwave synthesizer. After, 1000 µL of hydrolysate was centrifuged at 10000 rpm for 10 min at room temperature and 500 µL of supernatant were evaporated to dryness by using a centrifugal vacuum concentrator. The residue was dissolved in 500 µL of Milli-Q water, mixed vigorously and filtered through 0.22 µm nylon filters. For the determination of CML content, samples needed to be first reduced. In microwave glass tubes, dried samples containing 3-5 mg protein were weighed and 250 µL of Milli-Q water, 750 µL of sodium borate buffer (0.2 M, pH 9.2) and 500 µL 1 M sodium borohydride in 0.1 M NaOH were added. Samples were then incubated at room temperature for 4 h. Afterwards, 50 µL of the antifoaming reagent 1-octanol were added to reduce foaming that will occur during the subsequent addition of HCl. Samples were finally hydrolyzed by adding 1.45 mL of HCl 37% and using the same microwave protocol and conditions described above. After evaporation of acid, residues were dissolved in 500 µL of Milli-Q water and filtered through 0.22 µm syringe filters.

6.2.2.7 Analysis of furosine and AGEs

Analysis of furosine and AGEs (CML, CEL, MGO-Hs, GO-Hs) were performed according to the literature [184]. Clear hydrolyzates after filtering through 0.20 µm nylon filters were used for the analysis after proper dilutions. Dilutions and addition of internal standards were prepared so that the final sample solvent was acetonitrile: water (50:50, v/v). Five µL of the sample was injected into the Dionex UltiMate 3000 LC system (Thermo Fisher Scientific Inc., Waltham, USA) equipped with a Syncronis HILIC column (100 mm length × 2.1 mm internal diameter × 1.7 µm particle size, Thermo Fisher Scientific Inc., USA) maintained at 40°C. The UHPLC system was directly interfaced to an OrbiTrap Q Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) operated in positive ionization mode. The chromatographic conditions and mass spectrometry parameters are reported elsewhere [184]. The quantification of each compound was based on internal standard calibration by using a stable isotopically labeled internal standard.

6.2.2.8 Analysis of total Amino acids (AA)

Clear hydrolyzed filtrates were properly diluted with Milli-Q water. Working standard solutions were made by proper dilutions of the Amino acid Standard Stock solution (500 µM) with 0.1 M HCl. Concentration levels of amino acids in the calibration solutions were in the range of 0 to 200 µM. Both samples and working standard solutions were spiked with the Internal Standard (Aminocaproic acid solution, 50 µM) in a ratio of 1:1. Samples and working standard solutions were filtered through a 0.22 µm syringe membrane filter before HPLC analysis. For the analysis, the primary amino acids were derivatized by o-phthaldialdehyde in the presence of 3-mercapto propionic acid as described previously [326], [327]. Briefly, the amino acid composition analysis was carried out on an ultra-high-performance liquid chromatography–fluorescence detection (UHPLC-FLD) system (Thermo Ultimate 3000 RS, Thermo Scientific, MA, USA) equipped with an Agilent AdvanceBio AAA column (100 mm length × 3.0 mm internal diameter × 2.7 µm particle size; Agilent Technologies, CA, USA) fitted to a guard cartridge. Mobile phase A consisted of 10 mM Na_2HPO_4 in 10 mM $Na_2B_4O_7$ decahydrate (pH 8.2) whereas mobile phase B consisted of a mixture of acetonitrile, methanol and water at a ratio of 45:45:10 (v/v/v). A flow rate of 0.620 mL/min was used with the following gradient program: 0–0.35 min, 2% B; 0.35–13.4 min, 57% B; 13.4–13.5 min, 100% B; 13.5–15.7 min, 100% B; 15.7–15.8 min, 2% B; 15.8–18.0 min, 2% B. Fluorescence detection was carried out by setting an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The amino acids in the samples were quantified based on the internal standard calibration method using authentic amino acid calibration standards.

6.2.2.9 Preparation of water extract

One gram of plant-based and traditional meat samples was added to 20 mL of Milli-Q water and homogenized using an Ultra-turrax homogenizer (T 25 digital ULTRATURRAX®) for 2 min at 12000 rpm. The homogenates were cooled in the freezer (-20 °C) for 15 min and then centrifuged for 15 min at 7500 rpm at 0°C to collect any major matrix components in the bottom pellet and separate the top fat layer. Avoiding the fat layer and the pellet, 15 mL of the clear middle layer was retrieved and stored in the fridge (5°C) until further use. This water extract was used for acrylamide analyses.

6.2.2.10 Acrylamide analysis

In an eppendorf tube, 750 µL of water extract, 750 µL of Formic Acid (20 mM) containing acrylamide d-3 (53.3 ng/mL), 50 µL Carrez I and 50 µL of Carrez II were mixed vigorously using a vortex mixer for at least 1 min. Thereafter samples were centrifuged for 15 min at 15.000 rpm at 10°C. After centrifugation, 1 mL supernatant was filtered through a 0.22 µm nylon syringe filter. The filtrate was then passed through preconditioned MCX cartridges, where the first 8 drops were discarded, and the rest of the extract was collected into HPLC vials. Working concentrations of acrylamide were prepared in the range of 0.5-250 ng/mL and then 75 µL of each standard concentration, 75 µL of Formic Acid (20 mM) containing acrylamide d-3 (53.3 ng/mL) and 10 µL of Milli-Q water were mixed directly in HPLC vials.

Five µL of samples were injected in a Vanquish LC system (Thermo Fisher Scientific Inc., Waltham, USA) equipped with an Acquity UPLC® HSS T3 column (100 mm length x 2.1 mm internal diameter x 1.8 µm particle size; Waters, Taastrup, Denmark) at 40°C. Chromatographic separation was performed using isocratic condition (98:2) of mobile phase A consisting of 10 mM formic acid solution and mobile phase B consisting of 10 mM formic acid in acetonitrile for a total run of 10 min and at a flow rate of 0.250 mL/min. The LC system was directly interfaced with a TSQ Quantis triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA) operated in positive heated electrospray ionization mode with a voltage of 3500 V and using the following interface parameters: ion transfer tube as well as the vaporizer temperature was 275 ℃, sheath gas (60 arbitrary units), auxiliary gas (5 arb. unit), sweep gas (1 arb. unit). Data acquisition was performed by selected reaction monitoring (SRM) mode where acrylamide was monitored by the transitions of m/z values 72.08 → 55.04, 44.04 and 27.18 and acrylamide-d3 by m/z values of 75.01 → 58.05, 44.04 and 30.20. m/z 55.04 and m/z 58.05 were used as the quantifier ions for acrylamide and acrylamide-d3, respectively.

6.2.2.11 Statistical analysis

One-way Repeated Measures ANOVA tests with Tukey post-test were used to determine the statistical difference between samples. Data are presented as the means ± SD (standard deviation). All statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, San Diego CA, USA), and the statistical significance level was set at $p<0.05$.

6.2.3 Results

6.2.3.1 Total AA analysis

The total AA content of burger, chicken and schnitzel samples was found higher in traditional samples than their correspondent Brand A and Brand B samples. In sausages, the total AA content was instead found higher in Brand B samples than Brand A and traditional samples (Figure 13). This can be easily explained by the fact that the label protein content of Brand B is also higher than the one of Brand A and traditional samples.

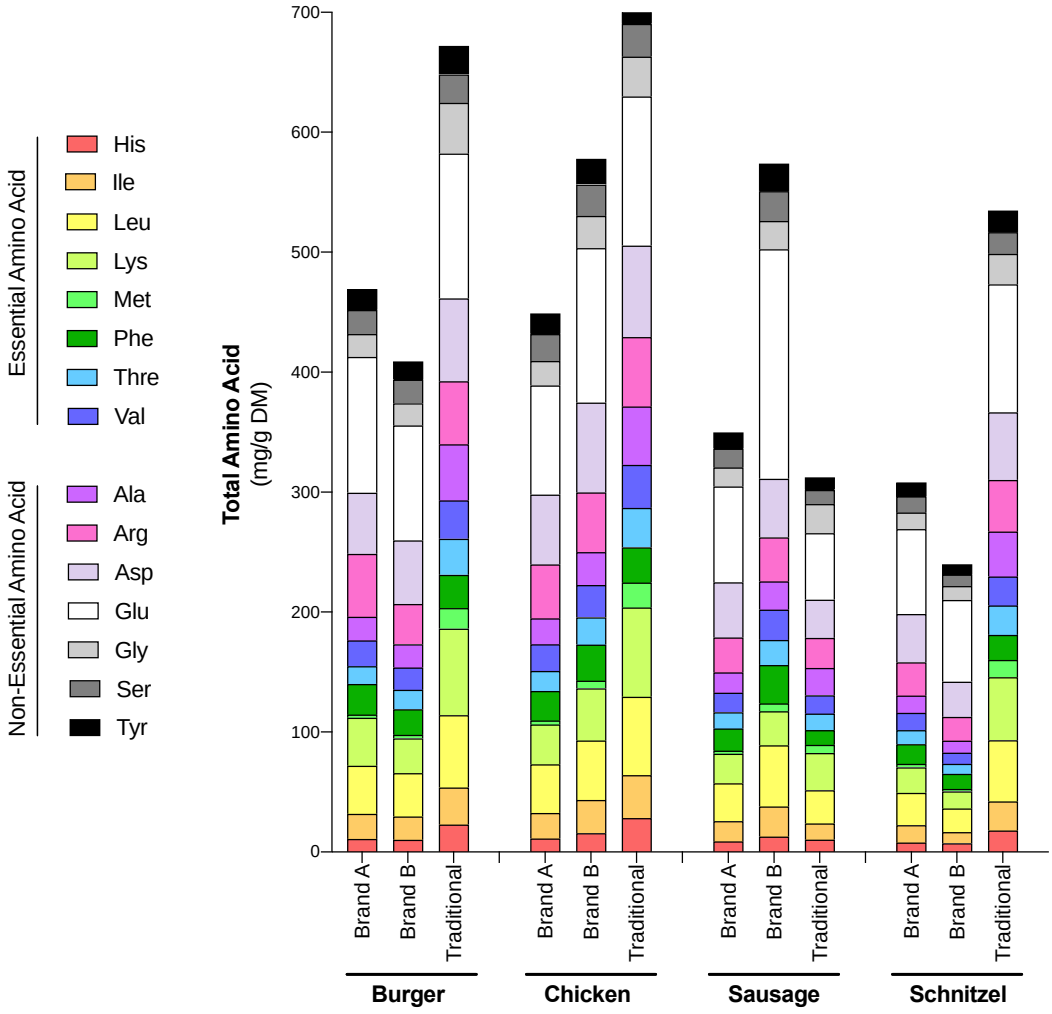

Figure 13. Total Amino Acid (AA) content (mg/g dry matter (DM)) of Brand A and Brand B plant-based meat alternatives and their correspondent animal-based counterparts.

6.2.3.2 AGEs and acrylamide analyses in traditional meat samples

The three most important Amadori products that are formed in foods are N-ε-fructosyllysine, N-ε-maltulosyllysine, and N-ε-lactulosyllysine, which are derived from glucose, maltose, and lactose, respectively. The determination of these Amadori compounds is collectively done after acid hydrolysis of food samples, by which they are all converted to furosine. Consequently, furosine represents an important marker of the early stages of the Maillard reaction in food [182]. Furosine was found significantly in high concentration in chicken samples than in burger, sausage and schnitzel samples. In addition, sausages showed significantly higher concentrations of furosine than burger samples. No statistical differences were observed between the other meat samples (Figure 14A). CML and CEL are the most common AGEs found in food and they are mainly generated by the reaction between lysine and glyoxal or methylglyoxal, respectively. Chicken samples showed significantly higher concentrations of CML than the other meat samples (Figure 14B). CEL was found to be significantly high in chicken than sausage and schnitzel samples (Figure 14C). Arg-derived AGEs include MGO- and GO-derived hydroimidazolones (MG-Hs and GO-Hs), which exist as three different isomers (H1-H2-H3) but have been quantified as MG-H3-equivalents and GO-H3 equivalents corresponding to the sum of MGO- or GO-isomers. Again, chicken samples showed the highest content of GO-Hs and MG-Hs in comparison with the other meat samples. In addition, both sausage and schnitzel showed higher concentrations of MG-Hs than burger samples (Figure 14D,E).

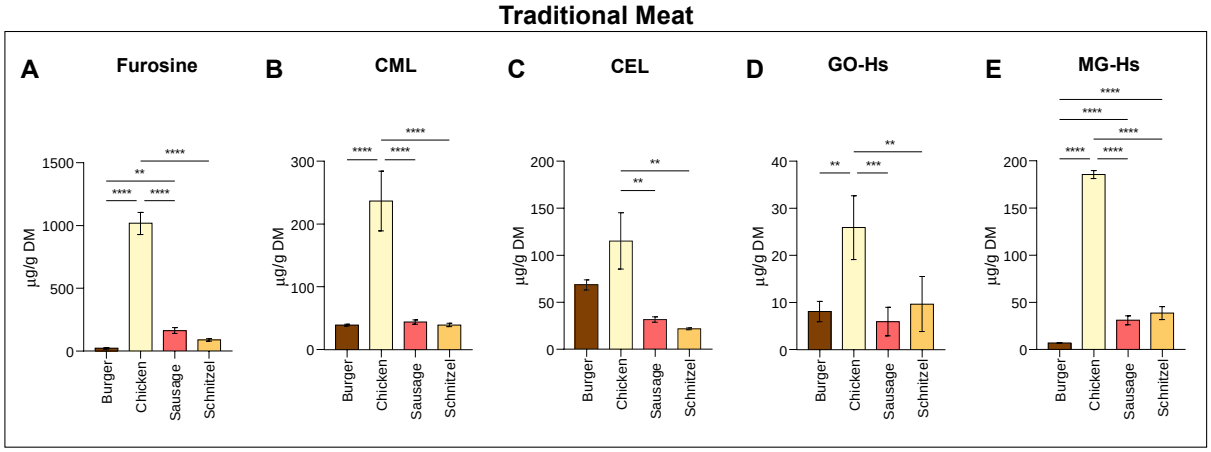

Figure 14. Furosine and AGEs content in traditional meat samples: (A) Furosine: chicken (p<0.0001 vs burger, sausage and schnitzel samples); sausage (p<0.01 vs burger samples). (B) CML: chicken (p<0.0001 vs burger, sausage and schnitzel samples). (C) CEL: chicken (p<0.01 vs sausage and schnitzel samples); (D) GO-Hs: chicken (p<0.01 vs burger and schnitzel samples; p<0.001 vs sausage samples). (E) MG-Hs: chicken (p<0.0001 vs burger, sausage, schnitzel samples); sausage (p<0.0001 vs burger samples); schnitzel (p<0.0001 vs burger samples).

No significant differences were detected in terms of acrylamide content in all meat samples but chicken and schnitzel samples seem to have the highest content of acrylamide (Figure 15).

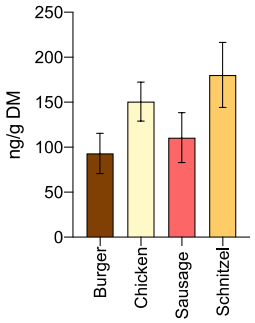

Figure 15. Acrylamide content in traditional meat samples

6.2.3.3 AGEs and acrylamide analyses in burger plant-based meat samples

Brand A burgers showed significantly higher content of furosine than Brand B and traditional burger samples (Figure 16A). Both Brand A and Brand B burger samples showed a significant increase in CML, GO-Hs and MG-Hs than traditional burger samples (Figure 16B,D,E). A significant decrease in CEL content was instead observed in both Brand A and Brand B samples than in traditional burger samples (Figure 16B,C). No differences were observed between Brand A, Brand B and Traditional burger samples in terms of acrylamide content (data not shown).

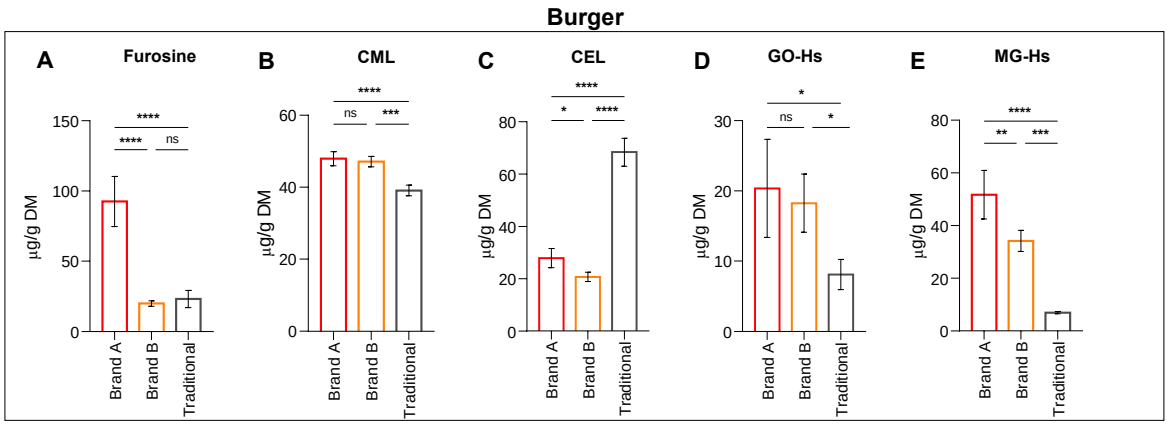

Figure 16. Furosine and AGEs content in plant-based burger samples (Brand A and Brand B) in comparison with traditional burger samples: (A) Furosine: Brand A (p<0.0001 vs Brand B and traditional samples); (B) CML: Brand A (p<0.0001 vs traditional samples); Brand B (p<0.001 vs traditional samples). (C) CEL: Brand A and Brand B (p<0.0001 vs traditional samples); Brand A (p<0.05 vs Brand B). (D) GO-Hs: Brand A and Brand B (p<0.01 vs traditional samples). (E) MG-Hs: Brand A (p<0.0001 vs traditional samples); Brand B (p<0.001 vs traditional samples); Brand A (p<0.001 vs Brand B samples).

6.2.3.4 AGEs and acrylamide analyses in chicken plant-based meat samples

Furosine was found to be significantly decreased in both Brand A and Brand B samples in comparison to the animal-based counterparts (Figure 17A). Baked of both Brand A and Brand B showed a significant decrease in terms of their content of CML (Figure 17B) whereas all Brand B samples showed a significant decrease in CEL, GO-Hs, MG-Hs concentrations. Interestingly, Brand A chicken samples contain similar content of CEL, GO-Hs, MG-Hs than traditional chicken samples (Figure 17C-E). No statistical differences were observed in terms of acrylamide between Brand A, Brand B and traditional meat samples (data not shown).

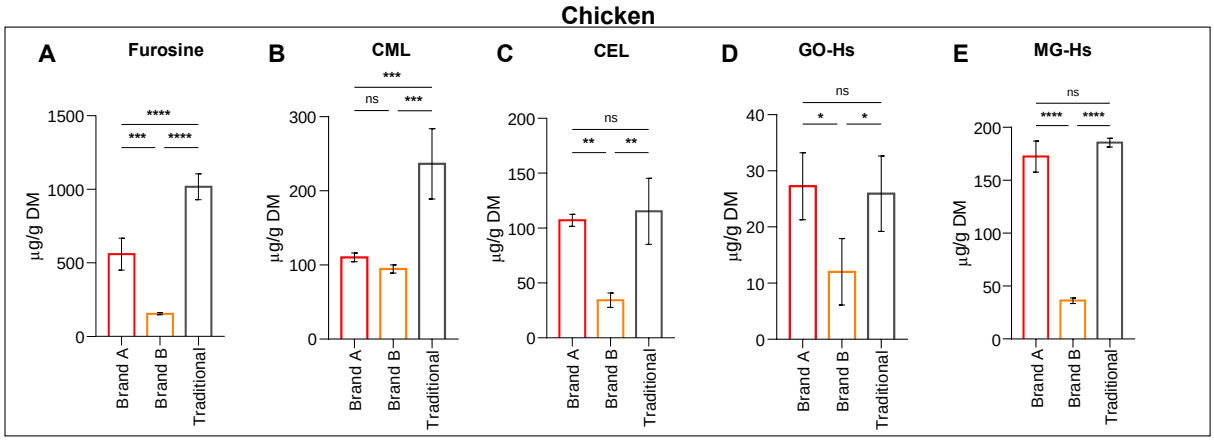

Figure 17. Furosine and AGEs content in plant-based chicken samples (Brand A and Brand B) in comparison with traditional chicken samples: (A) Furosine: Brand A and Brand B (p<0.0001 vs traditional samples); Brand A (p<0.001 vs Brand B) (B) CML: Brand A and Brand B (p<0.001 vs traditional samples). (C) CEL: Brand B (p<0.01 vs traditional and Brand A samples). (D) GO-Hs: Brand B (p<0.05 vs traditional and Brand A samples). (E) MG-Hs: Brand B (p<0.0001 vs traditional samples); Brand B (p<0.001 vs traditional and Brand A samples).

6.2.3.5 AGEs and acrylamide analyses in sausage plant-based meat samples

Brand B samples showed a significant increase in furosine, CML and MG-Hs concentration than traditional counterparts (Figure 18A,B,E). CEL content was found significantly reduced in Brand A and and Brand B samples in comparison with traditional samples while Brand A and Brand B samples showed a significant increase in GO-Hs content (Figure 18C,D). No statistical differences were observed in terms of acrylamide between sausages of Brand A, Brand B and traditional meat samples (data not shown).

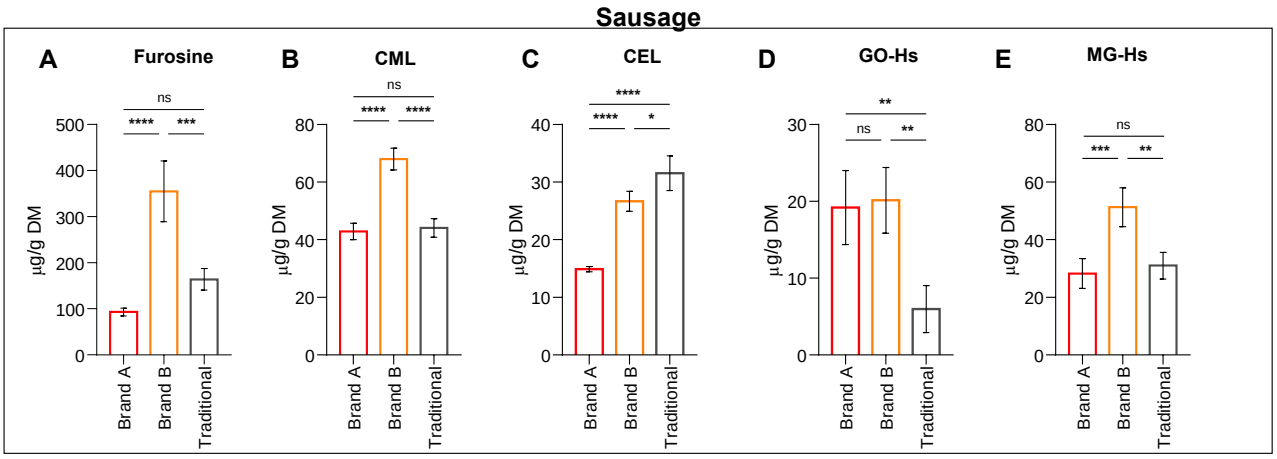

Figure 18. Furosine and AGEs content in plant-based sausage samples (Brand A and Brand B) in comparison with traditional sausage samples: (A) Furosine: Brand B (p<0.0001 vs traditional samples; p<0.001 Brand A samples); (B) CML: Brand B (p<0.0001 vs traditional and Brand A samples). (C) CEL: Brand B (p<0.05 vs traditional samples; p<0.0001 vs Brand A samples); Brand A (p<0.0001 vs traditional samples). (D) GO-Hs: Brand A and Brand B (p<0.01 vs traditional samples). (E) MG-Hs: Brand B (p<0.01 vs traditional samples; p<0.001 vs Brand A samples).

6.2.3.6 AGEs and acrylamide analyses in schnitzel plant-based meat samples

No differences between plant-based samples and traditional counterparts were observed in terms of furosine, CEL, GO-Hs and MG-Hs content. Only Brand A showed a significant increase in terms of CML than traditional samples (Figure 19). No statistical differences were observed in terms of acrylamide between Brand A, Brand B and traditional schnitzel samples (data not shown).

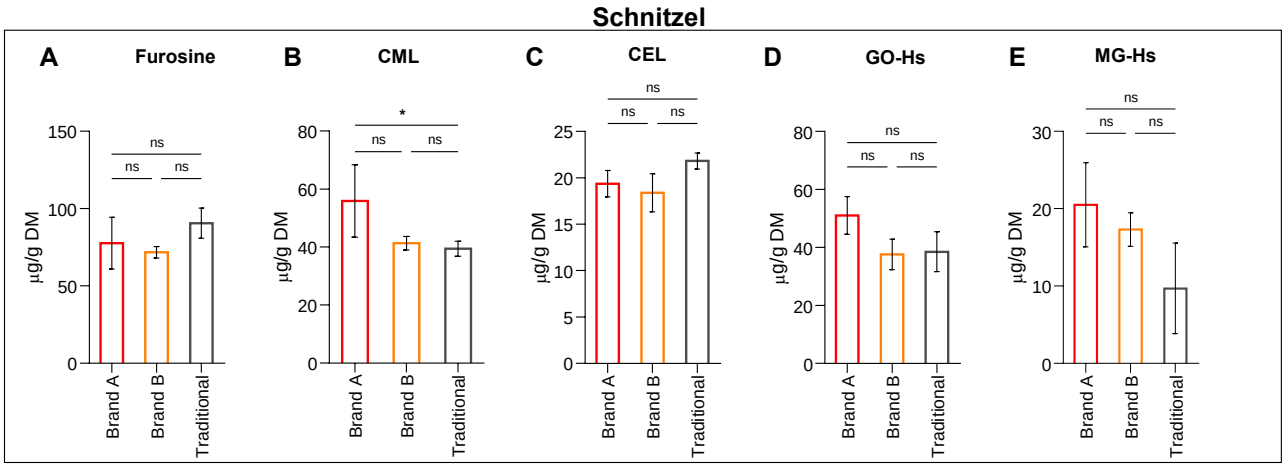

Figure 19. Furosine and AGEs content in plant-based schnitzel samples (Brand A and Brand B) in comparison with traditional schnitzel samples: (A) Furosine; (B) CML: Brand A (p<0.05 vs traditional samples); (C) CEL; (D) GO-Hs; (E) MG-Hs.

6.2.4 Discussion

Foods high in fat, sugars and protein exposed to high temperatures are significantly susceptible to the formation of MRPs and in particular to the formation of AGEs and other toxic intermediates of the Maillard Reaction such as acrylamide [167], [209]. Our body physiologically contributes to the detoxification of these compounds via anti-oxidants and specific enzymes [369], [370]. However, a high consumption of AGEs through an extended period prevents the body to eliminate them fast enough and, consequently, they start to accumulate, leading to oxidative stress, inflammation and the development of cardiovascular disease, diabetes, Alzheimer's disease, premature aging and kidney failure [369]. In this regard, people with high blood sugar such as diabetes patients are more susceptible to AGEs formation and in turn, are at a higher risk [371]. Recently, a worldwide health concern has become the occurrence of acrylamide in heat-processed foods, as the International Agency for Research on Cancer (IARC) has classified this substance as a likely human carcinogen [372]. Several studies have already investigated the content of AGEs and acrylamide in different kinds of food but it has emerged that meat usually contains the highest levels of AGEs [167] while acrylamide has been detected mostly in starchy foods such as potatoes and grain products that undergo high-temperature processing [208]. In the last few years, there has been an increasing interest in plant-based meat alternatives (PBMAs), because considered more sustainable and healthier when compared to animal-based counterparts. Nevertheless, plant-based foods are processed by various techniques during their production from harvesting of the plants until packaging, and very little is known about the formation of MRPs in these kinds of products [298]. One of the main challenges in PBMA products is to generate fibrous structures that resemble muscle tissue together with other features such as juiciness, tenderness and meaty flavor [373]. It is well known that the texture, flavor, and color of PBMAs strongly depend on the ingredients used for their production [374]. PBMAs are mainly composed of protein, fat, carbohydrates, and some minor compounds such as fibers and phytochemicals. Vitamins and minerals, as well as preservatives are often used to improve nutritional quality and shelf-life, respectively, whereas pigments are sometimes exploited to reproduce traditional meat colour. Among all these components, proteins are the ones that play a pivotal role in defining the physicochemical and sensory qualities of PBMAs [375], [376]. In this regard, several non-animal protein sources (e.g. cereals, vegetables, legumes, microalgae and fungi) have been

used to replace animal proteins for the production of a wide plethora of meat-free products such as burger patties, sausages, chicken filets, meat balls and schnitzels [377]. It has been estimated that the protein content of commercial PBMAs ranges from around 10 to 25% [378] even if the amino acid profile tends to be low in essential amino acids such as lysine and methionine [287]. Moreover, a high concentration of carbohydrates naturally occurs in PBMAs given that plant-based ingredients present considerable amounts of this nutrients [379]. Considering these differences from animal-based foods and the high processing techniques employed in the production of these products, it has become really important to analyze how MRPs formation occurs in PBMAs. In particular, this project aims at evaluating how food production and manipulation can affect the content of MRPs in different PBMAs in comparison with their animal correspondents.

In pursuit of this objective, we have opted to subject both traditional and PBMA samples to identical thermal treatment, specifically through the process of baking. Baking is a prevalent culinary practice employed on a daily basis, and also happens to be one of the techniques that contributes significantly to the production of AGEs and acrylamide [253], [380]. Acrylamide formation does not usually occur in meat samples, however, since we have included in our study schnitzel samples, which are breaded, the estimation of acrylamide especially in these products will be of great value. After the cooking process, we analyzed AGEs and acrylamide content only in traditional meat samples. From our results, we found that the highest content of furosine and AGEs was found in chicken samples. This could be in contradiction with data already reported in the literature where usually the highest content of AGEs has been found in beef [167]. These differences are not due to the different protein content as from our analysis on the total amino acid we found that, even if chicken samples have a higher content of total amino acid than sausage and schnitzel, their content is similar to the ones of burgers samples which are made only of beef. On the label of traditional chicken samples, also tapioca starch, xylose and dextrose are listed in the ingredients of traditional chicken samples (Table 8). Tapioca starch is a carbohydrate commonly added to food as a thickener and to improve texture and consistency in meat products [381]. On the other hand, xylose and dextrose, both sugars, are sometimes added to meat products to impact flavor, and in the case of dextrose, to enhance the water-holding capacity of poultry meat [382]. Our hypothesis is that the presence of both carbohydrates and sugars in addition to the high content of protein of our chicken samples and the increased temperatures employed in our study significantly favor Maillard Reaction leading to the high content of furosine and AGEs observed in our samples. Regarding

acrylamide, no statical differences have been observed between traditional meat samples even if schnitzel, as expected, showed the highest amount. Moving to the analysis of PBMAs, in plant-based burger samples the concentration of furosine and AGEs, with the exception of CEL, were all find increased than in traditional burger samples. Deng *et al.* analyzed CML content in several raw and cooked commercial plant-based burgers. In accordance with our results, their work demonstrated that a significant accumulation of CML occurred during thermal home-processing in the analyzed commercial plant-based burger, confirming that thermal home-processing procedures should be selected as targets for the control point of AGEs formation [368]. Therefore, adjusting the cooking condition by decreasing cooking temperatures and shortening heating duration can effectively inhibit the formation of AGEs. In their study, Deng *et al.* also found an increased content of CEL which instead we found decreased than traditional burger samples. The increase in CEL and CML content is not usually correlated. Zhu *et al.* found that the content of CML in braised chicken was higher than that of CEL [383]. These differences, as the ones observed in our samples, may be caused by different processing methods, as well as different reaction rates of lysine with GO or MGO.

Furosine and CML content of Brand A chicken samples were found decreased than traditional chicken samples while the content of CEL, GO-Hs and MG-Hs were found comparable to the traditional chicken samples. Interestingly, Brand B samples showed instead significantly lower content of furosine and AGEs than their traditional counterparts. These differences between Brand A and Brand B samples could be due to the difference in the protein source of the plant-based chicken samples. Indeed, Brand A is mainly made of pea protein while brand B by soy protein. Since the protein sources of these two products are different, their susceptibility to glycation may differ causing in turn a difference in AGEs content [384].

Regarding sausage samples, Brand A samples showed a lower content of CEL and increase levels of GO-Hs than traditional sausage samples. On the contrary, except for CEL content, Brand B showed a higher content of furosine, CML, GO-Hs and MG-Hs than traditional sausage samples. Brand A samples not only have a lower content of protein and carbohydrates than Brand B samples but interestingly contain coriander and apple extract among their ingredients which have been both associated with anti-glycation effects. Apple contains several polyphenols that can exert antiglycation effects through several mechanisms like, among others, competing with sugars to hinder the formation of linkages between sugar and protein, capturing or inhibiting the formation of reactive carbonyl species and preventing the

interaction between AGEs and receptors that are specific to AGEs [385]. In addition, also extracts of coriander have demonstrated to cause a marked inhibition of AGEs formation in *in vitro* assays[386]. Taken together, these results can explain why the content of furosine and AGEs in Brand A samples is lower or at least comparable to traditional samples. Schnitzel plant-based samples showed similar content of furosine and AGEs than traditional schnitzel samples suggesting that processing treatments as well as cooking temperature in this case does not influence with a great extent the content of AGEs.

Regarding acrylamide, no statistical differences have been observed between PBMAs and their correspondent traditional counterparts. Meat products have no benchmark levels in terms of acrylamide, hence there is no direct benchmark levels of acrylamide to compare the health risk posed by plant-based meat alternatives.

6.2.5 Conclusions

In conclusion, our results showed that the formation of AGEs occurs in PBMA products and, as with traditional meat samples, their formation can be influenced by various parameters including the protein content of the samples but also the source of protein. Furthermore, the presence of specific ingredients such as natural product extracts and antioxidants and the industrial or thermal home processing employed to cook food can be two additional factors that influence AGEs formation. This project represents a starting point to investigate the presence of harmful MRPs in food of plant origin. In the context of nutritional intervention for the prevention of chronic diseases, the novelty of this research approach is to highlight aspects of food that are still little considered. Additional data will be collected aimed at evaluating other intermediates of Maillard reaction such as α-dicarbonyl compounds, 5-hydroxymethylfurfural and furfural as well as investigating other cooking techniques. Consequently, new strategies to decrease their formation in food could be identified in order to update regulations that can be applied during production, manipulation, and thermal processes with the final goal to obtain plant-based foods with high safety standards.

Conclusions

The Maillard reaction is a chemical reaction between amino acids and reducing sugars that takes place during heating, browning, and storage of food. The reaction results in the formation of a complex mixture of compounds known as Maillard reaction products (MRPs). Despite their common occurrence in food, MRPs have only recently gained attention as potential contributors to human health and disease. While MRPs have been linked to a number of adverse health effects, including inflammation and oxidative stress, their role in human health remains unclear. To fully understand the impact of MRPs on health, it is crucial that more preclinical and clinical studies are conducted. Preclinical studies are particularly important for elucidating the biological effects of MRPs on different organs, tissues, and cells, providing insights into their potential mechanisms of action. Clinical studies are also essential for understanding the potential impact of MRPs accumulation on human health, especially their impact on vulnerable populations, such as elderly and individuals with multiple comorbidities who may be more susceptible to the harmful effects of MRPs.

Moreover, there is a need for standardizing MRPs content in food. At present, there is no standard unit of MRPs measurement, and this creates confusion and difficulty in comparing results from different studies. Moreover, standardized units of measurement for MRPs content in food will be beneficial for the general public as well. Providing clear benchmarks for MRPs content in food will allow the integration into the diet of food with low content of MRPs. Additionally, standardized units of measurement can help to promote transparency in the food industry, enabling consumers to make more informed decisions about the safety and quality of the food they consume.

In this frame, multidisciplinary studies that integrate pharmacological and chemical analytical approaches are essential for advancing our understanding of MRPs and their potential effects on human health. Chemical analysis can provide detailed information on the composition, structure, and behavior of MRPs in different food matrices. This can help to identify the specific types of MRPs that are most prevalent in different food products and the conditions under which they are most likely to form. Additionally, chemical analysis can

provide valuable information on the mechanisms by which MRPs form and how they may interact with other components in food. On the other hand, pharmacological studies can provide insights into the biological effects of different MRPs and their potential implications for human health. By examining the interactions between MRPs and cellular processes, pharmacological studies can help to identify potential mechanisms by which MRPs may contribute to disease states.

In conclusion, MRPs have the potential to significantly impact human health, and more research is needed to fully understand their effects. Preclinical and clinical studies, standardization of the measurement of MRPs, evaluation of MRPs content in different food matrices, and multidisciplinary studies are all essential for advancing our understanding of the role of MRPs in health and disease. The findings of such studies could have significant implications for public health and the development of new food processing and preparation strategies that promote healthy eating habits.

List of pubblications

During my PhD, I carried out several side projects investigating the role played by different natural extracts in human health. These projects led to the publication of two different manuscripts in international scientific journals:

1. **Phytochemical Analysis and Anti-Inflammatory Activity of Different Ethanolic Phyto-Extracts of Artemisia annua L. Biomolecules**

Abstract: Artemisia annua L. (AA) has shown for many centuries important therapeutic virtues associated with the presence of artemisinin (ART). The aim of this study was to identify and quantify ART and other secondary metabolites in ethanolic extracts of AA and evaluate the biological activity in the presence of an inflammatory stimulus. In this work, after the extraction of the aerial parts of AA with different concentrations of ethanol, ART was quantified by HPLC and HPLC-MS. In addition, anthocyanins, flavanols, flavanones, flavonols, lignans, low-molecular-weight phenolics, phenolic acids, stilbenes, and terpenes were identified and semi-quantitatively determined by UHPLC-QTOF-MS untargeted metabolomics. Finally, the viability of human neuroblastoma cells (SH-SY5Y) was evaluated in the presence of the different ethanolic extracts and in the presence of lipopolysaccharide (LPS). The results show that ART is more concentrated in AA samples extracted with 90% ethanol. Regarding the other metabolites, only the anthocyanins are more concentrated in the samples extracted with 90% ethanol. Finally, ART and all AA samples showed a protective action towards the pro-inflammatory stimulus of LPS. In particular, the anti-inflammatory effect of the leaf extract of AA with 90% ethanol was also confirmed at the molecular level since a reduction in TNF-α mRNA gene expression was observed in SH-SY5Y treated with LPS.

Abate G*, Zhang L*, **Pucci M***, Morbini G, Mac Sweeney E, Maccarinelli G, Ribaudo G, Gianoncelli A, Uberti D, Memo M, Lucini L, Mastinu A. Phytochemical Analysis and Anti-Inflammatory Activity of Different Ethanolic Phyto-Extracts of Artemisia annua L. Biomolecules. 2021 Jul 2;11(7):975. doi: 10.3390/biom11070975. PMID: 34356599; PMCID: PMC8301839.

2. Different Seasonal Collections of *Ficus carica L.* Leaves Diversely Modulate Lipid Metabolism and Adipogenesis in 3T3-L1 Adipocytes.

Abstract: Due to the high prevalence of obesity and type 2 diabetes, adipogenesis dysfunction and metabolic disorders are common features in the elderly population. Thus, the identification of novel compounds with anti-adipogenic and lipolytic effects is highly desirable to reduce diabetes complications. Plants represent an important source of bioactive compounds. To date, the antidiabetic potential of several traditional plants has been reported, among which *Ficus carica L.* is one of the most promising. Considering that plant metabolome changes in response to a number of factors including seasonality, the aim of this study was to evaluate whether Ficus carica leaves extracts collected in autumn (FCa) and spring (FCs) differently modulate lipid metabolism and adipogenesis in 3T3-L1 adipocytes. The 1H-NMR profile of the extracts showed that FCs have a higher content of caffeic acid derivatives, glucose, and sucrose than FCa. In contrast, FCa showed a higher concentration of malic acid and furanocoumarins, identified as psoralen and bergapten. In vitro testing showed that only FCa treatments were able to significantly decrease the lipid content (Ctrl vs. FCa 25 µg/mL, 50 µg/mL and 80 µg/mL; $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Furthermore, FCa treatments were able to downregulate the transcriptional pathway of adipogenesis and insulin sensitivity in 3T3-L1 adipocytes. In more detail, FCa 80 µg/mL significantly decreased the gene expression of PPARγ ($p < 0.05$), C/EBPα ($p < 0.05$), Leptin ($p<0.0001$), adiponectin ($p<0.05$) and GLUT4 ($p<0.01$). In conclusion, this study further supports an in-depth investigation of *F. carica* leaves extracts as a promising source of active compounds useful for targeting obesity and diabetes.

Pucci M, Mandrone M, Chiocchio I, Sweeney EM, Tirelli E, Uberti D, Memo M, Poli F, Mastinu A, Abate G. Different Seasonal Collections of *Ficus carica L.* Leaves Diversely Modulate Lipid Metabolism and Adipogenesis in 3T3-L1 Adipocytes. Nutrients. 2022 Jul 10;14(14):2833. doi: 10.3390/nu14142833. PMID: 35889791; PMCID: PMC9323846.

In addition, I contribute to the publication of the following papers:

3. Bayati, P.; Karimmojeni, H.; Razmjoo, J.; **Pucci, M**.; Abate, G.; Baldwin, T.C.; Mastinu, A. Physiological, Biochemical, and Agronomic Trait Responses of Nigella sativa Genotypes to Water Stress. Horticulturae 2022, 8, 193. https://doi.org/10.3390/horticulturae8030193

4. Ashrafi M, Azimi-Moqadam MR, MohseniFard E, Shekari F, Jafary H, Moradi P, **Pucci M**, Abate G, Mastinu A. Physiological and Molecular Aspects of Two Thymus Species Differently Sensitive to Drought Stress. BioTech (Basel). 2022 Mar 23;11(2):8. doi: 10.3390/biotech11020008. PMID: 35822781; PMCID: PMC9264393.

5. Mastinu, A.; Anyanwu, M.; Carone, M.; Abate, G.; Bonini, S.A.; Peron, G.; Tirelli, E.; **Pucci, M**.; Ribaudo, G.; Oselladore, E.; Premoli, M.; Gianoncelli, A.; Uberti, D.L.; Memo, M. The Bright Side of Psychedelics: Latest Advances and Challenges in Neuropharmacology. Int. J. Mol. Sci. 2023, 24, 1329. https://doi.org/10.3390/ijms24021329

Bibliography

1. Witkamp, R. F. and van Norren, K., “Let thy food be thy medicine….when possible,” *Eur. J. Pharmacol.*, vol. 836, no. May, pp. 102–114, 2018, doi: 10.1016/j.ejphar.2018.06.026.
2. World Health Organization, “Nutrition For Health and Development,” 2000.
3. Kalache, A.; de Hoogh, A. I.; Howlett, S. E.; Kennedy, B.; Eggersdorfer, M.; Marsman, D. S.; Shao, A.; and Griffiths, J. C., “Nutrition interventions for healthy ageing across the lifespan: a conference report,” *Eur. J. Nutr.*, vol. 58, no. s1, pp. 1–11, 2019, doi: 10.1007/s00394-019-02027-z.
4. Gómez-Pinilla, F., “the Effects of Gómez-Pinilla, F. (2008). the Effects of Nutrients on Brain Function. Nature Reviews. Neuroscience, 9(july), 568–578. https://doi.org/10.1038/nrn2421.BrainNutrients on Brain Function,” *Nat. Rev. Neurosci.*, vol. 9, no. july, pp. 568–578, 2008, doi: 10.1038/nrn2421.Brain.
5. Dominguez, L. J. and Barbagallo, M., “Nutritional prevention of cognitive decline and dementia,” *Acta Biomed.*, vol. 89, no. 2, pp. 276–290, 2018, doi: 10.23750/abm.v89i2.7401.
6. Rintamäki, R.; Rautio, N.; Peltonen, M.; Jokelainen, J.; Keinänen-Kiukaanniemi, S.; Oksa, H.; Saaristo, T.; Puolijoki, H.; Saltevo, J.; Tuomilehto, J.; Uusitupa, M.; and Moilanen, L., “Long-term outcomes of lifestyle intervention to prevent type 2 diabetes in people at high risk in primary health care,” *Prim. Care Diabetes*, vol. 15, no. 3, pp. 444–450, 2021, doi: 10.1016/j.pcd.2021.03.002.
7. Martínez-González, M. A.; Gea, A.; and Ruiz-Canela, M., “The Mediterranean Diet and Cardiovascular Health: A Critical Review,” *Circ. Res.*, vol. 124, no. 5, pp. 779–798, 2019, doi: 10.1161/CIRCRESAHA.118.313348.
8. Di Daniele, N.; Noce, A.; Vidiri, M. F.; Moriconi, E.; Marrone, G.; Annicchiarico-Petruzzelli, M.; D’urso, G.; Tesauro, M.; Rovella, V.; and De Lorenzo, A., “Impact of Mediterranean diet on metabolic syndrome, cancer and longevity,” *Oncotarget*, vol. 8, no. 5, pp. 8947–8979, 2017, [Online]. Available: www.impactjournals.com/oncotarget/

9. Cena, H. and Calder, P. C., “Defining a Healthy Diet : Evidence for the Role of,” *Nutrients*, vol. 12, no. 334, pp. 1–15, 2020, [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7071223/pdf/nutrients-12-00334.pdf
10. Neufeld, L. M.; Hendriks, S.; and Hugas, M., “Healthy Diet- a definition for the United Nations Food System Summit 2021.” 2021.
11. Trichopoulou, A., “Mediterranean diet as intangible heritage of humanity: 10 years on,” *Nutr. Metab. Cardiovasc. Dis.*, vol. 31, no. 7, pp. 1943–1948, 2021, doi: 10.1016/j.numecd.2021.04.011.
12. Colao, A.; Vetrani, C.; Muscogiuri, G.; Barrea, L.; Tricopolou, A.; and Piscitelli, P., “Towards a ‘ Planeterranean ’ Diet,” 2022.
13. Matafome, P.; Sena, C.; and Seiça, R., “Methylglyoxal, obesity, and diabetes,” *Endocrine*, vol. 43, no. 3, pp. 472–484, 2013, doi: 10.1007/s12020-012-9795-8.
14. Zheng, J.; Guo, H.; Ou, J.; Liu, P.; Huang, C.; Wang, M.; Simal-Gandara, J.; Battino, M.; Jafari, S. M.; Zou, L.; Ou, S.; and Xiao, J., “Benefits, deleterious effects and mitigation of methylglyoxal in foods: A critical review,” *Trends Food Sci. Technol.*, vol. 107, no. June 2020, pp. 201–212, 2021, doi: 10.1016/j.tifs.2020.10.031.
15. Wang, Y. and Ho, C. T., “Flavour chemistry of methylglyoxal and glyoxal,” *Chem. Soc. Rev.*, vol. 41, no. 11, pp. 4140–4149, 2012, doi: 10.1039/c2cs35025d.
16. Twarda-clapa, A.; Olczak, A.; Białkowska, A. M.; and Koziołkiewicz, M., “Advanced Glycation End-Products (AGEs): Formation, Chemistry, Classification, Receptors, and Diseases Related to AGEs Aleksandra,” 2022.
17. Sharma, C.; Kaur, A.; Thind, S. S.; Singh, B.; and Raina, S., “Advanced glycation End-products (AGEs): an emerging concern for processed food industries,” *J. Food Sci. Technol.*, vol. 52, no. 12, pp. 7561–7576, 2015, doi: 10.1007/s13197-015-1851-y.
18. Degen, J.; Vogel, M.; Richter, D.; Hellwig, M.; and Henle, T., “Metabolic transit of dietary Methylglyoxal,” *J. Agric. Food Chem.*, vol. 61, no. 43, pp. 10253–10260, 2013, doi: 10.1021/jf304946p.

19. Guilbaud, A.; Niquet-Leridon, C.; Boulanger, E.; and Tessier, F. J., “How can diet affect the accumulation of advanced glycation end-products in the human body?,” *Foods*, vol. 5, no. 4, pp. 1–14, 2016, doi: 10.3390/foods5040084.
20. Hernandez-Castillo, C. and Shuck, S. C., “Diet and Obesity-Induced Methylglyoxal Production and Links to Metabolic Disease,” *Chem. Res. Toxicol.*, vol. 34, no. 12, pp. 2424–2440, 2021, doi: 10.1021/acs.chemrestox.1c00221.
21. Tan, S. M.; Lindblom, R. S. J.; Ziemann, M.; Laskowski, A.; Granata, C.; Snelson, M.; Thallas-Bonke, V.; El-Osta, A.; Baeza-Garza, C. D.; Caldwell, S. T.; Hartley, R. C.; Krieg, T.; Cooper, M. E.; Murphy, M. P.; and Coughlan, M. T., “Targeting methylglyoxal in diabetic kidney disease using the mitochondria-targeted compound mitogamide,” *Nutrients*, vol. 13, no. 5, 2021, doi: 10.3390/nu13051457.
22. Nemet, I.; Varga-Defterdarović, L.; and Turk, Z., “Methylglyoxal in food and living organisms,” *Mol. Nutr. Food Res.*, vol. 50, no. 12, pp. 1105–1117, 2006, doi: 10.1002/mnfr.200600065.
23. Nagao, M.; Fujita, Y.; Wakabayashi, K.; Nukaya, H.; Kosuge, T.; and Sugimura, T., “Mutagens in Coffee and Other Beverages,” 1986.
24. De Revel, G. and Bertrand, A., “A Method for the Detection of Carbonvl 4 Compounds in Wine: Glyoxal and Me thylgl y oxal,” 1993.
25. Yamaguchi, M.; Ishida, J.; Xuan-Xuan, Z.; Yamaguchi, M.; Nakamura, M.; Junichiishtoa, I.; and Yoshitake, T., “Determination of glyoxal, methylglyoxal, diacethyl, and 2,3-pentanedione in fermented foods by high-performance liquid chromatography with fluorescence detection,” *J. Liq. Chromatogr.*, vol. 17, no. 1, pp. 203–211, Jan. 1994, doi: 10.1080/10826079408013445.
26. Degen, J.; Hellwig, M.; and Henle, T., “1,2-Dicarbonyl compounds in commonly consumed foods,” *J. Agric. Food Chem.*, vol. 60, no. 28, pp. 7071–7079, 2012, doi: 10.1021/jf301306g.
27. Hellwig, M.; Degen, J.; and Henle, T., “3-Deoxygalactosone, a ‘new’ 1,2-dicarbonyl compound in milk products,” *J. Agric. Food Chem.*, vol. 58, no. 19, pp. 10752–10760, 2010, doi: 10.1021/jf102388v.

28. Adams, C. J.; Manley-Harris, M.; and Molan, P. C., “The origin of methylglyoxal in New Zealand manuka (Leptospermum scoparium) honey,” *Carbohydr. Res.*, vol. 344, no. 8, pp. 1050–1053, May 2009, doi: 10.1016/j.carres.2009.03.020.
29. Atrott, J.; Haberlau, S.; and Henle, T., “Studies on the formation of methylglyoxal from dihydroxyacetone in Manuka (Leptospermum scoparium) honey,” *Carbohydr. Res.*, vol. 361, pp. 7–11, Nov. 2012, doi: 10.1016/j.carres.2012.07.025.
30. Hollnagel, A. and Kroh, L. W., “Formation of α-dicarbonyl fragments from mono- and disaccharides under caramelization and Maillard reaction conditions,” *Eur. Food Res. Technol.*, vol. 207, no. 1, pp. 50–54, 1998.
31. Zheng, J.; Ou, J.; and Ou, S., *Chemical Hazards in Thermally-Processed Foods*, no. October. 2019. doi: 10.1007/978-981-13-8118-8.
32. Thornalley, P. J.; Langborg, A.; and Minhas, H. S., “Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose,” 1999.
33. Onyango, A. N., “Small reactive carbonyl compounds as tissue lipid oxidation products; And the mechanisms of their formation thereby,” *Chemistry and Physics of Lipids*, vol. 165, no. 7. pp. 777–786, Oct. 2012. doi: 10.1016/j.chemphyslip.2012.09.004.
34. Saxena, R. K.; Anand, P.; Saran, S.; Isar, J.; and Agarwal, L., “Microbial production and applications of 1,2-propanediol,” *Indian Journal of Microbiology*, vol. 50, no. 1. pp. 2–11, 2010. doi: 10.1007/s12088-010-0017-x.
35. Sena, C. M.; Matafome, P.; Crisóstomo, J.; Rodrigues, L.; Fernandes, R.; Pereira, P.; and Seiça, R. M., “Methylglyoxal promotes oxidative stress and endothelial dysfunction,” *Pharmacol. Res.*, vol. 65, no. 5, pp. 497–506, 2012, doi: 10.1016/j.phrs.2012.03.004.
36. Hofmann, S. M.; Dong, H.; Li, Z.; Cai, W.; Altomonte, J.; Thung, S. N.; Zeng, F.; Fisher, E. a; and Vlassara, H., “Restricted intake of dietary glycoxidation products in the db/db mouse,” *Diabetes*, vol. 51, no. 7, pp. 2082–2089, 2002.
37. Hellwig, M.; Gensberger-Reigl, S.; Henle, T.; and Pischetsrieder, M., “Food-

derived 1,2-dicarbonyl compounds and their role in diseases," *Semin. Cancer Biol.*, vol. 49, no. March, pp. 1–8, 2018, doi: 10.1016/j.semcancer.2017.11.014.

38. Maasen, K.; Eussen, S. J. P. M.; Scheijen, J. L. J. M.; Van Der Kallen, C. J. H.; Dagnelie, P. C.; Opperhuizen, A.; Stehouwer, C. D. A.; Van Greevenbroek, M. M. J.; and Schalkwijk, C. G., "Higher habitual intake of dietary dicarbonyls is associated with higher corresponding plasma dicarbonyl concentrations and skin autofluorescence: The Maastricht Study," *Am. J. Clin. Nutr.*, vol. 115, no. 1, pp. 34–44, 2022, doi: 10.1093/ajcn/nqab329.
39. Okabe, E.; Tomo, T.; Tezono, K.; Kikuchi, H.; Kadota, J. I.; and Nasu, M., "Synergistic cytotoxicity of acidity and glucose degradation products in peritoneal dialysis fluid," *J. Artif. Organs*, vol. 7, no. 3, pp. 155–160, 2004, doi: 10.1007/s10047-004-0263-y.
40. Lee, H. K.; Seo, I. A.; Suh, D. J.; Lee, H. J.; and Park, H. T., "A novel mechanism of methylglyoxal cytotoxicity in neuroglial cells," *J. Neurochem.*, vol. 108, no. 1, pp. 273–284, 2009, doi: 10.1111/j.1471-4159.2008.05764.x.
41. Phillips, S. A. and Thornalley, P. J., "The formation of methylglyoxal from triose phosphates: Investigation using a specific assay for methylglyoxal," *Eur. J. Biochem.*, vol. 212, no. 1, pp. 101–105, 1993, doi: 10.1111/j.1432-1033.1993.tb17638.x.
42. Yuan, P. M. and Gracy, R. W., "The conversion of dihydroxyacetone phosphate to methylglyoxal and inorganic phosphate by methylglyoxal synthase," *Arch. Biochem. Biophys.*, vol. 183, no. 1, pp. 1–6, 1977, doi: 10.1016/0003-9861(77)90411-8.
43. Tressel, T.; Thompson, R.; Zieske, L. R.; Menendez, M. I.; and Davis, L., "Interaction between L-threonine dehydrogenase and aminoacetone synthetase and mechanism of aminoacetone production," *J. Biol. Chem.*, vol. 261, no. 35, pp. 16428–16437, 1986, doi: 10.1016/s0021-9258(18)66584-6.
44. Casazza, J. P.; Felver, M. E.; and Veech, R. L., "The metabolism of acetone in rat," *J. Biol. Chem.*, vol. 259, no. 1, pp. 231–236, 1984, doi: 10.1016/s0021-9258(17)43646-5.

45. Wetzels, S.; Wouters, K.; Schalkwijk, C. G.; Vanmierlo, T.; and Hendriks, J. J. A., "Methylglyoxal-derived advanced glycation endproducts in multiple sclerosis," *Int. J. Mol. Sci.*, vol. 18, no. 2, 2017, doi: 10.3390/ijms18020421.

46. Bondoc, F. Y.; Bao, Z.; Hu, W. Y.; Gonzalez, F. J.; Wang, Y.; Yang, C. S.; and Hong, J. Y., "Acetone catabolism by cytochrome P450 2E1: Studies with CYP2E1-null mice," *Biochem. Pharmacol.*, vol. 58, no. 3, pp. 461–463, 1999, doi: 10.1016/S0006-2952(99)00111-2.

47. Zhang, M. M.; Ong, C. Y.; Walker, M. J.; and McEwan, A. G., "Defence against methylglyoxal in Group A Streptococcus: a role for Glyoxylase I in bacterial virulence and survival in neutrophils?," pp. 1–26, 2015.

48. Turk, Z.; Nemet, I.; Varga-Defteardarovic, L.; and Car, N., "Elevated level of methylglyoxal during diabetic ketoacidosis and its recovery phase," *Diabetes Metab.*, vol. 32, no. 2, pp. 176–180, 2006, doi: 10.1016/S1262-3636(07)70266-5.

49. Maessen, D. E. M.; Stehouwer, C. D. A.; and Schalkwijk, C. G., "The role of methylglyoxal and the glyoxalase system in diabetes and other age-related diseases," *Clin. Sci.*, vol. 128, no. 12, pp. 839–861, 2015, doi: 10.1042/CS20140683.

50. Rabbani, N. and Thornalley, P. J., "Measurement of methylglyoxal by stable isotopic dilution analysis LC-MS/MS with corroborative prediction in physiological samples," *Nat. Protoc.*, vol. 9, no. 8, pp. 1969–1979, 2014, doi: 10.1038/nprot.2014.129.

51. Scheijen, J. L. J. M. and Schalkwijk, C. G., "Quantification of glyoxal, methylglyoxal and 3-deoxyglucosone in blood and plasma by ultra performance liquid chromatography tandem mass spectrometry: Evaluation of blood specimen," *Clin. Chem. Lab. Med.*, vol. 52, no. 1, pp. 85–91, 2014, doi: 10.1515/cclm-2012-0878.

52. Nakayama, K.; Nakayama, M.; Iwabuchi, M.; Terawaki, H.; Sato, T.; Kohno, M.; and Ito, S., "Plasma α-oxoaldehyde levels in diabetic and nondiabetic chronic kidney disease patients," *Am. J. Nephrol.*, vol. 28, no. 6, pp. 871–878, 2008, doi: 10.1159/000139653.

53. Lu, J.; Randell, E.; Han, Y. C.; Adeli, K.; Krahn, J.; and Meng, Q. H., “Increased plasma methylglyoxal level, inflammation, and vascular endothelial dysfunction in diabetic nephropathy,” *Clin. Biochem.*, vol. 44, no. 4, pp. 307–311, 2011, doi: 10.1016/j.clinbiochem.2010.11.004.

54. Farrera, D. O. and Galligan, J. J., “The Human Glyoxalase Gene Family in Health and Disease,” *Chem. Res. Toxicol.*, vol. 35, no. 10, pp. 1766–1776, 2022, doi: 10.1021/acs.chemrestox.2c00182.

55. de Bari, L.; Atlante, A.; Armeni, T.; and Kalapos, M. P., “Synthesis and metabolism of methylglyoxal, S-D-lactoylglutathione and D-lactate in cancer and Alzheimer’s disease. Exploring the crossroad of eternal youth and premature aging,” *Ageing Res. Rev.*, vol. 53, no. October 2018, p. 100915, 2019, doi: 10.1016/j.arr.2019.100915.

56. Shinohara, M.; Thornalley, P. J.; Giardino, I.; Beisswenger, P.; Thorpe, S. R.; Onorato, J.; and Brownlee, M., “Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis,” *J. Clin. Invest.*, vol. 101, no. 5, pp. 1142–1147, 1998, doi: 10.1172/JCI119885.

57. Thornalley, P. J., “Glyoxalase I - Structure, function and a critical role in the enzymatic defence against glycation,” *Biochem. Soc. Trans.*, vol. 31, no. 6, pp. 1343–1348, 2003, doi: 10.1042/bst0311343.

58. He, Y.; Zhou, C.; Huang, M.; Tang, C.; Liu, X.; Yue, Y.; Diao, Q.; Zheng, Z.; and Liu, D., “Glyoxalase system: A systematic review of its biological activity, related-diseases, screening methods and small molecule regulators,” *Biomed. Pharmacother.*, vol. 131, no. July, p. 110663, 2020, doi: 10.1016/j.biopha.2020.110663.

59. Brouwers, O.; Niessen, P. M.; Ferreira, I.; Miyata, T.; Scheffer, P. G.; Teerlink, T.; Schrauwen, P.; Brownlee, M.; Stehouwer, C. D.; and Schalkwijk, C. G., “Overexpression of glyoxalase-I reduces hyperglycemiainduced levels of advanced glycation end products and oxidative stress in diabetic rats,” *J. Biol. Chem.*, vol. 286, no. 2, pp. 1374–1380, 2011, doi: 10.1074/jbc.M110.144097.

60. Hanssen, N. M. J.; Stehouwer, C. D. A.; and Schalkwijk, C. G., “Methylglyoxal and glyoxalase I in atherosclerosis,” *Biochem. Soc. Trans.*, vol. 42, no. 2, pp. 443–449, 2014, doi: 10.1042/BST20140001.

61. Kuhla, B.; Boeck, K.; Lüth, H. J.; Schmidt, A.; Weigle, B.; Schmitz, M.; Ogunlade, V.; Münch, G.; and Arendt, T., “Age-dependent changes of glyoxalase I expression in human brain,” *Neurobiol. Aging*, vol. 27, no. 6, pp. 815–822, 2006, doi: 10.1016/j.neurobiolaging.2005.04.006.

62. Toriumi, K.; Miyashita, M.; Suzuki, K.; Tabata, K.; Horiuchi, Y.; Ishida, H.; Itokawa, M.; and Arai, M., “Role of glyoxalase 1 in methylglyoxal detoxification–the broad player of psychiatric disorders,” *Redox Biol.*, vol. 49, no. December 2021, p. 102222, 2022, doi: 10.1016/j.redox.2021.102222.

63. Kalapos, M. P., “Methylglyoxal in living organisms - Chemistry, biochemistry, toxicology and biological implications,” *Toxicol. Lett.*, vol. 110, no. 3, pp. 145–175, 1999, doi: 10.1016/S0378-4274(99)00160-5.

64. Jannapureddy, S.; Sharma, M.; Yepuri, G.; Schmidt, A. M.; and Ramasamy, R., “Aldose Reductase: An Emerging Target for Development of Interventions for Diabetic Cardiovascular Complications,” *Front. Endocrinol. (Lausanne).*, vol. 12, no. March, pp. 1–14, 2021, doi: 10.3389/fendo.2021.636267.

65. Jagt, D. L. V.; Robinson, B.; Taylor, K. K.; and Hunsaker, L. A., “Reduction of trioses by NADPH-dependent aldo-keto reductases. Aldose reductase, methylglyoxal, and diabetic complications,” *J. Biol. Chem.*, vol. 267, no. 7, pp. 4364–4369, 1992, doi: 10.1016/s0021-9258(18)42844-x.

66. Vander Jagt, D. L.; Hassebrook, R. K.; Hunsaker, L. A.; Brown, W. M.; and Royer, R. E., “Metabolism of the 2-oxoaldehyde methylglyoxal by aldose reductase and by glyoxalase-I: Roles for glutathione in both enzymes and implications for diabetic complications,” *Chem. Biol. Interact.*, vol. 130–132, pp. 549–562, 2001, doi: 10.1016/S0009-2797(00)00298-2.

67. Morgenstern, J.; Fleming, T.; Schumacher, D.; Eckstein, V.; Freichel, M.; Herzig, S.; and Nawroth, P., “Loss of glyoxalase 1 induces compensatory mechanism to achieve dicarbonyl detoxification in mammalian Schwann cells,” *J. Biol. Chem.*,

vol. 292, no. 8, pp. 3224–3238, 2017, doi: 10.1074/jbc.M116.760132.

68. Schumacher, D.; Morgenstern, J.; Oguchi, Y.; Volk, N.; Kopf, S.; Groener, J. B.; Nawroth, P. P.; Fleming, T.; and Freichel, M., "Compensatory mechanisms for methylglyoxal detoxification in experimental & clinical diabetes," *Mol. Metab.*, vol. 18, no. September, pp. 143–152, 2018, doi: 10.1016/j.molmet.2018.09.005.
69. Di Loreto, S.; Caracciolo, V.; Colafarina, S.; Sebastiani, P.; Gasbarri, A.; and Amicarelli, F., "Methylglyoxal induces oxidative stress-dependent cell injury and up-regulation of interleukin-1β and nerve growth factor in cultured hippocampal neuronal cells," *Brain Res.*, vol. 1006, no. 2, pp. 157–167, 2004, doi: 10.1016/j.brainres.2004.01.066.
70. Figarola, J. L.; Singhal, J.; Rahbar, S.; Awasthi, S.; and Singhal, S. S., "LR-90 prevents methylglyoxal-induced oxidative stress and apoptosis in human endothelial cells," *Apoptosis*, vol. 19, no. 5, pp. 776–788, 2014, doi: 10.1007/s10495-014-0974-3.
71. Poulsen, M. W.; Hedegaard, R. V.; Andersen, J. M.; de Courten, B.; Bügel, S.; Nielsen, J.; Skibsted, L. H.; and Dragsted, L. O., "Advanced glycation endproducts in food and their effects on health," *Food Chem. Toxicol.*, vol. 60, pp. 10–37, 2013, doi: 10.1016/j.fct.2013.06.052.
72. Rabbani, N.; Xue, M.; and Thornalley, P. J., "Methylglyoxal-induced dicarbonyl stress in aging and disease: First steps towards glyoxalase 1-based treatments," *Clin. Sci.*, vol. 130, no. 19, pp. 1677–1696, 2016, doi: 10.1042/CS20160025.
73. Shuck, S. C.; Wuenschell, G. E.; and Termini, J. S., "Product Studies and Mechanistic Analysis of the Reaction of Methylglyoxal with Deoxyguanosine," *Chem. Res. Toxicol.*, vol. 31, no. 2, pp. 105–115, 2018, doi: 10.1021/acs.chemrestox.7b00274.
74. Fan, X.; Subramaniam, R.; Weiss, M. F.; and Monnier, V. M., "Methylglyoxal-bovine serum albumin stimulates tumor necrosis factor alpha secretion in RAW 264.7 cells through activation of mitogen-activating protein kinase, nuclear factor κB and intracellular reactive oxygen species formation," *Arch. Biochem. Biophys.*, vol. 409, no. 2, pp. 274–286, 2003, doi: 10.1016/S0003-

9861(02)00599-4.

75. Westwood, M. E. and Thornalley, P. J., “Induction of synthesis and secretion of interleukin 1β in the human monocytic THP-1 cells by human serum albumins modified with methylglyoxal and advanced glycation endproducts,” *Immunol. Lett.*, vol. 50, no. 1–2, pp. 17–21, 1996, doi: 10.1016/0165-2478(96)02496-0.

76. Faure, P.; Troncy, L.; Lecomte, M.; Wiernsperger, N.; Lagarde, M.; Ruggiero, D.; and Halimi, S., “Albumin antioxidant capacity is modified by methylglyoxal,” *Diabetes Metab.*, vol. 31, no. 2, pp. 169–177, 2005, doi: 10.1016/S1262-3636(07)70183-0.

77. Yuen, A.; Laschinger, C.; Talior, I.; Lee, W.; Chan, M.; Birek, J.; Young, E. W. K.; Sivagurunathan, K.; Won, E.; Simmons, C. A.; and McCulloch, C. A., “Methylglyoxal-modified collagen promotes myofibroblast differentiation,” *Matrix Biol.*, vol. 29, no. 6, pp. 537–548, 2010, doi: 10.1016/j.matbio.2010.04.004.

78. Chong, S. A. C.; Lee, W.; Arora, P. D.; Laschinger, C.; Young, E. W. K.; Simmons, C. A.; Manolson, M.; Sodek, J.; and McCulloch, C. A., “Methylglyoxal inhibits the binding step of collagen phagocytosis,” *J. Biol. Chem.*, vol. 282, no. 11, pp. 8510–8520, 2007, doi: 10.1074/jbc.M609859200.

79. Dobler, D.; Ahmed, N.; Song, L.; Eboigbodin, K. E.; and Thornalley, P. J., “Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification,” *Diabetes*, vol. 55, no. 7, pp. 1961–1969, 2006, doi: 10.2337/db05-1634.

80. Galligan, J. J.; Wepy, J. A.; Streeter, M. D.; Kingsley, P. J.; Mitchener, M. M.; Wauchope, O. R.; Beavers, W. N.; Rose, K. L.; Wang, T.; Spiegel, D. A.; and Marnett, L. J., “Methylglyoxal-derived posttranslational arginine modifications are abundant histone marks,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 37, pp. 9228–9233, 2018, doi: 10.1073/pnas.1802901115.

81. Zheng, Q.; Omans, N. D.; Leicher, R.; Osunsade, A.; Agustinus, A. S.; Finkin-Groner, E.; D’Ambrosio, H.; Liu, B.; Chandarlapaty, S.; Liu, S.; and David, Y., “Reversible histone glycation is associated with disease-related changes in

chromatin architecture," *Nat. Commun.*, vol. 10, no. 1, 2019, doi: 10.1038/s41467-019-09192-z.

82. Kierdorf, K. and Fritz, G., "RAGE regulation and signaling in inflammation and beyond," *J. Leukoc. Biol.*, vol. 94, no. 1, pp. 55–68, 2013, doi: 10.1189/jlb.1012519.

83. Neeper, M.; Schmidt, A. M.; Brett, J.; Shi Du Yan; Wang, F.; Pan, Y. C. E.; Elliston, K.; Stern, D.; and Shaw, A., "Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins," *J. Biol. Chem.*, vol. 267, no. 21, pp. 14998–15004, 1992, doi: 10.1016/s0021-9258(18)42138-2.

84. Yue, Q.; Song, Y.; Liu, Z.; Zhang, L.; Yang, L.; and Li, J., "Receptor for Advanced Glycation End Products: A Pivotal Hub in Immune Diseases," *Molecules*, pp. 1–19, 2022, doi: 10.2210/rcsb_pdb/mom_2015_6.

85. Rouhiainen, A.; Kuja-Panula, J.; Tumova, S.; and Rauvala, H., "RAGE-mediated cell signaling," *Methods Mol. Biol.*, vol. 963, pp. 239–263, 2013, doi: 10.1007/978-1-62703-230-8_15.

86. Zhu, P.; Ren, M.; Yang, C.; Hu, Y. X.; Ran, J. M.; and Yan, L., "Involvement of RAGE, MAPK and NF-κB pathways in AGEs-induced MMP-9 activation in HaCaT keratinocytes," *Exp. Dermatol.*, vol. 21, no. 2, pp. 123–129, 2012, doi: 10.1111/j.1600-0625.2011.01408.x.

87. Kokkola, R.; Andersson, Å.; Mullins, G.; Östberg, T.; Treutiger, C. J.; Arnold, B.; Nawroth, P.; Andersson, U.; Harris, R. A.; and Harris, H. E., "RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages," *Scand. J. Immunol.*, vol. 61, no. 1, pp. 1–9, 2005, doi: 10.1111/j.0300-9475.2005.01534.x.

88. Ramasamy, R.; Yan, S. F.; and Schmidt, A. M., "Receptor for AGE (RAGE): Signaling mechanisms in the pathogenesis of diabetes and its complications," *Ann. N. Y. Acad. Sci.*, vol. 1243, no. 1, pp. 88–102, 2011, doi: 10.1111/j.1749-6632.2011.06320.x.

89. Wautier, M. P.; Chappey, O.; Corda, S.; Stern, D. M.; Schmidt, A. M.; and Wautier, J. L., "Activation of NADPH oxidase by AGE links oxidant stress to

altered gene expression via RAGE," *Am. J. Physiol. - Endocrinol. Metab.*, vol. 280, no. 5 43-5, 2001, doi: 10.1152/ajpendo.2001.280.5.e685.

90. Bierhaus, A.; Humpert, P. M.; Morcos, M.; Wendt, T.; Chavakis, T.; Arnold, B.; Stern, D. M.; and Nawroth, P. P., "Understanding RAGE, the receptor for advanced glycation end products," *J. Mol. Med.*, vol. 83, no. 11, pp. 876–886, 2005, doi: 10.1007/s00109-005-0688-7.
91. Sparvero, L. J.; Asafu-Adjei, D.; Kang, R.; Tang, D.; Amin, N.; Im, J.; Rutledge, R.; Lin, B.; Amoscato, A. A.; Zeh, H. J.; and Lotze, M. T., "RAGE (Receptor for advanced glycation endproducts), RAGE ligands, and their role in cancer and inflammation," *J. Transl. Med.*, vol. 7, pp. 1–21, 2009, doi: 10.1186/1479-5876-7-17.
92. Detzen, L.; Cheng, B.; Chen, C. Y.; Papapanou, P. N.; and Lalla, E., "Soluble Forms of the Receptor for Advanced Glycation Endproducts (RAGE) in Periodontitis," *Sci. Rep.*, vol. 9, no. 1, pp. 1–8, 2019, doi: 10.1038/s41598-019-44608-2.
93. Pullerits, R.; Bokarewa, M.; Dahlberg, L.; and Tarkowski, A., "Decreased levels of soluble receptor for advanced glycation end products in patients with rheumatoid arthritis indicating deficient inflammatory control.," *Arthritis Res. Ther.*, vol. 7, no. 4, pp. 817–824, 2005, doi: 10.1186/ar1749.
94. Falcone, C.; Emanuele, E.; D'Angelo, A.; Buzzi, M. P.; Belvito, C.; Cuccia, M.; and Geroldi, D., "Plasma levels of soluble receptor for advanced glycation end products and coronary artery disease in nondiabetic men," *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 5, pp. 1032–1037, 2005, doi: 10.1161/01.ATV.0000160342.20342.00.
95. Emanuele, E.; D'Angelo, A.; Tomaino, C.; Binetti, G.; Ghidoni, R.; Politi, P.; Bernardi, L.; Maletta, R.; Bruni, A. C.; and Geroldi, D., "Circulating levels of soluble receptor for advanced glycation end products and ligands of the receptor for advanced glycation end products in patients with acute liver failure," *Liver Transplant.*, vol. 21, no. 6, pp. 847–854, 2015, doi: 10.1002/lt.24129.
96. Vlassara, H. and Striker, G. E., "AGE restriction in diabetes mellitus: A paradigm

shift," *Nat. Rev. Endocrinol.*, vol. 7, no. 9, pp. 526–539, 2011, doi: 10.1038/nrendo.2011.74.

97. Vlassara, H. and Uribarri, J., "Advanced glycation end products (AGE) and diabetes: Cause, effect, or both?," *Curr. Diab. Rep.*, vol. 14, no. 1, 2014, doi: 10.1007/s11892-013-0453-1.

98. Chandra, K.; Singh, P.; Dwivedi, S.; and Jain, S., "Diabetes Mellitus and Oxidative Stress: A Co-relative and Therapeutic Approach," *J. Clin. Diagnostic Res.*, vol. 2, no. Glut 4, pp. 10–15, 2019, doi: 10.7860/jcdr/2019/40628.12878.

99. Katsarou, A.; Gudbjörnsdottir, S.; Rawshani, A.; Dabelea, D.; Bonifacio, E.; Anderson, B. J.; Jacobsen, L. M.; Schatz, D. A.; and Lernmark, A., "Type 1 diabetes mellitus," *Nat. Rev. Dis. Prim.*, vol. 3, pp. 1–18, 2017, doi: 10.1038/nrdp.2017.16.

100. Galicia-Garcia, U.; Benito-Vicente, A.; Jebari, S.; Larrea-Sebal, A.; Siddiqi, H.; Uribe, K. B.; Ostolaza, H.; and Martín, C., "Pathophysiology of type 2 diabetes mellitus," *Int. J. Mol. Sci.*, vol. 21, no. 17, pp. 1–34, 2020, doi: 10.3390/ijms21176275.

101. Schalkwijk, C. G. and Stehouwer, C. D. A., "Methylglyoxal, a highly reactive dicarbonyl compound, in diabetes, its vascular complications, and other age-related diseases," *Physiol. Rev.*, vol. 100, no. 1, pp. 407–461, 2020, doi: 10.1152/physrev.00001.2019.

102. Cook, L. J.; Davies, J.; Yates, A. P.; Elliott, A. C.; Lovell, J.; Joule, J. A.; Pemberton, P.; Thornalley, P. J.; and Best, L., "Effects of methylglyoxal on rat pancreatic β-cells," *Biochem. Pharmacol.*, vol. 55, no. 9, pp. 1361–1367, 1998, doi: 10.1016/S0006-2952(97)00619-9.

103. Jia, X.; Olson, D. J. H.; Ross, A. R. S.; Wu, L.; Jia, X.; Olson, D. J. H.; Ross, A. R. S.; and Wu, L., "Structural and functional changes in human insulin induced by methylglyoxal," *FASEB J.*, vol. 20, no. 9, pp. 1555–1557, 2006, doi: 10.1096/fj.05-5478fje.

104. Riboulet-Chavey, A.; Pierron, A.; Durand, I.; Murdaca, J.; Giudicelli, J.; and Van Obberghen, E., "Methylglyoxal impairs the insulin signaling pathways

independently of the formation of intracellular reactive oxygen species," *Diabetes*, vol. 55, no. 5, pp. 1289–1299, 2006, doi: 10.2337/db05-0857.

105. Lee, J.; Yun, J. S.; and Ko, S. H., "Advanced Glycation End Products and Their Effect on Vascular Complications in Type 2 Diabetes Mellitus," *Nutrients*, vol. 14, no. 15, pp. 1–13, 2022, doi: 10.3390/nu14153086.
106. Yorek, M. A.; Wu, I.; Lockhart, S.; Coppey, L. J.; Liew, C. W.; Qiang, G.; Burkart, A. M.; Hastings, S.; Pober, D.; Cahill, C.; Niewczas, M. A.; Israelsen, W. J.; Stillman, I. E.; Amenta, P. S.; Feener, E. P.; Vander, M. G.; Stanton, R. C.; and King, G. L., "Pyruvate kinase M2 activation may protect against the progression of diabetic glomerular pathology and mitochondrial dysfunction," *Nat. Med.*, vol. 23, no. 6, pp. 753–762, 2017, doi: 10.1038/nm.4328.Pyruvate.
107. Stitt, A. W., "The role of advanced glycation in the pathogenesis of diabetic retinopathy," *Exp. Mol. Pathol.*, vol. 75, no. 1, pp. 95–108, 2003, doi: 10.1016/S0014-4800(03)00035-2.
108. Sharma, Y.; Saxena, S.; Mishra, A.; Saxena, A.; and Natu, S. M., "Advanced glycation end products and diabetic retinopathy," *J. Ocul. Biol. Dis. Infor.*, vol. 5, no. 3–4, pp. 63–69, 2012, doi: 10.1007/s12177-013-9104-7.
109. Akagawa, M.; Mori, A.; Sakamoto, K.; and Nakahara, T., "Methylglyoxal impairs β2-adrenoceptor-mediated vasodilatory mechanisms in rat retinal arterioles," *Biol. Pharm. Bull.*, vol. 41, no. 2, pp. 272–276, 2018, doi: 10.1248/bpb.b17-00861.
110. Basta, G.; Schmidt, A. M.; and De Caterina, R., "Advanced glycation end products and vascular inflammation: Implications for accelerated atherosclerosis in diabetes," *Cardiovasc. Res.*, vol. 63, no. 4, pp. 582–592, 2004, doi: 10.1016/j.cardiores.2004.05.001.
111. Bento, C. F.; Fernandes, R.; Matafome, P.; Sena, C.; Seia, R.; and Pereira, P., "Methylglyoxal-induced imbalance in the ratio of vascular endothelial growth factor to angiopoietin 2 secreted by retinal pigment epithelial cells leads to endothelial dysfunction," *Exp. Physiol.*, vol. 95, no. 9, pp. 955–970, 2010, doi: 10.1113/expphysiol.2010.053561.

112. Bierhaus, A.; Fleming, T.; Stoyanov, S.; Leffler, A.; Babes, A.; Neacsu, C.; Sauer, S. K.; Eberhardt, M.; Schnölzer, M.; Lasischka, F.; Neuhuber, W. L.; Kichko, T. I.; Konrade, I.; Elvert, R.; Mier, W.; Pirags, V.; Lukic, I. K.; Morcos, M.; Dehmer, T.; Rabbani, N.; Thornalley, P. J.; Edelstein, D.; Nau, C.; Forbes, J.; Humpert, P. M.; Schwaninger, M.; Ziegler, D.; Stern, D. M.; Cooper, M. E.; Haberkorn, U.; Brownlee, M.; Reeh, P. W.; and Nawroth, P. P., "Methylglyoxal modification of Na v 1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy," *Nat. Med.*, vol. 18, no. 6, pp. 926–933, 2012, doi: 10.1038/nm.2750.

113. Griggs, R. B.; Laird, D. E.; Donahue, R. R.; Fu, W.; and Taylor, B. K., "Methylglyoxal requires AC1 and TRPA1 to produce pain and spinal neuron activation," *Front. Neurosci.*, vol. 11, no. DEC, pp. 1–11, 2017, doi: 10.3389/fnins.2017.00679.

114. Papadaki, M.; Holewinski, R. J.; Previs, S. B.; Martin, T. G.; Stachowski, M. J.; Li, A.; Blair, C. A.; Moravec, C. S.; Van Eyk, J. E.; Campbell, K. S.; Warshaw, D. M.; and Kirk, J. A., "Diabetes with heart failure increases methylglyoxal modifications in the sarcomere, which inhibit function," *JCI insight*, vol. 3, no. 20, pp. 1–17, 2018, doi: 10.1172/jci.insight.121264.

115. Hanssen, N. M. J.; Scheijen, J. L. J. M.; Jorsal, A.; Parving, H.-H.; Tarnow, L.; Rossing, P.; Stehouwer, C. D. A.; and Schalkwijk, C. G., "Higher plasma methylglyoxal levels are associated with incident cardiovascular disease in individuals with type 1 diabetes: a 12-year follow-up study," *Diabetes*, pp. 1–19, 2017.

116. Ogawa, S.; Nakayama, K.; Nakayama, M.; Mori, T.; Matsushima, M.; Okamura, M.; Senda, M.; Nako, K.; Miyata, T.; and Ito, S., "Methylglyoxal is a predictor in type 2 diabetic patients of intima-media thickening and elevation of blood pressure," *Hypertension*, vol. 56, no. 3, pp. 471–476, 2010, doi: 10.1161/HYPERTENSIONAHA.110.156786.

117. Blackburn, N. J. R.; Vulesevic, B.; McNeill, B.; Cimenci, C. E.; Ahmadi, A.; Gonzalez-Gomez, M.; Ostojic, A.; Zhong, Z.; Brownlee, M.; Beisswenger, P. J.;

Milne, R. W.; and Suuronen, E. J., “Methylglyoxal-derived advanced glycation end products contribute to negative cardiac remodeling and dysfunction post-myocardial infarction,” *Basic Res. Cardiol.*, vol. 112, no. 5, pp. 1–14, 2017, doi: 10.1007/s00395-017-0646-x.

118. Wang, X.; Desai, K.; Chang, T.; and Wu, L., “Vascular methylglyoxal metabolism and the development of hypertension,” *J. Hypertens.*, vol. 23, no. 8, pp. 1565–1573, 2005, doi: 10.1097/01.hjh.0000173778.85233.1b.
119. Aso, Y.; Inukai, T.; Tayama, K.; and Takemura, Y., “Serum concentrations of advanced glycation endproducts are associated with the development of atherosclerosis as well as diabetic microangiopathy in patients with type 2 diabetes,” *Acta Diabetol.*, vol. 37, no. 2, pp. 87–92, 2000, doi: 10.1007/s005920070025.
120. Kilhovd, B. K.; Juutilainen, A.; Lehto, S.; Rönnemaa, T.; Torjesen, P. A.; Birkeland, K. I.; Berg, T. J.; Hanssen, K. F.; and Laakso, M., “High serum levels of advanced glycation end products predict increased coronary heart disease mortality in nondiabetic women but not in nondiabetic men: A population-based 18-year follow-up study,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 25, no. 4, pp. 815–820, 2005, doi: 10.1161/01.ATV.0000158380.44231.fe.
121. Chiarelli, F.; De Martino, M.; Mezzetti, A.; Catino, M.; Morgese, G.; Cuccurullo, F.; and Verrotti, A., “Advanced glycation end products in children and adolescents with diabetes: Relation to glycemic control and early microvascular complications,” *J. Pediatr.*, vol. 134, no. 4, pp. 486–491, 1999, doi: 10.1016/S0022-3476(99)70208-8.
122. Miura, J.; Yamagishi, S. I.; Uchigata, Y.; Takeuchi, M.; Yamamoto, H.; Makita, Z.; and Iwamoto, Y., “Serum levels of non-carboxymethyllysine advanced glycation endproducts are correlated to severity of microvascular complications in patients with Type 1 diabetes,” *J. Diabetes Complications*, vol. 17, no. 1, pp. 16–21, 2003, doi: 10.1016/S1056-8727(02)00183-6.
123. Hegab, Z.; Gibbons, S.; Neyses, L.; and Mamas, M. A., “Role of advanced glycation end products in cardiovascular disease,” *World J. Cardiol.*, vol. 4, no. 4,

p. 90, 2012, doi: 10.4330/wjc.v4.i4.90.

124. Park, L.; Raman, K. G.; Lee, K. J.; Lu, Y.; Ferran, L. J.; Choe, W. S.; Stern, D.; and Schmidt, A. M., "Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts," *Nat. Med.*, vol. 4, no. 9, pp. 1025–1031, 1998, doi: 10.1038/2012.

125. He, T.; Zhou, H.; Li, C.; Chen, Y.; Chen, X.; Li, C.; Mao, J.; Lyu, J.; and Meng, Q. H., *Methylglyoxal suppresses human colon cancer cell lines and tumor growth in a mouse model by impairing glycolytic metabolism of cancer cells associated with down-regulation of c-Myc expression*, vol. 17, no. 9. 2016. doi: 10.1080/15384047.2016.1210736.

126. Paul-Samojedny, M.; Łasut, B.; Pudełko, A.; Fila-Daniłow, A.; Kowalczyk, M.; Suchanek-Raif, R.; Zieliński, M.; Borkowska, P.; and Kowalski, J., "Methylglyoxal (MGO) inhibits proliferation and induces cell death of human glioblastoma multiforme T98G and U87MG cells," *Biomed. Pharmacother.*, vol. 80, pp. 236–243, 2016, doi: 10.1016/j.biopha.2016.03.021.

127. Guo, Y.; Zhang, Y.; Yang, X.; Lu, P.; Yan, X.; Xiao, F.; Zhou, H.; Wen, C.; Shi, M.; Lu, J.; and Meng, Q. H., "Effects of methylglyoxal and glyoxalase I inhibition on breast cancer cells proliferation, invasion, and apoptosis through modulation of MAPKs, MMP9, and Bcl-2," *Cancer Biol. Ther.*, vol. 17, no. 2, pp. 169–180, 2016, doi: 10.1080/15384047.2015.1121346.

128. Antognelli, C.; Moretti, S.; Frosini, R.; Puxeddu, E.; Sidoni, A.; and Talesa, V. N., "Methylglyoxal acts as a Tumor-Promoting factor in Anaplastic Thyroid Cancer," *Cells*, vol. 8, no. 6, pp. 9–11, 2019, doi: 10.3390/cells8060547.

129. Lin, J. A.; Wu, C. H.; and Yen, G. C., *Methylglyoxal displays colorectal cancer-promoting properties in the murine models of azoxymethane and CT26 isografts*, vol. 115. Elsevier B.V., 2018. doi: 10.1016/j.freeradbiomed.2017.12.020.

130. Nokin, M. J.; Durieux, F.; Bellier, J.; Peulen, O.; Uchida, K.; Spiegel, D. A.; Cochrane, J. R.; Hutton, C. A.; Castronovo, V.; and Bellahcène, A., "Hormetic potential of methylglyoxal, a side-product of glycolysis, in switching tumours from growth to death," *Sci. Rep.*, vol. 7, no. 1, pp. 1–14, 2017, doi:

10.1038/s41598-017-12119-7.

131. Mattson, M. P., “Hormesis defined,” *Ageing Res. Rev.*, vol. 7, no. 1, pp. 1–7, 2008, doi: 10.1016/j.arr.2007.08.007.

132. Leone, A.; Nigro, C.; Nicolò, A.; Prevenzano, I.; Formisano, P.; Beguinot, F.; and Miele, C., “The Dual-Role of Methylglyoxal in Tumor Progression – Novel Therapeutic Approaches,” *Front. Oncol.*, vol. 11, no. March, pp. 1–12, 2021, doi: 10.3389/fonc.2021.645686.

133. Srikanth, V.; Westcott, B.; Forbes, J.; Phan, T. G.; Beare, R.; Venn, A.; Pearson, S.; Greenaway, T.; Parameswaran, V.; and Münch, G., “Methylglyoxal, cognitive function and cerebral atrophy in older people,” *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.*, vol. 68, no. 1, pp. 68–73, 2013, doi: 10.1093/gerona/gls100.

134. Hipkiss, A. R., “On the relationship between energy metabolism, proteostasis, aging and Parkinson’s disease: Possible causative role of methylglyoxal and alleviative potential of carnosine,” *Aging Dis.*, vol. 8, no. 3, pp. 334–345, 2017, doi: 10.14336/AD.2016.1030.

135. Gomes, R.; Sousa Silva, M.; Quintas, A.; Cordeiro, C.; Freire, A.; Pereira, P.; Martins, A.; Monteiro, E.; Barroso, E.; and Ponces Freire, A., “Argpyrimidine, a methylglyoxal-derived advanced glycation end-product in familial amyloidotic polyneuropathy,” *Biochem. J.*, vol. 385, no. 2, pp. 339–345, 2005, doi: 10.1042/BJ20040833.

136. Li, J.; Liu, D.; Sun, L.; Lu, Y.; and Zhang, Z., “Advanced glycation end products and neurodegenerative diseases: Mechanisms and perspective,” *J. Neurol. Sci.*, vol. 317, no. 1–2, pp. 1–5, 2012, doi: 10.1016/j.jns.2012.02.018.

137. Krautwald, M. and Münch, G., “Advanced glycation end products as biomarkers and gerontotoxins - A basis to explore methylglyoxal-lowering agents for Alzheimer’s disease?,” *Exp. Gerontol.*, vol. 45, no. 10, pp. 744–751, 2010, doi: 10.1016/j.exger.2010.03.001.

138. Angeloni, C.; Zambonin, L.; and Hrelia, S., “Role of methylglyoxal in alzheimer’s disease,” *Biomed Res. Int.*, vol. 2014, 2014, doi: 10.1155/2014/238485.

139. Lissner, L. J.; Rodrigues, L.; Wartchow, K. M.; Borba, E.; Bobermin, L. D.; Fontella,

F. U.; Hansen, F.; Quincozes-Santos, A.; Souza, D. O. G.; and Gonçalves, C. A., "Short-Term Alterations in Behavior and Astroglial Function After Intracerebroventricular Infusion of Methylglyoxal in Rats," *Neurochem. Res.*, vol. 46, no. 2, pp. 183–196, 2021, doi: 10.1007/s11064-020-03154-4.

140. Lissner, L. J.; Wartchow, K. M.; Rodrigues, L.; Bobermin, L. D.; Borba, E.; Dias, V. G.; Hansen, F.; Quincozes-Santos, A.; and Gonçalves, C. A., "Acute Methylglyoxal-Induced Damage in Blood–Brain Barrier and Hippocampal Tissue," *Neurotox. Res.*, vol. 40, no. 5, pp. 1337–1347, 2022, doi: 10.1007/s12640-022-00571-x.
141. Shi, D. Y.; Bierhaus, A.; Nawroth, P. P.; and Stern, D. M., "RAGE and Alzheimer's disease: A progression factor for amyloid-β- induced cellular perturbation?," *J. Alzheimer's Dis.*, vol. 16, no. 4, pp. 833–843, 2009, doi: 10.3233/JAD-2009-1030.
142. Yan, S. D. et al, "Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli.pdf," *Nature*. 1996.
143. Lue, L. F.; Walker, D. G.; Brachova, L.; Beach, T. G.; Rogers, J.; Schmidt, A. M.; Stern, D. M.; and Yan, S. Du, "Involvement of microglial receptor for advanced glycation endproducts (RAGE)in Alzheimer's disease: Identification of a cellular activation mechanism," *Exp. Neurol.*, vol. 171, no. 1, pp. 29–45, 2001, doi: 10.1006/exnr.2001.7732.
144. Sasaki, N.; Toki, S.; Chowei, H.; Saito, T.; Nakano, N.; Hayashi, Y.; Takeuchi, M.; and Makita, Z., "Immunohistochemical distribution of the receptor for advanced glycation end products in neurons and astrocytes in Alzheimer's disease," *Brain Res.*, vol. 888, no. 2, pp. 256–262, 2001, doi: 10.1016/S0006-8993(00)03075-4.
145. Fang, F.; Yu, Q.; Arancio, O.; Chen, D.; Gore, S. S.; Yan, S. S. Du; and Yan, S. F., "RAGE mediates Aβ accumulation in a mouse model of Alzheimers disease via modulation of β- and γ-secretase activity," *Hum. Mol. Genet.*, vol. 27, no. 6, pp. 1002–1014, 2018, doi: 10.1093/hmg/ddy017.
146. Xie, B.; Lin, F.; Peng, L.; Ullah, K.; Wu, H.; Qing, H.; and Deng, Y., "Methylglyoxal increases dopamine level and leads to oxidative stress in SH-SY5Y cells," *Acta Biochim. Biophys. Sin. (Shanghai).*, vol. 46, no. 11, pp. 950–956, 2014, doi:

10.1093/abbs/gmu094.

147. Xie, B.; Lin, F.; Ullah, K.; Peng, L.; Ding, W.; Dai, R.; Qing, H.; and Deng, Y., "A newly discovered neurotoxin ADTIQ associated with hyperglycemia and Parkinson's disease," *Biochem. Biophys. Res. Commun.*, vol. 459, no. 3, pp. 361–366, 2015, doi: 10.1016/j.bbrc.2015.02.069.
148. Song, D. W.; Xin, N.; Xie, B. J.; Li, Y. J.; Meng, L. Y.; Li, H. M.; Schläppi, M.; and Deng, Y. L., "Formation of a salsolinol-like compound, the neurotoxin, 1-acetyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, in a cellular model of hyperglycemia and a rat model of diabetes," *Int. J. Mol. Med.*, vol. 33, no. 3, pp. 736–742, 2014, doi: 10.3892/ijmm.2013.1604.
149. Richarme, G.; Mihoub, M.; Dairou, J.; Chi Bui, L.; Leger, T.; and Lamouri, A., "Parkinsonism-associated protein DJ-1/park7 is a major protein deglycase that repairs methylglyoxal- and glyoxal-glycated cysteine, arginine, and lysine residues," *J. Biol. Chem.*, vol. 290, no. 3, pp. 1885–1897, 2015, doi: 10.1074/jbc.M114.597815.
150. Mehdipour, M.; Etienne, J.; Liu, C.; Mehdipour, T.; Kato, C.; Conboy, M.; Conboy, I.; and Kiprov, D. D., "Attenuation of age-elevated blood factors by repositioning plasmapheresis: A novel perspective and approach," *Transfus. Apher. Sci.*, vol. 60, no. 3, p. 103162, 2021, doi: 10.1016/j.transci.2021.103162.
151. WHO, "Ageing and health," no. October, pp. 2020–2023, 2022.
152. Jaul, E. and Barron, J., "Age-Related Diseases and Clinical and Public Health Implications for the 85 Years Old and Over Population," *Front. Public Heal.*, vol. 5, no. December, pp. 1–7, 2017, doi: 10.3389/fpubh.2017.00335.
153. Gonzales, M. M.; Garbarino, V. R.; Pollet, E.; Palavicini, J. P.; Kellogg, D. L.; Kraig, E.; and Orr, M. E., "Biological aging processes underlying cognitive decline and neurodegenerative disease," *J. Clin. Invest.*, vol. 132, no. 10, pp. 1–10, 2022, doi: 10.1172/JCI158453.
154. Du, X.; Wang, X.; and Geng, M., "Alzheimer's disease hypothesis and related therapies," *Transl. Neurodegener.*, vol. 7, no. 1, pp. 1–7, 2018, doi: 10.1186/s40035-018-0107-y.

155. Serrano-Pozo, A.; Frosch, M. P.; Masliah, E.; and Hyman, B. T., "Neuropathological alterations in Alzheimer disease," *Cold Spring Harb. Perspect. Med.*, vol. 1, no. 1, pp. 1–23, 2011, doi: 10.1101/cshperspect.a006189.
156. Zhang, Y. W.; Thompson, R.; Zhang, H.; and Xu, H., "APP processing in Alzheimer's disease," *Mol. Brain*, vol. 4, no. 1, p. 3, 2011, doi: 10.1186/1756-6606-4-3.
157. Gu, L. and Guo, Z., "Alzheimer's Aβ42 and Aβ40 peptides form interlaced amyloid fibrils," *Bone*, vol. 23, no. 1, pp. 1–7, 2013, doi: 10.1111/jnc.12202.Alzheimer.
158. Thacker, D.; Willas, A.; Dear, A. J.; and Linse, S., "Role of Hydrophobicity at the N-Terminal Region of Aβ42 in Secondary Nucleation," *ACS Chem. Neurosci.*, 2022, doi: 10.1021/acschemneuro.2c00504.
159. Castro, M. A.; Hadziselimovic, A.; and Sanders, C. R., "The vexing complexity of the amyloidogenic pathway," *Protein Sci.*, vol. 28, no. 7, pp. 1177–1193, 2019, doi: 10.1002/pro.3606.
160. Boutajangout, A.; M. Sigurdsson, E.; and K. Krishnamurthy, P., "Tau as a Therapeutic Target for Alzheimers Disease," *Curr. Alzheimer Res.*, vol. 8, no. 6, pp. 666–677, 2011, doi: 10.2174/156720511796717195.
161. O'Brien, R. J. and Wong, P. C., "Amyloid precursor protein processing and alzheimer's disease," *Annu. Rev. Neurosci.*, vol. 34, pp. 185–204, 2011, doi: 10.1146/annurev-neuro-061010-113613.
162. *World Population Ageing 2019 Highlights*. 2019. doi: 10.18356/9df3caed-en.
163. Power, R.; Prado-Cabrero, A.; Mulcahy, R.; Howard, A.; and Nolan, J. M., "The Role of Nutrition for the Aging Population: Implications for Cognition and Alzheimer's Disease," *Annu. Rev. Food Sci. Technol.*, vol. 10, pp. 619–639, 2019, doi: 10.1146/annurev-food-030216-030125.
164. Klimova, B.; Dziuba, S.; and Cierniak-Emerych, A., "The Effect of Healthy Diet on Cognitive Performance Among Healthy Seniors – A Mini Review," *Front. Hum. Neurosci.*, vol. 14, no. August, pp. 1–9, 2020, doi: 10.3389/fnhum.2020.00325.
165. Zunkel, K.; Simm, A.; and Bartling, B., "Long-term intake of the reactive

metabolite methylglyoxal is not toxic in mice," *Food Chem. Toxicol.*, vol. 141, no. January, p. 111333, 2020, doi: 10.1016/j.fct.2020.111333.

166. Cai, W.; Uribarri, J.; Zhu, L.; Chen, X.; Swamy, S.; Zhao, Z.; Grosjean, F.; Simonaro, C.; Kuchel, G. A.; Schnaider-Beeri, M.; Woodward, M.; Striker, G. E.; and Vlassara, H., "Oral glycotoxins are a modifiable cause of dementia and the metabolic syndrome in mice and humans," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 13, pp. 4940–4945, 2014, doi: 10.1073/pnas.1316013111.
167. Uribarri, J.; Woodruff, S.; Goodman, S.; Cai, W.; Chen, X. U. E.; Pyzik, R.; Yong, A.; and Striker, G. E., "AGE's in Foods and practical ways to reduce them," *J Am Diet Assoc.*, vol. 110, no. 6, pp. 911–16, 2010, doi: 10.1016/j.jada.2010.03.018.Advanced.
168. Ogasawara, Y.; Tanaka, R.; Koike, S.; Horiuchi, Y.; Miyashita, M.; and Arai, M., "Determination of methylglyoxal in human blood plasma using fluorescence high performance liquid chromatography after derivatization with 1,2-diamino-4,5-methylenedioxybenzene," *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 1029–1030, pp. 102–105, 2016, doi: 10.1016/j.jchromb.2016.07.019.
169. van Dongen, K. C. W.; Linkens, A. M. A.; Wetzels, S. M. W.; Wouters, K.; Vanmierlo, T.; van de Waarenburg, M. P. H.; Scheijen, J. L. J. M.; de Vos, W. M.; Belzer, C.; and Schalkwijk, C. G., "Dietary advanced glycation endproducts (AGEs) increase their concentration in plasma and tissues, result in inflammation and modulate gut microbial composition in mice; evidence for reversibility," *Food Res. Int.*, vol. 147, no. January, p. 110547, 2021, doi: 10.1016/j.foodres.2021.110547.
170. Edelblum, K. L. and Turner, J. R., "The Tight Junction in Inflammatory Disease: Communication Breakdown," vol. 9, no. 6, pp. 715–720, 1993, doi: 10.1016/j.coph.2009.06.022.The.
171. Capaldo, C. T.; Farkas, A. E.; Hilgarth, R. S.; Krug, S. M.; Wolf, M. F.; Benedik, J. K.; Fromm, M.; Koval, M.; Parkos, C.; and Nusrat, A., "Proinflammatory cytokine-induced tight junction remodeling through dynamic self-assembly of claudins," *Mol. Biol. Cell*, vol. 25, no. 18, pp. 2710–2719, 2014, doi: 10.1091/mbc.E14-02-

0773.

172. Schmitt, M.; Schewe, M.; Sacchetti, A.; Feijtel, D.; van de Geer, W. S.; Teeuwssen, M.; Sleddens, H. F.; Joosten, R.; van Royen, M. E.; van de Werken, H. J. G.; van Es, J.; Clevers, H.; and Fodde, R., "Paneth Cells Respond to Inflammation and Contribute to Tissue Regeneration by Acquiring Stem-like Features through SCF/c-Kit Signaling," *Cell Rep.*, vol. 24, no. 9, pp. 2312-2328.e7, 2018, doi: 10.1016/j.celrep.2018.07.085.
173. Dudakov, J. A.; Hanash, A. M.; and Van Den Brink, M. R. M., "Interleukin-22: Immunobiology and pathology," *Annu. Rev. Immunol.*, vol. 33, no. February, pp. 747–785, 2015, doi: 10.1146/annurev-immunol-032414-112123.
174. Zenewicz, L. A.; Yancopoulos, G. D.; Valenzuela, D. M.; Murphy, A. J.; Stevens, S.; and Flavell, R. A., "Innate and Adaptive Interleukin-22 Protects Mice from Inflammatory Bowel Disease," *Immunity*, vol. 29, no. 6, pp. 947–957, 2008, doi: 10.1016/j.immuni.2008.11.003.
175. Mukherjee, S. and Hooper, L. V., "Antimicrobial Defense of the Intestine," *Immunity*, vol. 42, no. 1, pp. 28–39, 2015, doi: 10.1016/j.immuni.2014.12.028.
176. Bevins, C. L. and Salzman, N. H., "Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis," *Nat. Rev. Microbiol.*, vol. 9, no. 5, pp. 356–368, 2011, doi: 10.1038/nrmicro2546.
177. Taguchi, K.; Fukami, K.; Elias, B. C.; and Brooks, C. R., "Trimethylamine N-Oxide in Chronic Kidney Disease," pp. 1–16, 2021.
178. Zhang, S.; Jiao, T.; Chen, Y.; Gao, N.; Zhang, L.; and Jiang, M., "Methylglyoxal induces systemic symptoms of irritable bowel syndrome," *PLoS One*, vol. 9, no. 8, 2014, doi: 10.1371/journal.pone.0105307.
179. Wang, J.; Cai, W.; Yu, J.; Liu, H.; He, S.; Zhu, L.; and Xu, J., "Dietary Advanced Glycation End Products Shift the Gut Microbiota Composition and Induce Insulin Resistance in Mice," *Diabetes, Metab. Syndr. Obes. Targets Ther.*, vol. 15, no. February, pp. 427–437, 2022, doi: 10.2147/DMSO.S346411.
180. Terry, N. and Margolis, K. G., "Serotonergic Mechanisms Regulating the GI Tract: Experimental Evidence and Therapeutic Relevance," *Handb. Exp. Pharmacol.*,

no. January, pp. 251–263, 2015, doi: 10.1007/164.

181. Fennema, O., “Chemical Changes in Food during Processing- An Overview,” 1985, pp. 1–16.

182. Poojary, M. M. and Lund, M. N., “Chemical Stability of Proteins in Foods: Oxidation and the Maillard Reaction,” *Annu. Rev. Food Sci. Technol.*, vol. 13, no. 1, pp. 35–58, 2022, doi: 10.1146/annurev-food-052720-104513.

183. Nielsen, S. D.; Knudsen, L. J.; Bækgaard, L. T.; Rauh, V.; and Larsen, L. B., “Influence of Lactose on the Maillard Reaction and Dehydroalanine-Mediated Protein Cross-Linking in Casein and Whey,” *Foods*, vol. 11, no. 7, 2022, doi: 10.3390/foods11070897.

184. Akıllıoğlu, H. G. and Lund, M. N., “Quantification of advanced glycation end products and amino acid cross-links in foods by high-resolution mass spectrometry: Applicability of acid hydrolysis,” *Food Chem.*, vol. 366, no. December 2020, 2022, doi: 10.1016/j.foodchem.2021.130601.

185. Friedman, M., “Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins,” *J. Agric. Food Chem.*, vol. 47, no. 4, pp. 1295–1319, 1999, doi: 10.1021/jf981000+.

186. Zhao, Y.; Luo, X.; Li, J.; Xu, M.; and Tu, Y., “Formation of lysinoalanine in egg white under alkali treatment,” *Poult. Sci.*, vol. 95, no. 3, pp. 660–667, 2016, doi: 10.3382/ps/pev372.

187. Koyama, S.; Oka, D.; Tsujii, Y.; Takano, K.; and Handa, A., “Effects of lanthionine and lysinoalanine on heat-induced gelation of egg white,” *Food Sci. Technol. Res.*, vol. 26, no. 6, pp. 789–795, 2020, doi: 10.3136/fstr.26.789.

188. Cattaneo, S.; Masotti, F.; and Pellegrino, L., “Chemical modifications of casein occurring during industrial manufacturing of milk protein powders,” *Eur. Food Res. Technol.*, vol. 235, no. 2, pp. 315–323, 2012, doi: 10.1007/s00217-012-1760-5.

189. Calabrese, M. G.; Mamone, G.; Caira, S.; Ferranti, P.; and Addeo, F., “Quantitation of lysinoalanine in dairy products by liquid chromatography-mass spectrometry with selective ion monitoring,” *Food Chem.*, vol. 116, no. 3, pp.

799–805, 2009, doi: 10.1016/j.foodchem.2009.03.041.

190. Cartus, A. T., *d-Amino acids and cross-linked amino acids as food contaminants*. Woodhead Publishing Limited, 2012. doi: 10.1533/9780857095794.2.286.

191. Cattaneo, S.; Masotti, F.; and Pellegrino, L., "Liquid infant formulas: Technological tools for limiting heat damage," *J. Agric. Food Chem.*, vol. 57, no. 22, pp. 10689–10694, 2009, doi: 10.1021/jf901800v.

192. Sun, J.; Akıllıoğlu, H. G.; Aasmul-Olsen, K.; Ye, Y.; Lund, P.; Zhao, X.; Brunse, A.; Nielsen, C. F.; Chatterton, D. E. W.; Sangild, P. T.; Lund, M. N.; and Bering, S. B., "Ultra-High Temperature Treatment and Storage of Infant Formula Induces Dietary Protein Modifications, Gut Dysfunction, and Inflammation in Preterm Pigs," *Mol. Nutr. Food Res.*, vol. 66, no. 20, pp. 1–13, 2022, doi: 10.1002/mnfr.202200132.

193. Wada, Y. and Lönnerdal, B., "Effects of Industrial Heating Processes of Milk-Based Enteral Formulas on Site-Specific Protein Modifications and Their Relationship to in Vitro and in Vivo Protein Digestibility," *J. Agric. Food Chem.*, vol. 63, no. 30, pp. 6787–6798, 2015, doi: 10.1021/acs.jafc.5b02189.

194. Commission, T. H. E.; The, O. F.; and Communities, E., "European Commission (EC 2002)," no. 3, pp. 92–93, 2002.

195. Gökmen, V.; Serpen, A.; Açar, Ö. Ç.; and Morales, F. J., "Significance of furosine as heat-induced marker in cookies," *J. Cereal Sci.*, vol. 48, no. 3, pp. 843–847, 2008, doi: 10.1016/j.jcs.2008.06.007.

196. Lund, M. N. and Ray, C. A., "Control of Maillard Reactions in Foods: Strategies and Chemical Mechanisms," *J. Agric. Food Chem.*, vol. 65, no. 23, pp. 4537–4552, 2017, doi: 10.1021/acs.jafc.7b00882.

197. Erbersbobler, H. F. and Hupe, A., "Determination of lysine damage and calculation of lysine," *Zeitschrift für Ernährugswiss.*, vol. 30, pp. 46–49, 1991.

198. Delgado-Andrade, C.; Rufián-Henares, J. A.; and Morales, F. J., "Fast method to determine furosine in breakfast cereals by capillary zone electrophoresis," *Eur. Food Res. Technol.*, vol. 221, no. 5, pp. 707–711, 2005, doi: 10.1007/s00217-005-0030-1.

199. Morales, V.; Luz Sanz, M.; Marťin-Álvareza, P. J.; and Corzoa, N., “Combined use of HMF and furosine to assess fresh honey quality,” *J. Sci. Food Agric.*, vol. 89, no. 8, pp. 1332–1338, 2009, doi: 10.1002/jsfa.3590.

200. Van Renterghem, R. and De Block, J., “Furosine in consumption milk and milk powders,” *Int. Dairy J.*, vol. 6, no. 4, pp. 371–382, 1996, doi: 10.1016/0958-6946(95)00060-7.

201. Seiquer, I.; Díaz-alguacil, J.; Delgado-andrade, C.; Lo, M.; and Galdo, G., “Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11–14,” no. April, pp. 1082–1088, 2018.

202. Erbersdobler, H. F. and Somoza, V., “Forty years of furosine - Forty years of using Maillard reaction products as indicators of the nutritional quality of foods,” *Mol. Nutr. Food Res.*, vol. 51, no. 4, pp. 423–430, 2007, doi: 10.1002/mnfr.200600154.

203. Troise, A. D.; Fiore, A.; Wiltafsky, M.; and Fogliano, V., “Quantification of Nε-(2-Furoylmethyl)-l-lysine (furosine), Nε-(Carboxymethyl)-l-lysine (CML), Nε-(Carboxyethyl)-l-lysine (CEL) and total lysine through stable isotope dilution assay and tandem mass spectrometry,” *Food Chem.*, vol. 188, pp. 357–364, 2015, doi: 10.1016/j.foodchem.2015.04.137.

204. Saeed, Y.; Wang, J. Q.; and Zheng, N., “Furosine induces DNA damage and cell death in selected human cell lines: a strong toxicant to kidney Hek-293 cells,” *Food Sci. Biotechnol.*, vol. 26, no. 4, pp. 1093–1101, 2017, doi: 10.1007/s10068-017-0131-1.

205. Li, H. Y.; Xing, L.; Wang, J. Q.; and Zheng, N., “Toxicology studies of furosine in vitro/in vivo and exploration of the related mechanism,” *Toxicol. Lett.*, vol. 291, no. February, pp. 101–111, 2018, doi: 10.1016/j.toxlet.2018.02.018.

206. Li, H.; Wang, Y.; Yang, H.; Zhang, Y.; Xing, L.; Wang, J.; and Zheng, N., “Furosine, a maillard reaction product, triggers necroptosis in hepatocytes by regulating the ripk1/ripk3/mlkl pathway,” *Int. J. Mol. Sci.*, vol. 20, no. 10, 2019, doi: 10.3390/ijms20102388.

207. Mottram, D. S.; Wedzicha, B. L.; and Dodson, A. T., “Acrylamide is formed in the

Maillard reaction.," *Nature*, vol. 76, no. October, pp. 83–88, 2002.

208. Stojanovska, S. and Tomovska, J., "Factors influence to formation of acrylamide in food," *J. Hyg. Eng. Des.*, no. September, pp. 10–15, 2015.
209. Nguyen, H. T.; Van Der Fels-Klerx, H. J.; Peters, R. J. B.; and Van Boekel, M. A. J. S., "Acrylamide and 5-hydroxymethylfurfural formation during baking of biscuits: Part I: Effects of sugar type," *Food Chem.*, vol. 192, pp. 575–585, 2016, doi: 10.1016/j.foodchem.2015.07.016.
210. Tareke, E.; Rydberg, P.; Karlsson, P.; Eriksson, S.; and Törnqvist, M., "Analysis of acrylamide, a carcinogen formed in heated foodstuffs," *J. Agric. Food Chem.*, vol. 50, no. 17, pp. 4998–5006, 2002, doi: 10.1021/jf020302f.
211. Capuano, E. and Fogliano, V., "Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies," *LWT - Food Sci. Technol. J.*, vol. 44, no. 4, pp. 793–810, 2011, doi: 10.1016/j.lwt.2010.11.002.
212. Dearfield, K. L.; Abernathy, C. O.; Ottley, M. S.; Brantner, J. H.; and Hayes, P. F., "Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity, and carcinogenicity," *Mutat. Res. Genet. Toxicol.*, vol. 195, no. 1, pp. 45–77, 1988, doi: 10.1016/0165-1110(88)90015-2.
213. Dearfield, K. L.; Douglas, G. R.; Ehling, U. H.; Moore, M. M.; Sega, G. A.; and Brusick, D. J., "Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk," *Mutat. Res. - Fundam. Mol. Mech. Mutagen.*, vol. 330, no. 1–2, pp. 71–99, 1995, doi: 10.1016/0027-5107(95)00037-J.
214. Friedman, M., "Chemistry, biochemistry, and safety of acrylamide. A review," *J. Agric. Food Chem.*, vol. 51, no. 16, pp. 4504–4526, 2003, doi: 10.1021/jf030204+.
215. "Toxicology and carcinogenesis studies of acrylamide (CASRN 79-06-1) in F344/N rats and B6C3F1 mice (feed and drinking water studies)," 2012.
216. Virk-Baker, M. K.; Nagy, T. R.; Barnes, S.; and Groopman, J., "Dietary acrylamide and human cancer: A systematic review of literature," *Nutr. Cancer*, vol. 66, no. 5, pp. 774–790, 2014, doi: 10.1080/01635581.2014.916323.

217. Lipworth, L.; Sonderman, J. S.; Tarone, R. E.; and McLaughlin, J. K., “Review of epidemiologic studies of dietary acrylamide intake and the risk of cancer,” *Eur. J. Cancer Prev.*, vol. 21, no. 4, pp. 375–386, 2012, doi: 10.1097/CEJ.0b013e3283529b64.
218. “Scientific Opinion on acrylamide in food,” *EFSA J.*, vol. 13, no. 6, 2015, doi: 10.2903/j.efsa.2015.4104.
219. Benford, D.; Bignami, M.; Chipman, J. K.; and Ramos Bordajandi, L., “Assessment of the genotoxicity of acrylamide,” *EFSA J.*, vol. 20, no. 5, 2022, doi: 10.2903/j.efsa.2022.7293.
220. EU, “Commission Regulation (EU) 2017/2158: establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food,” *Off. J. Eur. Union*, vol. L 304, no. 315, pp. 24–44, 2017, [Online]. Available: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R2158&from=EN
221. Zhang, W.; Poojary, M. M.; Olsen, K.; Ray, C. A.; and Lund, M. N., “Formation of α-Dicarbonyls from Dairy Related Carbohydrates with and without Nα-Acetyl- l -Lysine during Incubation at 40 and 50 °c,” *J. Agric. Food Chem.*, vol. 67, no. 22, pp. 6350–6358, 2019, doi: 10.1021/acs.jafc.9b01532.
222. Aktağ, I. G. and Gökmen, V., “A survey of the occurrence of α-dicarbonyl compounds and 5-hydroxymethylfurfural in dried fruits, fruit juices, puree and concentrates,” *J. Food Compos. Anal.*, vol. 91, no. April, p. 103523, 2020, doi: 10.1016/j.jfca.2020.103523.
223. Hellwig, M. and Henle, T., “Maillard Reaction Products in Different Types of Brewing Malt,” *J. Agric. Food Chem.*, vol. 68, no. 48, pp. 14274–14285, 2020, doi: 10.1021/acs.jafc.0c06193.
224. Zhang, W.; Poojary, M. M.; Rauh, V.; Ray, C. A.; Olsen, K.; and Lund, M. N., “Quantitation of α-Dicarbonyls and Advanced Glycation Endproducts in Conventional and Lactose-Hydrolyzed Ultrahigh Temperature Milk during 1 Year of Storage,” *J. Agric. Food Chem.*, vol. 67, no. 46, pp. 12863–12874, 2019, doi: 10.1021/acs.jafc.9b05037.

225. Arribas-Lorenzo, G. and Morales, F. J., “Analysis, distribution, and dietary exposure of glyoxal and methylglyoxal in cookies and their relationship with other heat-induced contaminants,” *J. Agric. Food Chem.*, vol. 58, no. 5, pp. 2966–2972, 2010, doi: 10.1021/jf902815p.

226. De Revel, G.; Pripis-Nicolau, L.; Barbe, J. C.; and Bertrand, A., “The detection of α-dicarbonyl compounds in wine by formation of quinoxaline derivatives,” *J. Sci. Food Agric.*, vol. 80, no. 1, pp. 102–108, 2000, doi: 10.1002/(SICI)1097-0010(20000101)80:1<102::AID-JSFA493>3.0.CO;2-Y.

227. Lim, H. H. and Shin, H. S., “In-solution derivatization and detection of glyoxal and methylglyoxal in alcoholic beverages and fermented foods by headspace solid-phase microextraction and gas chromatography–mass spectrometry,” *J. Food Compos. Anal.*, vol. 92, no. June, p. 103584, 2020, doi: 10.1016/j.jfca.2020.103584.

228. Wang, X. J.; Gao, F.; Li, L. C.; Hui, X.; Li, H.; and Gao, W. Y., “Quantitative analyses of α-dicarbonyl compounds in food samples by HPLC using 4-(2,3-dimethyl-6-quinoxalinyl)-1,2-benzenediamine as a derivatizing reagent,” *Microchem. J.*, vol. 141, no. 2017, pp. 64–70, 2018, doi: 10.1016/j.microc.2018.05.006.

229. Papetti, A.; Mascherpa, D.; and Gazzani, G., “Free α-dicarbonyl compounds in coffee, barley coffee and soy sauce and effects of in vitro digestion,” *Food Chem.*, vol. 164, pp. 259–265, 2014, doi: 10.1016/j.foodchem.2014.05.022.

230. Mavric, E.; Wittmann, S.; Barth, G.; and Henle, T., “Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand,” *Mol. Nutr. Food Res.*, vol. 52, no. 4, pp. 483–489, 2008, doi: 10.1002/mnfr.200700282.

231. Berk, E.; Hamzalloglu, A.; and Gökmen, V., “Investigations on the Maillard Reaction in Sesame (Sesamum indicum L.) Seeds Induced by Roasting,” *J. Agric. Food Chem.*, vol. 67, no. 17, pp. 4923–4930, 2019, doi: 10.1021/acs.jafc.9b01413.

232. Kasai, H.; Iwamoto-Tanaka, N.; and Fukada, S., “DNA modifications by the

mutagen glyoxal: Adduction to G and C, deamination of C and GC and GA cross-linking," *Carcinogenesis*, vol. 19, no. 8, pp. 1459–1465, 1998, doi: 10.1093/carcin/19.8.1459.

233. Olsen, R.; Molander, P.; Øvrebø, S.; Ellingsen, D. G.; Thorud, S.; Thomassen, Y.; Lundanes, E.; Greibrokk, T.; Backman, J.; Sjöholm, R.; and Kronberg, L., "Reaction of glyoxal with 2'-deoxyguanosine, 2'-deoxyadenosine, 2'-deoxycytidine, cytidine, thymidine, and calf thymus DNA: Identification of DNA adducts," *Chem. Res. Toxicol.*, vol. 18, no. 4, pp. 730–739, 2005, doi: 10.1021/tx0496688.
234. Murata-Kamiya, N.; Kamiya, H.; Kaji, H.; and Kasai, H., "Mutations induced by glyoxal and methylglyoxal in mammalian cells.," *Nucleic Acids Symp. Ser.*, no. 44, pp. 3–4, 2000, doi: 10.1093/nass/44.1.3.
235. Suwa, Y.; Nagao, M.; Kosugi, A.; and Sugimura, T., "Sulfite suppresses the mutagenic property of coffee," *Mutat. Res. Toxicol.*, vol. 102, no. 4, pp. 383–391, 1982, doi: 10.1016/0165-1218(82)90101-X.
236. Nishi, Y.; Miyakawa, Y.; and Kato, K., "Chromosome aberrations induced by pyrolysates of carbohydrates in Chinese hamster V79 cells," *Mutat. Res. Lett.*, vol. 227, no. 2, pp. 117–123, 1989, doi: 10.1016/0165-7992(89)90007-9.
237. Tucker, J. D.; Taylor, R. T.; Christensen, M. L.; Strout, C. L.; Hanna, M. L.; and Carrano, A. V., "Cytogenetic response to 1,2-dicarbonyls and hydrogen peroxide in Chinese hamster ovary AUXB1 cells and human peripheral lymphocytes," *Mutat. Res. Toxicol.*, vol. 224, no. 2, pp. 269–279, 1989, doi: 10.1016/0165-1218(89)90166-3.
238. Garberg, P.; Akerblom, E.-L.; and Bolcsfoldi, G., "Evaluation of a genotoxicity test measuring DNA-strand breaks in mouse lymphoma cells by alkaline unwinding and hydroxyapatite elution Per Garberg *, Eva-Lena Akerblom and George Bolcsfoldi," *Mutat. Res.*, vol. 203, pp. 155–176, 1988.
239. Roberts, M. J.; Wondrak, G. T.; Laurean, D. C.; Jacobson, M. K.; and Jacobson, E. L., "DNA damage by carbonyl stress in human skin cells," *Mutat. Res. - Fundam. Mol. Mech. Mutagen.*, vol. 522, no. 1–2, pp. 45–56, 2003, doi: 10.1016/S0027-5107(02)00232-4.

240. Svendsen, C.; Høie, A. H.; Alexander, J.; Murkovic, M.; and Husøy, T., "The food processing contaminant glyoxal promotes tumour growth in the multiple intestinal neoplasia (Min) mouse model," *Food Chem. Toxicol.*, vol. 94, pp. 197–202, 2016, doi: 10.1016/j.fct.2016.06.006.

241. Zaccone, E. J.; Thompson, J. A.; Ponnoth, D. S.; Cumpston, A. M.; Goldsmith, W. T.; Jackson, M. C.; Kashon, M. L.; Frazer, D. G.; Hubbs, A. F.; Shimko, M. J.; and Fedan, J. S., "Popcorn flavoring effects on reactivity of rat airways in vivo and in vitro," *J. Toxicol. Environ. Heal. - Part A Curr. Issues*, vol. 76, no. 11, pp. 669–689, 2013, doi: 10.1080/15287394.2013.796302.

242. Xie, W.; Kim, K. H.; Vince, R.; and More, S. S., "The Amyloid Aggregation Accelerator Diacetyl Prevents Cognitive Decline in Alzheimer's Mouse Models," *Chem. Res. Toxicol.*, vol. 34, no. 5, pp. 1355–1366, 2021, doi: 10.1021/acs.chemrestox.1c00089.

243. Niwa, T., "3-Deoxyglucosone: Metabolism, analysis, biological activity, and clinical implication," *J. Chromatogr. B Biomed. Sci. Appl.*, vol. 731, no. 1, pp. 23–36, 1999, doi: 10.1016/S0378-4347(99)00113-9.

244. Hitimi, K.; Satoshi, M.; Takeshi, O.; Bing-Fen, L.; Aysuko, U.; Hiroshi, K.; Kotaro, S.; Hiroyuki, Y.; Kayo, I.; and Masato, K., "Relation Between Serum 3-Deoxyglucosone and Development of Diabetic Microangiopathy," *Diabetes Care*, vol. 26, no. 6, 2003.

245. Lal, S.; Kappler, F.; Walker, M.; Orchard, T. J.; Beisswenger, P. J.; Szwergold, B. S.; Brown, T. R.; and Res, B., "Quantitation of 3-Deoxyglucosone Levels in Human Plasma either by ultrafiltration or by addition of ethanol as," *Arch. Biochem. Biophys.*, vol. 342, no. 2, pp. 254–260, 1997.

246. Hamada, Y.; Nakamura, J.; Fujisawa, H.; Yago, H.; Nakashima, E.; Koh, N.; and Hotta, N., "Effects of glycemic control on plasma 3-deoxyglucosone levels in NIDDM patients," *Diabetes Care*, vol. 20, no. 9, pp. 1466–1469, 1997, doi: 10.2337/diacare.20.9.1466.

247. Beisswenger, P. J.; Howell, S. K.; O'Dell, R. M.; Wood, M. E.; Touchette, A. D.; and Szwergold, B. S., "α-Dicarbonyls increase in the postprandial period and

reflect the degree of hyperglycemia," *Diabetes Care*, vol. 24, no. 4, pp. 726–732, 2001, doi: 10.2337/diacare.24.4.726.

248. Sakiyama, H.; Takahashi, M.; Yamamoto, T.; Teshima, T.; Lee, S. H.; Miyamoto, Y.; Misonou, Y.; and Taniguchi, N., "The internalization and metabolism of 3-deoxyglucosone in human umbilical vein endothelial cells," *J. Biochem.*, vol. 139, no. 2, pp. 245–253, 2006, doi: 10.1093/jb/mvj017.
249. T, K.; T, A.; S, U.; T, M.; and S, U., "Effect of 3-deoxyglucosone on the activities of enzymes responsible for glucose metabolism in mouse liver," *Biol. Pharm. Bull*, vol. 7, no. 11, pp. 101–104, 1996, [Online]. Available: http://www.mendeley.com/research/geology-volcanic-history-eruptive-style-yakedake-volcano-group-central-japan/%0Ahttps://www.sciencedirect.com/science/article/pii/S0038080620318011%0Ahttps://www.jstage.jst.go.jp/article/jjshs1925/72/6/72_6_525/_article/-c
250. Sassi-Gaha, S.; Loughlin, D. T.; Kappler, F.; Schwartz, M. L.; Su, B.; Tobia, A. M.; and Artlett, C. M., "Two dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts," *Matrix Biol.*, vol. 29, no. 2, pp. 127–134, 2010, doi: 10.1016/j.matbio.2009.09.007.
251. Ameur, L. A.; Trystram, G.; and Birlouez-Aragon, I., "Accumulation of 5-hydroxymethyl-2-furfural in cookies during the backing process: Validation of an extraction method," *Food Chem.*, vol. 98, no. 4, pp. 790–796, 2006, doi: 10.1016/j.foodchem.2005.07.038.
252. Gökmen, V. and Şenyuva, H. Z., "Study of colour and acrylamide formation in coffee, wheat flour and potato chips during heating," *Food Chem.*, vol. 99, no. 2, pp. 238–243, 2006, doi: 10.1016/j.foodchem.2005.06.054.
253. Purlis, E., "Browning development in bakery products - A review," *J. Food Eng.*, vol. 99, no. 3, pp. 239–249, 2010, doi: 10.1016/j.jfoodeng.2010.03.008.
254. Gökmen, V.; Açar, Ö. Ç.; Köksel, H.; and Acar, J., "Effects of dough formula and baking conditions on acrylamide and hydroxymethylfurfural formation in cookies," *Food Chem.*, vol. 104, no. 3, pp. 1136–1142, 2007, doi:

10.1016/j.foodchem.2007.01.008.

255. Hamzalıoğlu, A. and Gökmen, V., “5-Hydroxymethylfurfural accumulation plays a critical role on acrylamide formation in coffee during roasting as confirmed by multiresponse kinetic modelling,” *Food Chem.*, vol. 318, no. November 2019, 2020, doi: 10.1016/j.foodchem.2020.126467.

256. Choudhary, A.; Kumar, V.; Kumar, S.; Majid, I.; Aggarwal, P.; and Suri, S., “5-Hydroxymethylfurfural (HMF) formation, occurrence and potential health concerns: recent developments,” *Toxin Rev.*, vol. 40, no. 4, pp. 545–561, 2021, doi: 10.1080/15569543.2020.1756857.

257. Basaran, B.; Anlar, P.; Yılmaz Oral, Z. F.; Polat, Z.; and Kaban, G., “Risk assessment of acrylamide and 5-hydroxymethyl-2-furfural (5-HMF) exposure from bread consumption: Turkey,” *J. Food Compos. Anal.*, vol. 107, no. January, p. 104409, 2022, doi: 10.1016/j.jfca.2022.104409.

258. Shapla, U. M.; Solayman, M.; Alam, N.; Khalil, M. I.; and Gan, S. H., “5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health,” *Chem. Cent. J.*, vol. 12, no. 1, pp. 1–18, 2018, doi: 10.1186/s13065-018-0408-3.

259. Morales, F. J., “Hydroxymethylfurfural (HMF) and related compounds,” in *Process-Induced Food Toxicants: Occurrence, Formation, Mitigation, and Health Risks*, 2009, pp. 135–174.

260. National Toxicology Program, “Toxicology and Carcinogenesis Studies of 5- (Hydroxymethyl) -2-Furfural in F344 / N Rats and B6C3F1 Mice (Gavage Studies),” 2010.

261. Bauer-Marinovic, M.; Taugner, F.; Florian, S.; and Glatt, H., “Toxicity studies with 5-hydroxymethylfurfural and its metabolite 5-sulphooxymethylfurfural in wild-type mice and transgenic mice expressing human sulphotransferases 1A1 and 1A2,” *Arch. Toxicol.*, vol. 86, no. 5, pp. 701–711, 2012, doi: 10.1007/s00204-012-0807-5.

262. Pastoriza de la Cueva, S.; Álvarez, J.; Végvári, Á.; Montilla-Gómez, J.; López, O. C.-; Delgado-Andrade, C.; and Rufián-Henares, J. A., “Relationship between HMF

intake and SMF formation in vivo: an animal and human study," *Mol. Nutr. Food Res.*, pp. 1–12, 2016, doi: 10.1002/mnfr.201600773.This.

263. European Commission, "Scientific Opinion on Flavouring Group Evaluation 13 Revision 3 (FGE.13Rev3): furfuryl and furan derivatives with and without additional side-chain substituents and heteroatoms from chemical group 14," *EFSA J.*, vol. 19, no. 2, 2021, doi: 10.2903/j.efsa.2021.6386.
264. Codex Alimentarius Commission, "Codex Alimentarius Commission Standards," *Codex Stan 12-1981*, pp. 1–8, 2001.
265. European Commission, "Opinion of the Scientific Committee on Food on furfural and furfural diethylacetal," no. December 2002, p. 16, 2003.
266. "Scientific Opinion on Flavouring Group Evaluation 13, Revision 2 (FGE.13Rev2): Furfuryl and furan derivatives with and without additional side-chain substituents and heteroatoms from chemical group 14," *EFSA J.*, vol. 9, no. 8, 2011, doi: 10.2903/j.efsa.2011.2313.
267. Lo, T. W. C.; Westwood, M. E.; McLellan, A. C.; Selwood, T.; and Thornalley, P. J., "Binding and modification of proteins by methylglyoxal under physiological conditions: A kinetic and mechanistic study with Nα-acetylarginine, Nα-acetylcysteine, and Nα-acetyllysine, and bovine serum albumin," *J. Biol. Chem.*, vol. 269, no. 51, pp. 32299–32305, 1994, doi: 10.1016/s0021-9258(18)31635-1.
268. Glomb, M. A. and Monnier, V. M., "Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction," *Journal of Biological Chemistry*, vol. 270, no. 17. pp. 10017–10026, 1995. doi: 10.1074/jbc.270.17.10017.
269. Ahmed, M. U.; Thorpe, S. R.; and Baynes, J. W., "Identification of N(ε)-carboxymethyllysine as a degradation product of fructoselysine in glycated protein," *J. Biol. Chem.*, vol. 261, no. 11, pp. 4889–4894, 1986, doi: 10.1016/s0021-9258(19)89188-3.
270. Hellwig, M.; Witte, S.; and Henle, T., "Free and Protein-Bound Maillard Reaction Products in Beer: Method Development and a Survey of Different Beer Types," *J. Agric. Food Chem.*, vol. 64, no. 38, pp. 7234–7243, 2016, doi:

10.1021/acs.jafc.6b02649.

271. Scheijen, J. L. J. M.; Clevers, E.; Engelen, L.; Dagnelie, P. C.; Brouns, F.; Stehouwer, C. D. A.; and Schalkwijk, C. G., “Analysis of advanced glycation endproducts in selected food items by ultra-performance liquid chromatography tandem mass spectrometry: Presentation of a dietary AGE database,” *Food Chem.*, vol. 190, pp. 1145–1150, 2016, doi: 10.1016/j.foodchem.2015.06.049.
272. Taş, N. G. and Gökmen, V., “Effect of Roasting and Storage on the Formation of Maillard Reaction and Sugar Degradation Products in Hazelnuts (Corylus avellana L.),” *J. Agric. Food Chem.*, vol. 67, no. 1, pp. 415–424, 2019, doi: 10.1021/acs.jafc.8b05048.
273. Zhang, G.; Huang, G.; Xiao, L.; and Mitchell, A. E., “Determination of advanced glycation endproducts by LC-MS/MS in raw and roasted almonds (Prunus dulcis),” *J. Agric. Food Chem.*, vol. 59, no. 22, pp. 12037–12046, 2011, doi: 10.1021/jf202515k.
274. Poojary, M. M.; Zhang, W.; Olesen, S. B.; Rauh, V.; and Lund, M. N., “Green Tea Extract Decreases Arg-Derived Advanced Glycation Endproducts but Not Lys-Derived AGEs in UHT Milk during 1-Year Storage,” *J. Agric. Food Chem.*, vol. 68, no. 48, pp. 14261–14273, 2020, doi: 10.1021/acs.jafc.0c05995.
275. Glomb, M. A. and Lang, G., “Isolation and characterization of glyoxal - Arginine modifications,” *J. Agric. Food Chem.*, vol. 49, no. 3, pp. 1493–1501, 2001, doi: 10.1021/jf001082d.
276. Henle, T.; Walter, A. W.; Haeßner, R.; and Klostermeyer, H., “Detection and identification of a protein-bound imidazolone resulting from the reaction of arginine residues and methylglyoxal,” *Z. Lebensm. Unters. Forsch.*, vol. 199, no. 1, pp. 55–58, 1994, doi: 10.1007/BF01192954.
277. Klöpfer, A.; Spanneberg, R.; and Glomb, M. A., “Formation of arginine modifications in a model system of N α- Tert -butoxycarbonyl (Boc)-arginine with methylglyoxal,” *J. Agric. Food Chem.*, vol. 59, no. 1, pp. 394–401, 2011, doi: 10.1021/jf103116c.
278. Poojary, M. M.; Zhang, W.; Greco, I.; De Gobba, C.; Olsen, K.; and Lund, M. N.,

"Liquid chromatography quadrupole-Orbitrap mass spectrometry for the simultaneous analysis of advanced glycation end products and protein-derived cross-links in food and biological matrices," *J. Chromatogr. A*, vol. 1615, p. 460767, 2020, doi: 10.1016/j.chroma.2019.460767.

279. Boughton, B. A.; Reddy, P.; Boland, M. P.; Roessner, U.; and Yates, P., "Non-protein amino acids in Australian acacia seed: Implications for food security and recommended processing methods to reduce djenkolic acid," *Food Chem.*, vol. 179, pp. 109–115, 2015, doi: 10.1016/j.foodchem.2015.01.072.
280. Li, M.; Zeng, M.; He, Z.; Zheng, Z.; Qin, F.; Tao, G.; Zhang, S.; and Chen, J., "Increased accumulation of protein-bound N (carboxymethyl)lysine in tissues of healthy rats after chronic oral n (carboxymethyl)lysine," *J. Agric. Food Chem.*, vol. 63, no. 5, pp. 1658–1663, 2015, doi: 10.1021/jf505063t.
281. Delgado-Andrade, C., "Carboxymethyl-lysine: Thirty years of investigation in the field of AGE formation," *Food Funct.*, vol. 7, no. 1, pp. 46–57, 2016, doi: 10.1039/c5fo00918a.
282. Key, T. J.; Appleby, P. N.; Crowe, F. L.; Bradbury, K. E.; Schmidt, J. A.; and Travis, R. C., "Cancer in British vegetarians: Updated analyses of 4998 incident cancers in a cohort of 32,491 meat eaters, 8612 fish eaters, 18,298 vegetarians, and 2246 vegans," *Am. J. Clin. Nutr.*, vol. 100, no. SUPPL. 1, pp. 378–385, 2014, doi: 10.3945/ajcn.113.071266.
283. Kim, H.; Caulfield, L. E.; Garcia-Larsen, V.; Steffen, L. M.; Coresh, J.; and Rebholz, C. M., "Plant-Based Diets Are Associated With a Lower Risk of Incident Cardiovascular Disease, Cardiovascular Disease Mortality, and All-Cause Mortality in a General Population of Middle-Aged Adults," *J. Am. Heart Assoc.*, vol. 8, no. 16, 2019, doi: 10.1161/JAHA.119.012865.
284. Satija, A.; Bhupathiraju, S. N.; Rimm, E. B.; Spiegelman, D.; Chiuve, S. E.; Borgi, L.; Willett, W. C.; Manson, J. A. E.; Sun, Q.; and Hu, F. B., "Plant-Based Dietary Patterns and Incidence of Type 2 Diabetes in US Men and Women: Results from Three Prospective Cohort Studies," *PLoS Med.*, vol. 13, no. 6, pp. 1–18, 2016, doi: 10.1371/journal.pmed.1002039.

285. Tuso, P. J.; Ismail, M. H.; Ha, B. P.; and Bartolotto, C., “Nutritional update for physicians: plant-based diets.,” *Perm. J.*, vol. 17, no. 2, pp. 61–66, 2013, doi: 10.7812/TPP/12-085.

286. McDermott, A., “Looking to ‘junk’ food to design healthier options,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 118, no. 41, pp. 1–4, 2021, doi: 10.1073/pnas.2116665118.

287. van Vliet, S.; Kronberg, S. L.; and Provenza, F. D., “Plant-Based Meats, Human Health, and Climate Change,” *Front. Sustain. Food Syst.*, vol. 4, no. October, 2020, doi: 10.3389/fsufs.2020.00128.

288. Drewnowski, A., “Plant-based milk alternatives in the USDA Branded Food Products Database would benefit from nutrient density standards,” *Nat. Food*, vol. 2, no. 8, pp. 567–569, 2021, doi: 10.1038/s43016-021-00334-5.

289. Vanga, S. K. and Raghavan, V., “How well do plant based alternatives fare nutritionally compared to cow’s milk?,” *J. Food Sci. Technol.*, vol. 55, no. 1, pp. 10–20, 2018, doi: 10.1007/s13197-017-2915-y.

290. Rizzo, G.; Laganà, A. S.; Rapisarda, A. M. C.; La Ferrera, G. M. G.; Buscema, M.; Rossetti, P.; Nigro, A.; Muscia, V.; Valenti, G.; Sapia, F.; Sarpietro, G.; Zigarelli, M.; and Vitale, S. G., “Vitamin B12 among Vegetarians: Status, Assessment and Supplementation,” *Nutrients*, vol. 8, no. 12, pp. 1–23, 2016, doi: 10.3390/nu8120767.

291. Antoine, T.; Icard-Vernière, C.; Scorrano, G.; Salhi, A.; Halimi, C.; Georgé, S.; Carrière, F.; Mouquet-Rivier, C.; and Reboul, E., “Evaluation of vitamin D bioaccessibility and mineral solubility from test meals containing meat and/or cereals and/or pulses using in vitro digestion,” *Food Chem.*, vol. 347, p. 128621, 2021, doi: 10.1016/j.foodchem.2020.128621.

292. Gibson, R. S.; Heath, A. L. M.; and Szymlek-Gay, E. A., “Is iron and zinc nutrition a concern for vegetarian infants and young children in industrialized countries?,” *Am. J. Clin. Nutr.*, vol. 100, no. SUPPL. 1, 2014, doi: 10.3945/ajcn.113.071241.

293. Pointke, M. and Pawelzik, E., “Plant-Based Alternative Products: Are They Healthy Alternatives? Micro- and Macronutrients and Nutritional Scoring,”

Nutrients, vol. 14, no. 3, 2022, doi: 10.3390/nu14030601.

294. Banach, J. L.; van der Berg, J. P.; Kleter, G.; van Bokhorst-van de Veen, H.; Bastiaan-Net, S.; Pouvreau, L.; and van Asselt, E. D., "Alternative proteins for meat and dairy replacers: Food safety and future trends," *Crit. Rev. Food Sci. Nutr.*, vol. 0, no. 0, pp. 1–18, 2022, doi: 10.1080/10408398.2022.2089625.
295. Xiang, J.; Liu, F.; Wang, B.; CHen, L.; Liu, W.; and Tan, S., "A Literature Review on Maillard Reaction Based on Milk Products : Advantages , Disadvantages , and Avoidance Strategies," *Foods*, vol. 10, p. 1998, 2021.
296. Tamanna, N. and Mahmood, N., "Food processing and maillard reaction products: Effect on human health and nutrition," *Int. J. Food Sci.*, vol. 2015, 2015, doi: 10.1155/2015/526762.
297. Modi, B.; Timilsina, H.; Bhandari, S.; Achhami, A.; Pakka, S.; Shrestha, P.; Kandel, D.; Gc, D. B.; Khatri, S.; Chhetri, P. M.; and Parajuli, N., "Current Trends of Food Analysis, Safety, and Packaging," *Int. J. Food Sci.*, vol. 2021, 2021, doi: 10.1155/2021/9924667.
298. Alcorta, A.; Porta, A.; Tárrega, A.; Alvarez, M. D.; and Pilar Vaquero, M., "Foods for plant-based diets: Challenges and innovations," *Foods*, vol. 10, no. 2, pp. 1–23, 2021, doi: 10.3390/foods10020293.
299. Rabbani, N.; Al-Motawa, M.; and Thornalley, P. J., "Protein glycation in plants—an under-researched field with much still to discover," *Int. J. Mol. Sci.*, vol. 21, no. 11, 2020, doi: 10.3390/ijms21113942.
300. Day, L.; Cakebread, J. A.; and Loveday, S. M., "Food proteins from animals and plants: Differences in the nutritional and functional properties," *Trends Food Sci. Technol.*, vol. 119, no. June 2021, pp. 428–442, 2022, doi: 10.1016/j.tifs.2021.12.020.
301. Akillioglu, H. G. and Gökmen, V., "Mitigation of acrylamide and hydroxymethyl furfural in instant coffee by yeast fermentation," *Food Res. Int.*, vol. 61, pp. 252–256, 2014, doi: 10.1016/j.foodres.2013.07.057.
302. Haug, A.; Høstmark, A. T.; and Harstad, O. M., "Bovine milk in human nutrition - A review," *Lipids Health Dis.*, vol. 6, pp. 1–16, 2007, doi: 10.1186/1476-511X-6-

25.

303. Smith, N. W.; Fletcher, A. J.; Hill, J. P.; and McNabb, W. C., “Modeling the Contribution of Milk to Global Nutrition,” *Front. Nutr.*, vol. 8, no. January, pp. 1–7, 2022, doi: 10.3389/fnut.2021.716100.
304. Da Silva, M. S. and Rudkowska, I., “Dairy nutrients and their effect on inflammatory profile in molecular studies Marine,” *Mol. Nutr. Food Res.*, pp. 1–44, 2014, doi: 10.1002/mnfr.201400569.This.
305. Khan, I. T.; Bule, M.; Ullah, R.; Nadeem, M.; Asif, S.; and Niaz, K., “The antioxidant components of milk and their role in processing, ripening, and storage: Functional food,” *Vet. World*, vol. 12, no. 1, pp. 12–33, 2019, doi: 10.14202/vetworld.2019.12-33.
306. Rai, C.; Nandini, C. D.; and Priyadarshini, P., “Composition and structure elucidation of bovine milk glycosaminoglycans and their anti-adipogenic activity via modulation PPAR-γ and C/EBP-α,” *Int. J. Biol. Macromol.*, vol. 193, no. PA, pp. 137–144, 2021, doi: 10.1016/j.ijbiomac.2021.10.076.
307. Ratajczak, A. E.; Zawada, A.; Rychter, A. M.; Dobrowolska, A.; and Krela-Kaźmierczak, I., “Milk and dairy products: Good or bad for human bone? practical dietary recommendations for the prevention and management of osteoporosis,” *Nutrients*, vol. 13, no. 4, 2021, doi: 10.3390/nu13041329.
308. Guzmán-Luna, P.; Mauricio-Iglesias, M.; Flysjö, A.; and Hospido, A., “Analysing the interaction between the dairy sector and climate change from a life cycle perspective: A review,” *Trends Food Sci. Technol.*, vol. 126, pp. 168–179, 2022, doi: 10.1016/j.tifs.2021.09.001.
309. Krzywonos, M. and Piwowar-Sulej, K., “Plant-Based Innovations for the Transition to Sustainability: A Bibliometric and in-Depth Content Analysis,” *Foods*, vol. 11, no. 19, 2022, doi: 10.3390/foods11193137.
310. Silva, A. R. A.; Silva, M. M. N.; and Ribeiro, B. D., “Health issues and technological aspects of plant-based alternative milk,” *Food Res. Int.*, vol. 131, no. December 2019, p. 108972, 2020, doi: 10.1016/j.foodres.2019.108972.
311. Mäkinen, O. E.; Wanhalinna, V.; Zannini, E.; and Arendt, E. K., “Foods for Special

Dietary Needs: Non-dairy Plant-based Milk Substitutes and Fermented Dairy-type Products," *Crit. Rev. Food Sci. Nutr.*, vol. 56, no. 3, pp. 339–349, 2016, doi: 10.1080/10408398.2012.761950.

312. Sethi, S.; Tyagi, S. K.; and Anurag, R. K., "Plant-based milk alternatives an emerging segment of functional beverages: a review," *J. Food Sci. Technol.*, vol. 53, no. 9, pp. 3408–3423, 2016, doi: 10.1007/s13197-016-2328-3.
313. Omoni, A. O. and Aluko, R. E., "Soybean foods and their benefits: Potential mechanisms of action," *Nutr. Rev.*, vol. 63, no. 8, pp. 272–283, 2005, doi: 10.1301/nr.2005.aug.272-283.
314. Welch, R. W., "Oats in human nutrition.," *Arch. Tierernahr.*, vol. 39, no. 7, pp. 603–610, 1989, doi: 10.1080/17450398909428330.
315. Truswell, A. S., "Cereal grains and coronary heart disease," *Eur. J. Clin. Nutr.*, vol. 56, no. 1, pp. 1–14, 2002, doi: 10.1038/sj.ejcn.1601283.
316. Kodad, O.; Socias i Company, R.; and Alonso, J. M., "Genotypic and environmental effects on tocopherol content in almond," *Antioxidants*, vol. 7, no. 1, pp. 1–9, 2018, doi: 10.3390/antiox7010006.
317. Biswas, S.; Sircar, D.; Mitra, A.; and de, B., "Phenolic constituents and antioxidant properties of some varieties of Indian rice," *Nutr. Food Sci.*, vol. 41, no. 2, pp. 123–135, 2011, doi: 10.1108/00346651111117391.
318. Bocker, R. and Silva, E. K., "Innovative technologies for manufacturing plant-based non-dairy alternative milk and their impact on nutritional, sensory and safety aspects," *Futur. Foods*, vol. 5, no. July 2021, p. 100098, 2022, doi: 10.1016/j.fufo.2021.100098.
319. Tangyu, M.; Muller, J.; Bolten, C. J.; and Wittmann, C., "Fermentation of plant-based milk alternatives for improved flavour and nutritional value," *Appl. Microbiol. Biotechnol.*, vol. 103, no. 23–24, pp. 9263–9275, 2019, doi: 10.1007/s00253-019-10175-9.
320. Trindler, C.; Annika Kopf-Bolanz, K.; and Denkel, C., "Aroma of peas, its constituents and reduction strategies – Effects from breeding to processing," *Food Chem.*, vol. 376, no. August 2021, p. 131892, 2022, doi:

10.1016/j.foodchem.2021.131892.

321. McClements, D. J., “Development of Next-Generation Nutritionally Fortified Plant-Based Milk Substitutes: Structural Design Principles,” pp. 271–314, 2020, doi: 10.1142/9781848161672_0006.

322. Aydar, E. F.; Tutuncu, S.; and Ozcelik, B., “Plant-based milk substitutes: Bioactive compounds, conventional and novel processes, bioavailability studies, and health effects,” *J. Funct. Foods*, vol. 70, no. April, p. 103975, 2020, doi: 10.1016/j.jff.2020.103975.

323. Liu, S.; Sun, H.; Ma, G.; Zhang, T.; Wang, L.; Pei, H.; Li, X.; and Gao, L., “Insights into flavor and key influencing factors of Maillard reaction products: A recent update,” *Front. Nutr.*, vol. 9, 2022, doi: 10.3389/fnut.2022.973677.

324. ALjahdali, N. and Carbonero, F., “Impact of Maillard reaction products on nutrition and health: Current knowledge and need to understand their fate in the human digestive system,” *Crit. Rev. Food Sci. Nutr.*, vol. 59, no. 3, pp. 474–487, 2019, doi: 10.1080/10408398.2017.1378865.

325. Walther, B.; Guggisberg, D.; Badertscher, R.; Egger, L.; Portmann, R.; Dubois, S.; Haldimann, M.; Kopf-Bolanz, K.; Rhyn, P.; Zoller, O.; Veraguth, R.; and Rezzi, S., “Comparison of nutritional composition between plant-based drinks and cow’s milk,” *Front. Nutr.*, vol. 9, no. October, 2022, doi: 10.3389/fnut.2022.988707.

326. Vinther Schmidt, C.; Olsen, K.; and Mouritsen, O. G., “Umami potential of fermented beverages: Sake, wine, champagne, and beer,” *Food Chem.*, vol. 360, no. January, p. 128971, 2021, doi: 10.1016/j.foodchem.2020.128971.

327. Hildebrand, G.; Poojary, M. M.; O’Donnell, C.; Lund, M. N.; Garcia-Vaquero, M.; and Tiwari, B. K., “Ultrasound-assisted processing of Chlorella vulgaris for enhanced protein extraction,” *J. Appl. Phycol.*, vol. 32, no. 3, pp. 1709–1718, 2020, doi: 10.1007/s10811-020-02105-4.

328. Akıllıoğlu, H. G.; Chatterton, D. E. W.; and Lund, M. N., “Maillard reaction products and amino acid cross-links in liquid infant formula: Effects of UHT treatment and storage,” *Food Chem.*, vol. 396, no. January, 2022, doi: 10.1016/j.foodchem.2022.133687.

329. Prag, A. A. and Henriksen, C. B., “Transition from animal-based to plant-based food production to reduce greenhouse gas emissions from agriculture-the case of Denmark,” *Sustain.*, vol. 12, no. 19, pp. 1–20, 2020, doi: 10.3390/su12198228.
330. Tomé, D. and Bos, C., “Lysine requirement through the human life cycle,” *J. Nutr.*, vol. 137, no. 6, pp. 0–3, 2007, doi: 10.1093/jn/137.6.1642s.
331. Millward, D. J., “Knowledge gained from studies of leucine consumption in animals and humans,” *J. Nutr.*, vol. 142, no. 12, 2012, doi: 10.3945/jn.111.157370.
332. Brosnan, J. and ME, B., “The Sulfur-Containing Amino Acids: An Overview,” *J. Nutr.*, vol. 136, no. 6, pp. 16365–16405, 2006, [Online]. Available: https://www.pkdiet.com/pdf/eaa/aminoacidwrksp.pdf
333. Sousa, A. and Bolanz, K. A. K., “Nutritional Implications of an Increasing Consumption of Non-Dairy Plant-Based Beverages Instead of Cow’s Milk in Switzerland,” *Adv. Dairy Res.*, vol. 05, no. 04, 2017, doi: 10.4172/2329-888x.1000197.
334. Vistoli, G.; De Maddis, D.; Cipak, A.; Zarkovic, N.; Carini, M.; and Aldini, G., “Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): An overview of their mechanisms of formation,” *Free Radic. Res.*, vol. 47, no. S1, pp. 3–27, 2013, doi: 10.3109/10715762.2013.815348.
335. Antonova, K.; Vikhnina, M.; Soboleva, A.; Mehmood, T.; Heymich, M. L.; Leonova, T.; Bankin, M.; Lukasheva, E.; Gensberger-Reigl, S.; Medvedev, S.; Smolikova, G.; Pischetsrieder, M.; and Frolov, A., “Analysis of chemically labile glycation adducts in seed proteins: Case study of methylglyoxal-derived hydroimidazolone 1 (MG-H1),” *Int. J. Mol. Sci.*, vol. 20, no. 15, 2019, doi: 10.3390/ijms20153659.
336. Boschin, G.; D’Agostina, A.; Rinaldi, A.; and Arnoldi, A., “Lysinoalanine content of formulas for enteral nutrition,” *J. Dairy Sci.*, vol. 86, no. 7, pp. 2283–2287, 2003, doi: 10.3168/jds.S0022-0302(03)73820-X.
337. Rombouts, I.; Lambrecht, M. A.; Carpentier, S. C.; and Delcour, J. A., “Identification of lanthionine and lysinoalanine in heat-treated wheat gliadin

and bovine serum albumin using tandem mass spectrometry with higher-energy collisional dissociation," *Amino Acids*, vol. 48, no. 4, pp. 959–971, 2016, doi: 10.1007/s00726-015-2139-2.

338. Deleu, L. J.; Lambrecht, M. A.; Van de Vondel, J.; and Delcour, J. A., "The impact of alkaline conditions on storage proteins of cereals and pseudo-cereals," *Curr. Opin. Food Sci.*, vol. 25, pp. 98–103, 2019, doi: 10.1016/j.cofs.2019.02.017.
339. Sargautis, D.; Kince, T.; and Sargautiene, V., "Review: Current trends in oat protein recovery and utilization in aqueous food systems," *Res. Rural Dev.*, vol. 36, pp. 77–83, 2021, doi: 10.22616/rrd.27.2021.011.
340. Maasen, K.; Scheijen, J. L. J. M.; Opperhuizen, A.; Stehouwer, C. D. A.; Van Greevenbroek, M. M.; and Schalkwijk, C. G., "Quantification of dicarbonyl compounds in commonly consumed foods and drinks; presentation of a food composition database for dicarbonyls," *Food Chem.*, vol. 339, no. September 2020, p. 128063, 2021, doi: 10.1016/j.foodchem.2020.128063.
341. Gökmen, V. and Acar, J., "Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography," vol. 847, pp. 69–74, 1999.
342. Ferrer, E.; Alegría, A.; Courtois, G.; and Farré, R., "High-performance liquid chromatographic determination of Maillard compounds in store-brand and name-brand ultra-high-temperature-treated cows' milk," *J. Chromatogr. A*, vol. 881, no. 1–2, pp. 599–606, 2000, doi: 10.1016/S0021-9673(00)00218-1.
343. Morales, F. J.; Romero, C.; and Jimenez-Pérez, S., "An enhanced liquid chromatographic method for 5-hydroxymethylfurfural determination in UHT milk," *Chromatographia*, vol. 33, no. 1–2, pp. 45–48, 1992, doi: 10.1007/BF02276850.
344. Morales, F. J.; Romero, C.; and Jiménez-Pérez, S., "Chromatographic Determination of Bound Hydroxymethylfurfural as an Index of Milk Protein Glycosylation," *J. Agric. Food Chem.*, vol. 45, no. 5, pp. 1570–1573, 1997, doi: 10.1021/jf960930v.
345. Teixidó, E.; Santos, F. J.; Puignou, L.; and Galceran, M. T., "Analysis of 5-

hydroxymethylfurfural in foods by gas chromatography-mass spectrometry," *J. Chromatogr. A*, vol. 1135, no. 1, pp. 85–90, 2006, doi: 10.1016/j.chroma.2006.09.023.

346. Teixidó, E.; Núñez, O.; Santos, F. J.; and Galceran, M. T., "5-Hydroxymethylfurfural content in foodstuffs determined by micellar electrokinetic chromatography," *Food Chem.*, vol. 126, no. 4, pp. 1902–1908, 2011, doi: 10.1016/j.foodchem.2010.12.016.
347. Xiao, L.; Lee, J.; Zhang, G.; Ebeler, S. E.; Wickramasinghe, N.; Seiber, J.; and Mitchell, A. E., "HS-SPME GC/MS characterization of volatiles in raw and dry-roasted almonds (Prunus dulcis)," *Food Chem.*, vol. 151, pp. 31–39, 2014, doi: 10.1016/j.foodchem.2013.11.052.
348. Valdés, A.; Beltrán, A.; Karabagias, I.; Badeka, A.; Kontominas, M. G.; and Garrigós, M. C., "Monitoring the oxidative stability and volatiles in blanched, roasted and fried almonds under normal and accelerated storage conditions by DSC, thermogravimetric analysis and ATR-FTIR," *Eur. J. Lipid Sci. Technol.*, vol. 117, no. 8, pp. 1199–1213, 2015, doi: 10.1002/ejlt.201400384.
349. Agila, A. and Barringer, S., "Effect of Roasting Conditions on Color and Volatile Profile Including HMF Level in Sweet Almonds (Prunus dulcis)," *J. Food Sci.*, vol. 77, no. 4, pp. 1–8, 2012, doi: 10.1111/j.1750-3841.2012.02629.x.
350. Yaylayan, V. A. and Stadler, R. H., "Acrylamide formation in food: A mechanistic perspective," *J. AOAC Int.*, vol. 88, no. 1, pp. 262–267, 2005, doi: 10.1093/jaoac/88.1.262.
351. Keramat, J.; LeBail, A.; Prost, C.; and Soltanizadeh, N., "Acrylamide in Foods: Chemistry and Analysis. A Review," *Food Bioprocess Technol.*, vol. 4, no. 3, pp. 340–363, 2011, doi: 10.1007/s11947-010-0470-x.
352. Granvogl, M. and Schieberle, P., "Thermally generated 3-aminopropionamide as a transient intermediate in the formation of acrylamide," *J. Agric. Food Chem.*, vol. 54, no. 16, pp. 5933–5938, 2006, doi: 10.1021/jf061150h.
353. Michalak, J.; Gujska, E.; Czarnowska, M.; and F, N., "Study on the acrylamide content in selected groups of food products," *Polish J. Commod. Sci.*, vol. 4(41),

no. December 2016, pp. 60–66, 2014.

354. Cichońska, P. and Ziarno, M., “Legumes and legume-based beverages fermented with lactic acid bacteria as a potential carrier of probiotics and prebiotics,” *Microorganisms*, vol. 10, no. 1, 2022, doi: 10.3390/microorganisms10010091.
355. Yang, M. and Li, L., “Physicochemical, textural and sensory characteristics of probiotic soy yogurt prepared from germinated soybean,” *Food Technol. Biotechnol.*, vol. 48, no. 4, pp. 490–496, 2010.
356. Tian, B.; Xie, B.; Shi, J.; Wu, J.; Cai, Y.; Xu, T.; Xue, S.; and Deng, Q., “Physicochemical changes of oat seeds during germination,” *Food Chem.*, vol. 119, no. 3, pp. 1195–1200, 2010, doi: 10.1016/j.foodchem.2009.08.035.
357. Shkembi, B. and Huppertz, T., “Glycemic Responses of Milk and Plant-Based Drinks : Food Matrix Effects,” pp. 1–18, 2023.
358. Jeske, S.; Zannini, E.; and Arendt, E. K., “Evaluation of Physicochemical and Glycaemic Properties of Commercial Plant-Based Milk Substitutes,” *Plant Foods Hum. Nutr.*, vol. 72, no. 1, pp. 26–33, 2017, doi: 10.1007/s11130-016-0583-0.
359. Englyst, K. N.; Vinoy, S.; Englyst, H. N.; and Lang, V., “Glycaemic index of cereal products explained by their content of rapidly and slowly available glucose,” *Br. J. Nutr.*, vol. 89, no. 3, pp. 329–339, 2003, doi: 10.1079/bjn2002786.
360. Quezada-Calvillo, R.; Robayo-Torres, C. C.; Ao, Z.; Hamaker, B. R.; Quaroni, A.; Brayer, G. D.; Sterchi, E. E.; Baker, S. S.; and Nichols, B. L., “Luminal substrate ‘brake’ on mucosal maltase-glucoamylase activity regulates total rate of starch digestion to glucose,” *J. Pediatr. Gastroenterol. Nutr.*, vol. 45, no. 1, pp. 32–43, 2007, doi: 10.1097/MPG.0b013e31804216fc.
361. Pereira, P. M. de C. C. and Vicente, A. F. dos R. B., “Meat nutritional composition and nutritive role in the human diet,” *Meat Sci.*, vol. 93, no. 3, pp. 586–592, 2013, doi: 10.1016/j.meatsci.2012.09.018.
362. Bastos, D. M.; Monaro, É.; Siguemoto, É.; and Séfora, M., “Maillard Reaction Products in Processed Food : Pros and Cons,” *Food Ind. Process.*, pp. 281–300, 2007.
363. Takeuchi, M., “Serum Levels of Toxic AGEs (TAGE) May Be a Promising Novel

Biomarker for the Onset/Progression of Lifestyle-Related Diseases," *Diagnostics*, vol. 6, no. 2, p. 23, 2016, doi: 10.3390/diagnostics6020023.

364. Bakhsh, A.; Lee, S.-J.; Lee, E.-Y.; Hwang, Y.-H.; and Joo, S.-T., "Traditional Plant-based Meat Alternatives, Current, and Future Perspective: A Review," *J. Agric. Life Sci.*, vol. 55, no. 1, pp. 1–11, 2021, doi: 10.14397/jals.2021.55.1.1.
365. "Plant-Based-Meat Global Market Report 2023," 2023.
366. Santo, R. E.; Kim, B. F.; Goldman, S. E.; Dutkiewicz, J.; Biehl, E. M. B.; Bloem, M. W.; Neff, R. A.; and Nachman, K. E., "Considering Plant-Based Meat Substitutes and Cell-Based Meats: A Public Health and Food Systems Perspective," *Front. Sustain. Food Syst.*, vol. 4, no. August, pp. 1–23, 2020, doi: 10.3389/fsufs.2020.00134.
367. Rubio, N. R.; Xiang, N.; and Kaplan, D. L., "Plant-based and cell-based approaches to meat production," *Nat. Commun.*, vol. 11, no. 1, pp. 1–11, 2020, doi: 10.1038/s41467-020-20061-y.
368. Deng, P.; Chen, Y.; Xie, S.; Xue, C.; He, Z.; Chen, Q.; Wang, Z.; Qin, F.; Chen, J.; and Zeng, M., "Accumulation of Heterocyclic Amines and Advanced Glycation End Products in Various Processing Stages of Plant-Based Burgers by UHPLC-MS/MS," *J. Agric. Food Chem.*, 2022, doi: 10.1021/acs.jafc.2c06393.
369. D'Cunha, N. M.; Sergi, D.; Lane, M. M.; Naumovski, N.; Gamage, E.; Rajendran, A.; Kouvari, M.; Gauci, S.; Dissanayka, T.; Marx, W.; and Travica, N., "The Effect of Dietary Advanced Glycation End-Products on Neurocognitive and Mental Disorders," pp. 1–22, 2022.
370. Rifai, L. and Saleh, F. A., "A Review on Acrylamide in Food: Occurrence, Toxicity, and Mitigation Strategies," *Int. J. Toxicol.*, vol. 39, no. 2, pp. 93–102, 2020, doi: 10.1177/1091581820902405.
371. Sergi, D.; Boulestin, H.; Campbell, F. M.; and Williams, L. M., "The Role of Dietary Advanced Glycation End Products in Metabolic Dysfunction," *Mol. Nutr. Food Res.*, vol. 65, no. 1, pp. 1–11, 2021, doi: 10.1002/mnfr.201900934.
372. Programme, F. S., "Health Implications of Acrylamide in Food," no. June, 2002.
373. Zhang, J.; Chen, Q.; Kaplan, D. L.; and Wang, Q., "High-moisture extruded

protein fiber formation toward plant-based meat substitutes applications: Science, technology, and prospect," *Trends Food Sci. Technol.*, vol. 128, no. August, pp. 202–216, 2022, doi: 10.1016/j.tifs.2022.08.008.

374. Singh, M.; Trivedi, N.; Enamala, M. K.; Kuppam, C.; Parikh, P.; Nikolova, M. P.; and Chavali, M., "Plant-based meat analogue (PBMA) as a sustainable food: a concise review," *Eur. Food Res. Technol.*, vol. 247, no. 10, pp. 2499–2526, 2021, doi: 10.1007/s00217-021-03810-1.
375. McClements, D. J.; Weiss, J.; Kinchla, A. J.; Nolden, A. A.; and Grossmann, L., "Methods for testing the quality attributes of plant-based foods: Meat-and processed-meat analogs," *Foods*, vol. 10, no. 2, 2021, doi: 10.3390/foods10020260.
376. Cutroneo, S.; Angelino, D.; Tedeschi, T.; Pellegrini, N.; and Martini, D., "Nutritional Quality of Meat Analogues: Results From the Food Labelling of Italian Products (FLIP) Project," *Front. Nutr.*, vol. 9, no. April, pp. 1–12, 2022, doi: 10.3389/fnut.2022.852831.
377. Kyriakopoulou, K.; Keppler, J. K.; and Van Der Goot, A. J., "Functionality of Ingredients and Additives in Plant-Based Meat Analogues," *Foods*, vol. 10, no. 3, p. 9147, 2020, [Online]. Available: https://doi.org/10.3390/foods10030600%0Ahttps://www.avebe.com/vegan-meat-analogues-2/
378. Bohrer, B. M., "An investigation of the formulation and nutritional composition of modern meat analogue products," *Food Sci. Hum. Wellness*, vol. 8, no. 4, pp. 320–329, 2019, doi: 10.1016/j.fshw.2019.11.006.
379. Romão, B.; Botelho, R. B. A.; Nakano, E. Y.; Raposo, A.; Han, H.; Vega-Muñoz, A.; Ariza-Montes, A.; and Zandonadi, R. P., "Are Vegan Alternatives to Meat Products Healthy? A Study on Nutrients and Main Ingredients of Products Commercialized in Brazil," *Front. Public Heal.*, vol. 10, no. May, pp. 1–12, 2022, doi: 10.3389/fpubh.2022.900598.
380. Inan-Eroglu, E.; Ayaz, A.; and Buyuktuncer, Z., "Formation of advanced glycation endproducts in foods during cooking process and underlying mechanisms: A

comprehensive review of experimental studies," *Nutr. Res. Rev.*, vol. 33, no. 1, pp. 77–89, 2020, doi: 10.1017/S0954422419000209.

381. Bühler, J. M.; Schlangen, M.; Möller, A. C.; Bruins, M. E.; and Der, A. J. Van, "Starch in Plant-Based Meat Replacers : A New Approach to Using Endogenous Starch from Cereals and Legumes," vol. 2100157, pp. 1–12, 2022, doi: 10.1002/star.202100157.
382. Isabel Guerrero-Legarreta (Ed.), *Handbook of Poultry Science and Technology*.
383. Zhu, Z.; Fang, R.; Huang, M.; Wei, Y.; and Zhou, G., "Oxidation combined with Maillard reaction induced free and protein-bound Nε-carboxymethyllysine and Nε-carboxyethyllysine formation during braised chicken processing," *Food Sci. Hum. Wellness*, vol. 9, no. 4, pp. 383–393, 2020, doi: 10.1016/j.fshw.2020.05.013.
384. Schreuders, F. K. G.; Dekkers, B. L.; Bodnár, I.; Erni, P.; Boom, R. M.; Jan, A.; and Goot, V. Der, "Comparing structuring potential of pea and soy protein with gluten for meat analogue preparation," *J. Food Eng.*, vol. 261, no. May, pp. 32–39, 2019, doi: 10.1016/j.jfoodeng.2019.04.022.
385. Yu, C. H. J.; Migicovsky, Z.; Song, J.; and Rupasinghe, H. P. V., "(Poly)phenols of apples contribute to in vitro antidiabetic properties: Assessment of Canada's Apple Biodiversity Collection," *Plants People Planet*. 2022. doi: 10.1002/ppp3.10315.
386. Ramkissoon, J. S.; Mahomoodally, M. F.; Ahmed, N.; and Subratty, A. H., "Antioxidant and anti-glycation activities correlates with phenolic composition of tropical medicinal herbs," *Asian Pac. J. Trop. Med.*, vol. 6, no. 7, pp. 561–569, 2013, doi: 10.1016/S1995-7645(13)60097-8.

MODULO DI EMBARGO DELLA TESI
(da compilare solo se si richiede un periodo di segretazione della tesi)

Il/La sottoscritto/a…Mariachiara Pucci…… Nato/a il…26/01/1994…….
a (indicare anche l'eventuale paese estero)…Livorno……..
provincia di (ovvero sigla del paese estero)…Livorno…….
Dottorato di Ricerca in …Scienze Biomediche e Medicina Translazionale…….
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DICHIARA

- che il contenuto della tesi **non può essere immediatamente consultabile per il seguente motivo**

Motivi di segretezza e/o di proprietà dei risultati relativi a ricerche effettuate

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La motivazione deve essere dettagliata e controfirmata obbligatoriamente dal Tutor e/o Relatore
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- che sarà comunque consultabile immediatamente l'abstract della tesi, che viene caricato in Esse3, profilo studente.

Luogo e Data
Brescia, 03/02/2023

Firma del Dichiarante
Mariachiara Pucci

Controfirma del Tutor e/o Relatore del Dottorato
per la motivazione di embargo e il periodo.

Daniela Uberti