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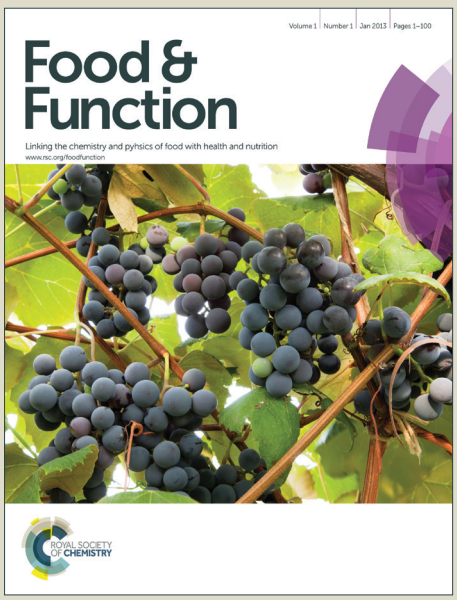

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ARTICLE

Antioxidant activity of bovine alpha lactalbumin Maillard products and evaluation of their *in vitro* gastro-duodenal digestive proteolysis

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Food processing offers various pathways to tailor food functionality and digestibility. This work sought to study the impact of thermally-induced Maillard reaction between bovine alpha-lactalbumin (α-la) and fructose or fructo-oligosacchrides on physicochemical properties, antioxidant capacity and *in vitro* digestive fate under simulated adult and infant conditions. Colloidal stability (measured by DLS) was decreased as a result of the Maillard glycation, while antioxidant capacity (determined by FRAP) and surface hydrophobicity (H0 measurements) were elevated. Semi-dynamic *in vitro* digestion of Maillard conjugates revealed a mixed trend as a result of postulated competing effects of glycation on α-la's susceptibility to proteolysis; steric hindrance accompanied by protein unfolding could hinder or promote the availability of enzymatic cleavage sites. Results also showed thermal processing altered the digestive breakdown profile of α-la under infant conditions contrary to negligible effects observed under adult conditions. Evaluation of the antioxidant capacity during digestion (via DPPH assay) revealed that adult digesta possessed increased antioxidant activity throughout the gastric phase compared to infant digesta, whereas infant digesta of conjugates exhibited an increase in antioxidant capacity in the duodenum compared to adult. Moreover, during infant digestion of conjugates, an increase in antioxidant capacity was observed in the later stages of the digestion.. Overall, this work demonstrates that controlled thermal processing of bovine α-la could potentially modulate its functionality and digestibility, particularly as it pertains to its ability to interfere with oxidative reactions in the lumen, possibly through the generation of bioactive peptides.

1. Introduction

Alimentary proteins are known to play a major role in human nutrition and health, not only as macronutrients but also as important sources of bioactive peptides[1-3]. Furthermore, proteins have been extensively used by the food industry as functional ingredients, such as foaming, emulsifying and gelling agents whose properties depend on both intrinsic (e.g. molecular structure, composition) and extrinsic (e.g. temperature, chemical environment, pH) factors[4]. Reported strategies to rationally modulate protein functionality have addressed both physical and chemical pathways, for example leading to controlled assembly into proteinaceous fibrils, nano-particles or even chemical functionalization through covalent grafting with polysaccharide moieties[5-7].

To this end, the Maillard reaction naturally occurs in many thermally processed foods and has been extensively studied for its possible ramifications to protein structure and functionality[6, 8]. In the era of sustainability and green chemistry, harnessing the practically inevitable Maillard reaction to rationally modify proteins has been advocated[6, 9-11]. Amongst the many protein sources, milk proteins are not only considered major nutritional components but also increasingly recognized as rich viable sources of bioactive peptides[1, 12]. In this respect, α-lactalbumin (α-la), a 14 kDa single-chain calcium and zinc binding milk protein, has been shown to portray various bioactive properties as well as being the origin of a variety of bioactive peptides possessing diverse activities (e.g. immune-modulating, antimicrobial, antiviral, antioxidant, etc.)[13, 14]. Furthermore, previous studies[6, 15-17] revealed that α-la glycated with different

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carbohydrates via a controlled Maillard reaction exhibited modulated solubility, emulsifying activity and antioxidative capacity as well as a marked impact on antigenicity, compared to the native protein. The impact of the Maillard reaction on protein characteristics and functionality was found to be dependent on reaction conditions. Moreover, α-la is quantitatively one of the most abundant proteins in whey, making up to approximately 20-25% of the whey proteins, and also making up a significant proportion of human milk proteins (~1.2 mg/ml)[13, 18]. Therefore, α-la is supplemented to infant formulae which undergo various processing operations, including thermal processing during which the Maillard reaction unintentionally occurs and may modulate its structure and functionality. In terms of digestibility, α-la has been found to be susceptible to proteolysis by trypsin and pepsin[19]. Certain processing operations, e.g. binding of zinc ions to α-la or its esterification, tend to increase its susceptibility to proteolysis[20]. On the other hand, α-la *in vitro* gastric proteolysis has been shown to be retarded in the presence of physiological phosphatidylcholine[21]. *In vivo* studies in infant formula-fed piglets suggest that α-la is highly resistant to proteolytic breakdown and can persist for more than 1 h after meal ingestion. This resistance to proteolysis is believed to stem from the highly compact conformation assumed by α-la for the presence of four intramolecular disulfide bridges which hinder enzyme accessibility to cleavage sites[13, 14]. Moreover, the susceptibility of α-la and other milk proteins, such as β-lactoglobulin, has been shown to vary between infant and adult models[22, 23]. In addition, breast milk and infant formula have been found to reduce oxidative stress in premature infants caused by the rapid production and accumulation of radicals as well as reactive oxygen species during the transition from the relatively hypoxic uterus environment to a hyperoxic external environment while administering supplemental oxygen therapy. This ability was attributed to the antioxidant properties of milk proteins and their derived peptides[24]. Altogether, the importance of understanding protein digestibility is elemental to rational food design as intact proteins may also serve as prodrug-like precursors liberating their latent bioactive peptides only after ingestion[25].

The overall goal of this research was to study the impact of the Maillard reaction on the functionality of bovine α-la based Maillard conjugates, particularly as it pertains to their digestive fate in adults and infants. Some studies have shown contradicting effects whereby the Maillard reaction can at times increase or decrease protein susceptibility to digestion[26-28]. Practically, this study included fabrication of α-la MRPs using fructose and fructo-oligosacchrides (FOS) and interrogation of their physicochemical characteristics and *in vitro* digestive fate utilizing adult and infant digestion models. Specific emphasis was drawn to evaluating MRPs antioxidant performance throughout the digestive tract.

2. Materials and methods

2.1 Materials

Bovine α-la (97.2% protein) was kindly donated by Davisco Foods Internaional Inc. (Le Sueur, MN). Fructose (≥99% purity), ammonium thiocyanate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich (Rehovot, Israel). Oligofructose (Orafti®P95, 95% oligofructose, also contains sucrose, fructose and glucose) (FOS) was kindly donated by BENEO (Universal Network LTD, Rosh HaAyin, Israel). All solutions were prepared with deionized water (DW) (filtration unit on site) and all reagents were of analytical grade.

2.2 Methods

2.2.1 Preparation of Maillard reaction products (MRPs)

α-la based MRPs were prepared by mixing the protein with fructose or FOS as detailed in previous work[29, 30]. Briefly, fructose-based MRPs were produced at a mole ratio of 1:3 (α-la:fructose) while a practical 3:1 %w/w ratio of α-la:FOS was used to prepare the FOS-based MRPs. Maillard conjugates were prepared by dissolving the protein powder with the carbohydrate while mixing in DW at ambient temperature for 4 h. Solutions were adjusted to pH 7.0 with 1 M NaOH, lyophilized and pulverized into fine powders which were incubated at 60 ^{0}C under water restricted conditions (79% RH achieved over saturated potassium bromide) for 12 and 24 h (FOS-based MRPs) or 12 and 36 h (fructose-based MRPs). Control samples were produced similarly without the addition of carbohydrates. Finally, resulting samples were dialyzed at 4°C over 12 h against 5 volumes of 2 L DW, to remove unreacted carbohydrates (dialysis tubing 6-8 kDa nominal MW cut off, Cellu Sep, Seguin, TX). Dialyzed samples were then lyophilized, pulverized and kept in a desiccator until further analysis. Heated protein-carbohydrate mixtures were termed as Maillard reaction products (MRPs) of α-la and indexed α-la-Fru and α-la-FOS with numerical indices denoting heating duration.

2.2.2 Evaluation of MRP formation and properties

2.2.2.1 UV absorbance

Initial evaluation of MRP formation was performed by a comparative absorbance analysis in the range 200-600 nm of 0.2% (w/w) sample solutions adjusted to pH 7 using UV/visible spectrophotometer (OPTIZEN POP, MECASYS, Daejeon, Korea) with DW as a blank reference. As previously reported[31], the most significant differences were observed at 305 nm, and thus are presented.

2.2.2.2 Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed to monitor the formation of high MW moieties normally produced during the course of non-enzymatic browning, as previously described[29, 30]. Briefly, electrophoresis was carried out using a gradient gel (4–15% Mini-PROTEAN® TGX™ Precast Gel) at 145 V for 1 h in Tris/Glycine/SDS running buffer (Bio-Rad Laboratories, Rishon LeZion, Israel). After electrophoresis the gels were stained by Coomassie brilliant blue R250 and imaged with a Microtek 9800XL Plus scanner (Microtek, Carson, CA).

2.2.2.3 Color measurement

The evaluation of color which may be produced during the Maillard reaction was carried out using a Minolta CR-300 chroma meter (Konica Minolta Sensing Americas, Ramsey, NJ) according to the CIE Lab scale[32]. The system provides the value of three color components; L* (black-white component, luminosity), a* (+red to –green component) and b* (+yellow to –blue component). Powder samples of heated controls and MRPs were measured in a 5 cm diameter plastic petri dish and compared to the native sample [33]. The ΔE index was calculated using equation 1:

[1] $\Delta E = ((L_S-L_N)^2 + (a_S-a_N)^2 + (b_N-b_N)^2)^{1/2}$.

Where S defines sample values and N denotes values measured for the native protein.

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2.2.3 Colloidal characterization, antioxidant capacity and protein surface hydrophobicity of MRPs

2.2.3.1 Size of MRPs under various pH values

Previous reports show MRPs exhibit altered colloidal behavior under varying pH conditions[29, 30, 34, 35] . Thus, this study sought to evaluate the mean particle size of 0.2% (w/w) sample solutions in DW as affected by pH (between 3.0 to 10.0). Colloid size was determined through monitoring the dynamic light scattering (DLS) of samples using a Nano-ZS DLS instrument (Malvern Instruments, Worcestershire, UK). Data collected was used to calculate the particle size distribution of samples and the mean particle size using the Stokes–Einstein equation, as previously described[29].

2.2.3.2 Determining the antioxidant capacity of MRPs

The modifications arising from the Maillard reaction have been reported to influence the antioxidant capacity of proteins[6, 16, 36]. For this reason, the reducing ability of samples was measured using the ferric reducing/antioxidant power (FRAP) assay, as previously reported[29, 30, 37]. Briefly, 200 µL of freshly prepared FRAP reagent (mixture of 1 mL of 10 mM TPTZ, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, 2.4 mL H_2O and 10 mL of 300 mM sodium acetate, pH 3.6) and 10 µL of 5% (w/w) sample were mixed. Absorbance of samples was measured at 593 nm after 10 min using a 96-well plate (OptiMax™ microplate reader, Siloam Biosciences Inc., Cincinnati, OH). Ascorbic acid stock solutions 0-1000 µM were used to generate the calibration curve. Results were expressed as µmole equivalents of ascorbic acid per gram protein.

2.2.3.3 Surface hydrophobicity

Another important property that may be affected by the Maillard reaction is protein's surface hydrophobicity[38], which was studied via the 8-anilino-1-naphtalenesulfonic acid (ANS) assay. Samples were dissolved in phosphate buffer (0.2 M, pH 7.5) in different concentrations (0.05, 0.10, 0.15, 0.20, 0.25 mg/mL). An aliquot of ANS solution (20 µL, 8.0 mM in phosphate buffer) was added to 4 mL of sample, mixed well by vortex and left in the dark for 15 min before sample fluorescence was recorded. Fluorescence intensity (FI) was measured at wavelengths of 325 nm (excitation) and 420 nm (emission) in line with previous studies[39]. The initial slope of the FI versus concentration plot (calculated by linear regression analysis) was used as an index of protein hydrophobicity defined as H_0 value.

***2.2.4 In vitro* digestive fate of MRPs**

***2.2.4.1 In vitro* semi-dynamic gastro-duodenal adult and infant digestion**

Previous reports have demonstrated that the Maillard reaction may modify proteolysis and that the digestibility of proteins and MRPs may differ between adults and infants[22, 30]. Thus, gastro-duodenal proteolysis of MRPs and control samples was evaluated in a semi-dynamic model mimicking the gut of a healthy adult or infant[40]. All digestion experiments were performed in duplicates (at least), on two separate occasions in a single blind manner where experimenters handled coded samples. Samples were marked with two digits and one letter code, which were decoded only after SDS-PAGE analyses .

In vitro digestions were performed in a water jacketed reactor maintained at 37 °C and continuously stirred at ~250 rpm (6.1418.220, Metrohm, Herisau, Switzerland) controlled through a dual auto titration unit (Titrando 902) regulated by a computerized program ("TIAMO" software) gradually altering the reactor's pH to mimic adult or infant gastric pH post-prandial profiles[22, 41-43]. After gastric digestion phase, a static duodenal phase was performed for 1 h at pH = 6.5 following neutralization of gastric digesta with 1 M ammonium bicarbonate. Prior to *in vitro* digestion, a 60 mg sample was dissolved in 20 mL of simulated duodenal fluid (SDF) (0.15 M NaCl, pH 6.5) and mixed in a ratio of 1:2 v/v with simulated gastric fluid (SGF) (0.15 M NaCl, pH 1.2), after which pH was adjusted to 4.5 for adult digestion or 6.5 for infant digestion. Porcine gastric mucosa pepsin solution (pre-dissolved in SGF 0.15 M NaCl, pH 4.5 for adult or pH 6.5 for infant) was added to reach a final concentration of 170 (adult) or 22.75 (infant) U pepsin per mg of protein in the digestion mix, and the auto titration program was initiated. Gastric digesta aliquots were withdrawn before pepsin addition and at different time points along the gastric phase: for the adult model after 1, 5, 10, 30 and 60 min, and for the infant model after 5, 10, 60, 120 and 180 min. These were rapidly neutralized with 1 M ammonium bicarbonate and placed on ice, and were denoted: G0 for a sample before pepsin addition, G1 for a sample withdrawn 1 min after pepsin addition etc. After 60 min (adult) or 180 min (infant) of gastric phase, sodium taurocholate, sodium glycodeoxycholate, porcine trypsin and porcine α-chymotrypsin solutions (pre-dissolved in SDF 0.15 M, pH 6.5) were added and the pH was adjusted and maintained at 6.5 by the software for additional 60 min. A second burst of bile salts was added in the adult model 30 min after the beginning of the duodenal phase. Concentrations of enzymes and bile salts in the digestion mixture were as follows: 4 mM (adult) or 1 mM (infant) sodium taurocholate, 4 mM (adult) or 1 mM (infant) sodium glycodeoxycholate, 34.5 (adult) or 3.45 (infant) U trypsin per mg of protein, and 0.4 (adult) or 0.04 (infant) U α-chymotrypsin per mg of protein. Duodenal digesta aliquots were withdrawn 3, 6, 30 and 60 min after the beginning of the duodenal phase, rapidly mixed with the serine protease inhibitor phenylmethanesulfonylfluoride (PMSF) in excess and placed on ice. Duodenal samples were denoted similarly to gastric samples, only with "D" instead of "G" (for example: D3 indicating a sample taken during the duodenal phase 3 min after addition of enzymes). Samples were stored at -20 °C until further analysis.

2.2.4.2 Monitoring proteolysis products by SDS-PAGE

Comparison of peptide breakdown profiles of digesta samples was based on SDS-PAGE as described in previous works[30].

2.2.4.3 Determining antioxidant capacity throughout gastro-duodenal digestion (DPPH)

Previous studies have shown that proteins and peptides may possess antioxidant properties, which vary during digestion due to their enzymatic breakdown[44]. Thus, radical scavenging activity of digested fractions was determined using the DPPH assay as previously described[13, 45, 46], with slight modifications. Digesta aliquots (200 µL) were added to 800 µL 0.25 mM DPPH in methanol. The solution was mixed vigorously and incubated at room temperature in the dark for 24 h, after which absorbance was measured at 517 nm. The percentage of DPPH radical scavenging activity was calculated as follows:

Radical-scavenging activity (%) = [1 – (A517 nm_{sample} - A517 $nm_{control}$) / A517 nm_{blank}] x 100.

2.2.5 Experimental design and statistical analysis.

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Experiments were carried out in triplicates and results are presented as the calculated mean and standard deviation. Statistical analyses were performed using Microsoft Excel 2010 data analysis toolpack and relied on t-tests assuming equal variances and ANOVA single factor.

3. Results and Discussion

3.1 Evaluation of the Maillard reaction and impact of thermal processing

The Maillard reaction has been described to occur even unintentionally during thermal processing of products containing a protein and a reducing carbohydrate, leading to protein glycation before the progression of the reaction to the formation of pools of different chemicals[8, 47]. In its early stages, the Maillard reaction is known to affect protein's physicochemical properties such as MW, charge and tertiary structure. Identification and categorization of MRPs is typically monitored through various methods, such as SDS-PAGE, absorbance and color formation, which provide indications as to the occurrence and extent of the Maillard reaction[6, 31, 48].

The data collected in this study for α-la, controls and MRPs are summarized in **Figure 1**. Alterations in the UV absorbance at 305 nm **(Figure 1A)** indicated the occurrence of the Maillard reaction, as glycated samples at 36 h for fructose and 24 h for FOS displayed a significant ($p<0.05$) increase in UV absorbance compared to the native protein and concurring with previous reports[29, 49, 50]. No notable changes in UV absorbance were noted in samples of α-la heated in the absence of a carbohydrate. However, the differences in UV absorbance were subtle and SDS-PAGE analysis (**Figure 1B**) provided further evidence to the formation of species with MW exceeding that of α-la band at ~14 kDa. These were noticeable in the form of band smearing and the intensification of higher MW bands, some bands (denoted in **Figure 1**) possibly corresponding to dimers and trimers. Differences in band intensities are clearly observed when comparing α-la-Fru 36 h to α-la 36 h and α-la-FOS 24 h to α-la 24 h, corroborating the critical role of the sugar moiety in altering the properties of α-la and supporting the occurrence of the Maillard reaction. Interestingly, results also provided evidence of the presence of glyco-α-la at MW of ~15-16 kDa as previously reported[51]. The intensification of specific higher MW bands (denoted in **Figure 1)** may arise from the production of covalently linked protein dimers and trimers due to Maillard mediated cross linking, as shown previously[6, 52]. Alternatively, dimers and trimes could form due to thermally induced disulphide-bond shuffling between protein molecules, as previously reported[53]. This would also provide a plausible explanation to the subtle changes noted in the control samples of α-la heated on its own. It is also important to note that due to the low MW of fructose and the relatively low degree of polymerization of the FOS used (DP between 2 to 8), one can assume that mildly glycated α-la could overlap the native α-la band, because of the limited ability to resolve the mildly glycated protein from the unglycated fraction by the SDS-PAGE running conditions[30]. Altogether these experiments substantiated that prolonged heating increased the generation of MRPs and that fructose exacerbated the reaction to a larger extent than FOS. This concurs with previous reports which suggest an inverted correlation between sugar moiety size and its reactivity via the Maillard reaction. Thus, shorter carbonic chains exist more readily in open chain forms which are more reactive[54].

Color measurements (**Figure 1C)** further supported the previous results as conjugated samples clearly presented a significantly higher ($p<0.05$) color development relatively to the native protein in comparison with control samples. As mentioned earlier, this color development is characteristic for the Maillard reaction and may arise from the formation of various colored molecules that are produced during different stages of the reaction[6, 8]. In addition, color development was not significantly different in fructose and FOS MRPs heated for 12 h, despite the use of higher protein:carbohydrate ratio in FOS MRPs fabrication. Moreover, color evolution was significantly more noticeable in fructose based conjugates heated for 36 h than FOS based conjugates heated for 24 h, as expected from previous results and carbohydrate inherent reactivity. Altogether, experiments verified that the Maillard reaction occurred and showed that glycation time and carbohydrate moiety type are major factors affecting the progression and therefore products of the Maillard reaction.

3.2 Protein colloidal stability, antioxidant capacity and surface hydrophobicity

The techno-functionality of α-la as well as other proteins is known to be highly linked to protein's responsiveness to its environment, reactivity and potential interaction with other molecular species. The impact of the Maillard reaction on α-la behavior under a variety of pH conditions was initially evaluated by determining protein mean size using DLS. Concomitantly, the antioxidant capacity and surface hydrophobicity were analyzed and all these results are summarized in **Figure 2**. Size analyses (**Figure 2A**) revealed that the major influence of pH on protein colloidal stability is confined to the surrounding of α-la isoelectric point (pI ~ 5) concurring with previous reports[29, 34]. Furthermore, ζ-potential of all samples did not show that Maillard-induced glycation alters the isoelectric point of α-la (data not shown) concurring with a previous report[34]. Overall, size results highlighted that the thermal processing of α-la without the addition of carbohydrates hampered the protein's colloidal stability which was expressed in elevated colloid sizes. This is believed to arise from inter-molecular interactions affecting molecules tendency to associate following heat induced rearrangements and disulfide bond shuffling, exposing hydrophobic regions and inducing aggregation, as previously reported[53, 55]. Prolonged heating of these samples appeared to lead to a subsequent size reduction. This apparently peculiar observation is believed to be a result of extensive protein-protein associations generating larger insoluble aggregates that may sediment and therefore evade measurement by DLS. This notion was indeed

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experimentally supported in the noticeable accumulation of sediments in the aforementioned samples. Therefore, the smaller particle sizes measured are believed to be moderately aggregated protein fractions which have yet to sediment. In terms of the effects of the Maillard conjugation, a particle size increase was also observed compared to the native α-la. Here, fructose based conjugates formed after 12 h of heating yielded samples with improved colloidal stability, i.e. reduced particle sizes. Further heating up to 36 h, again hampered the sample colloidal stability. This is believed to arise from the mixed impact of glycation whereby steric repulsion by the added moiety is weighed against its impact on protein structure and unfolding. Thus, steric repulsion hinders protein-protein association while unfolding exacerbates such associations and the balance between both competing mechanisms is affected by the extent of the Maillard-induced glycation. Similarly, FOS had a mixed effect on α-la stability; yet, a trend of hampered stability compared to the native α-la was noted. Surface hydrophobicity was also measured for these samples in order to substantiate the aforementioned notion. Fluorescent probe ANS assay enabled determination of H_0 values (**Figure 2B**) which are known indicators of sample surface hydrophobicity[39]. These experiments affirmed that extended thermal processing and/or Maillard conjugation significantly ($p<0.05$) increased hydrophobicity. The increase in H_0 could be attributed to a combined contribution of two counteracting factors. On one hand, partial denaturation of the protein upon heating accompanied by disulfide bond shuffling and removal of calcium ions lead to the externalization of hydrophobic regions and thus elevated H_0[53, 56]. On the other hand, the decrease in H_0 could be the outcome of extensive exposure of hydrophobic elements, inducing close inter-molecular interactions leading to the effective burial of hydrophobic regions in the aqueous samples.

Maillard-induced glycation has also been implicated in altered antioxidant capacity. Thus, the reducing ability of samples was measured using the FRAP assay[57, 58]. Results (**Figure 2C**) showed a significant ($p<0.05$) increase in antioxidant capacity of conjugates markedly exceeding that of the native protein or controls. Moreover, the increase in antioxidant ability was dependent on carbohydrate moiety type and heating time. α-la-Fru conjugates heated for 12 h possessed comparable capability as FOS MRPs, despite the minute amount of reacting fructose in the reaction. This result could be attributed to the difference in reactivity patterns between the carbohydrates, as shown in previous reports[29, 30] and discussed earlier.

Overall, the different results obtained using fructose and FOS suggests thermal processing and Maillard-induced glycation have various competing effects originating from the different moiety physicochemical properties (e.g. MW and reactivity). For example, chain length may be a major factor affecting protein colloidal stability and surface hydrophobicity by the tendency of the carbohydrate to attach at the outer rim or the inner regions of the protein. Location of glycation site is expected to be detrimental to protein rearrangements and steric hindrance. Such expected structural and chemical alterations could affect MRP susceptibility to proteolysis which is pivotal in any protein's digestive fate[28, 59].

3.3 Evaluation of MRP digestive fate in an adult gastro-duodenal model

Human digestion is a complex multistage process essential for growth, cell maintenance and energy[60]. In this respect, various colloidal aspects of protein digestibility have been interrogated in the past[61]. Furthermore, protein gastro-duodenal proteolysis is believed to be a key step in liberating encrypted peptides which may carry extra-nutritional benefits and a myriad of bioactivities[3, 62]. In this part of the work native α-la, controls and MRPs were subjected to a semi-dynamic *in vitro* gastro-duodenal model recreating the physiology of a healthy adult. Previous studies have established that α-la is rapidly degraded by pepsin within 30 sec to 2 min but slowly digested by trypsin either in pure form or in whole whey[13, 21, 63]. SDS-PAGE analysis of digesta collected during semi-dynamic *in vitro* digestion (**Figure 3**), further established this trend and also provided a look into the differential degradation of the various α-la-based MRPs. Control experiments (data not shown) demonstrated that thermal processing did not markedly alter α-la digestion patterns. As can be seen in **Figures 3B-E**, Maillard-induced conjugation with fructose or FOS resulted in increased persistence of peptides (up to ~8 kDa) during digestion compared to the native protein. While fructose conjugates (**Figures 3B and 3C**) presented a ~16 kDa fraction that resisted digestion for 90 min compared to 66 min in the native protein, this was not the case in FOS MRPs. It is important to note that some peptic fragments evaded even 60 min of duodenal digestion, thus, raising their bioaccessibility and the possibility to confer health benefits, such as radical scavenging. Again, the observations of these experiments could be the outcome of competing effects of protein steric hindrance versus unfolding that may hinder or promote the availability of enzymatic cleavage sites, as detailed previously[30].

3.4 Evaluation of MRP digestive fate in an infant gastro-duodenal model

Infants are a sensitive population whose nutritional needs necessitate high intake of protein. The bioaccessibility of antimicrobial peptides and the essential amino acids Trp and Cys during digestion have led to enrichment of infant formulas with α-la[13, 18]. The current study also tested the proteolysis of α-la and its MRPs in an infant model as such MRPs may unintentionally form during infant formula production. This was inspired by a recent study showing Maillard conjugation could alter the formation of bioactive peptides during infant digestion[30]. In this part of the work, a relevant infant digestion model was employed to monitor the progression of proteolysis by SDS-PAGE (**Figures 4 and 5**). First, these experiments demonstrated that native α-la persisted longer under infant digestion conditions (**Figure 4A**) and diverged from that occurring in the adult model (**Figure 3A**). These experiments also revealed a distinct breakdown pattern of peptides that endured longer periods of infant digestion across a vast range of

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MW. Similar findings were also found during *in vitro* digestion of other proteins such as lactoferrin, β-casein and ovalbumin[22, 30, 64]. Interestingly, results showed thermal processing did affect the peptic breakdown profile of α-la in the infant gut unlike the negligible effect observed in the adult model. These differences probably arise from alterations in the physiological parameters varying between adults and infants[22, 30]. Heating for up to 24 h led to accelerated dissipation of large ≥ 55 kDa fragments, persistence of intermediate fragments ~25 kDa < MW < ~35 kDa and earlier formation of small peptides ≤ 10 kDa (60 min compared to 120 min in adult model) in comparison with the native protein. Extensive heating up to 36 h resulted in the same pattern for intermediate fragments, but in delayed dissipation of large and small fragments. The Maillard reaction between α-la- and fructose was found to hinder proteolysis as expressed across the whole peptic breakdown profile (**Figures 5A and 5B**), i.e. fragments exhibited delayed proteolysis compared to the native protein. Interestingly, these fragments endured for more than 60 min of duodenal digestion; thus, raising the possibility of peptide fractions persisting further down the digestive tract and perhaps even reaching the large intestine. Overall, glycation through the Maillard reaction was found to have two contradicting effects as demonstrated in **Figures 3 and 5** and as suggested by others as well[28, 59, 65, 66]. On one hand, glycation may interfere with refolding of the protein leading to structural changes exposing new enzymatic cleavage sites. On the other hand, conjugation is accompanied by covalent binding of a carbohydrate moiety to the protein backbone thereby limiting enzymatic accessibility through steric hindrance. These two counteracting effects could explain the different digestive patterns of the various MRPs. In contrast to fructose based conjugates, α-la-FOS MRPs heated for 12 h (**Figure 5C**) had an apparent delayed proteolysis of the breakdown peptides compared to the native protein, while increasing heating duration to 24 h (**Figure 5D**) resulted in an opposite effect whereby proteolysis of breakdown peptides was accelerated. Interestingly, a similar impact was also recently reported for lactoferrin-FOS MRPs digested in an *in vitro* static gastro-duodenal infant model[30]. Altogether, glycation via the Maillard reaction was shown to alter protein's digestibility to varying extents as a consequence of controllable thermal processing. This concurs with the known effects of thermal processing on the nutritional value of alimentary proteins[67, 68]. Yet, the observation of this study further raises the need to better the understanding of the possible ramifications of heating and Maillard-type glycation on the proteolytic breakdown of MRPs if the rational design of protein digestibility and delivery of bioactive peptides is to be sought.

3.5 Evaluating the antioxidant capacity of Maillard conjugates throughout digestion

To date, many studies have focused on evaluating the antioxidant activity of proteins in relation to food's shelf life overlooking its potential antioxidant capacity during and as a consequence of gastrointestinal digestion. Milk proteins exert their antioxidant activity either directly or after degradation to different peptides[25]. In this part of the work, samples collected during the *in vitro* gastro-duodenal adult and infant digestions were analyzed using the DPPH assay to evaluate their antioxidant capacity under simulated gastric and duodenal conditions (**Figure 6**). Peptides possessing antioxidant capability are generally small in size, with MW not exceeding 3 kDa[13], therefore, DPPH radical scavenging assay was found to be the most suitable for such small peptides and amino acids. First, analyses revealed that adult (**Figure 6A**) gastric digesta possessed significantly ($p<0.05$) higher antioxidant capacity compared to samples collected from simulated infant digestion (**Figure 6B**), excluding FOS based conjugates. This pattern could be explained by the rapid pepsinolysis portrayed in adult digestion compared to infant digestion (**Figures 3, 4 and 5**) which led to the generation of antioxidant peptides. Although FOS based conjugates were also rapidly digested under adult conditions, no significant difference was found in antioxidant capacity between adult and infant gastric digesta. In the end of the duodenal phase, there was also no significant difference in control samples between adult and infant. However, a significant ($p<0.05$) increase in antioxidant capacity was observed in conjugate samples of infant digesta vs adult digesta. This could be attributed to different enzymatic cleavage patterns in MRPs compared to controls, as previously shown for lactoferrin[30]. In light of the limitations of *in vitro* methods, further work is needed to confirm these findings *in vivo*. In adult digestion (**Figure 6A**), a significant ($p<0.05$) decrease was observed in antioxidant activity after 10 min of gastric proteolysis compared to G0 with no significant changes in the later stages of the simulated digestion. Interestingly, infant digesta of MRPs (**Figure 6B**) exhibited a significant ($p<0.05$) increase in antioxidant capacity under duodenal conditions, with fructose based conjugates also possessing a significantly higher antioxidant capacity at the end of the gastric phase compared to G60. This pattern could be explained by the subtle pepsinolysis in the infant gastric phase resulting in sustained release of peptides where enzymes liberated encrypted antioxidant peptides. Based on SDS-PAGE of digesta (**Figures 4 and 5**), it may be hypothesized that these peptides originate from the intermediate fraction (25-35 kDa) which was broken down in fructose MRPs starting from G180 and in FOS MRPs in the duodenal phase. This is in accordance with previous studies suggesting that peptides generated from the digestion of milk proteins possess antioxidant activities[25, 69]. Antioxidant milk-derived peptides are usually composed of 5 to 11 amino acids including the hydrophobic amino acids Pro, His, Tyr, or Trp in the sequence[25], which could serve as hydrogen donors. Despite the short sequence of α-la (123 residues), it is composed of numerous aromatic amino acid residues including 4 Phe, 4 Tyr and 4 Trp which increase its potential to produce antioxidative peptides[70]. The generation of antioxidant peptides is highly dependent on proteases' ability to cleave specific peptide bonds thereby releasing the specific bioactive peptides. Thus, altered proteolysis would very well explain the observed differences in antioxidant capacity noted in the various digesta samples. Overall, these results suggest

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that the Maillard reaction could not only improve the antioxidant activity of α-la but also its antioxidant capacity throughout digestion in the stomach and duodenum. In turn, liberation of antioxidant peptides in the lumen could interfere with oxidative stress therein. Moreover, studies characterizing the antioxidant peptides and *in vivo* experimentsare needed to ascertain this possible beneficial effect and to deepen our understanding of antioxidant peptide formation in the gastrointestinal tract.

Conclusions

This study has shown that Maillard induced glycation with fructose or FOS alters α-la's physiochemical properties to different extents depending on the carbohydrate moiety type. Functionally, conjugation was found to increase α-la's inherent antioxidant capacity in terms of ferric reducing power. Thermal processing also increased α-la's surface hydrophobicity which in turn decreased its colloidal stability to pH. These changes were also expressed in changes in α-la's susceptibility to digestive proteolysis with varying effects noted under conditions mirroring the digestive functions of adults and infants. Under simulated adult digestive conditions, glycation delayed protein degradation and therefore enabled some peptic fragments to evade even 1h of duodenal digestion. Glycation was found to have a more pronounced impact on gastro-duodenal proteolysis of α-la under infant conditions. In light of these findings, it is suggested that Maillard glycation of α-la could increase the bioaccessibility of peptides in the human GIT and especially in infants thereby amplifying the ability to confer health benefits. Specifically, this study demonstrated that thermally induced Maillard conjugation with FOS had the most pronounced effect on the antioxidant profile of the peptic fragments during *in vitro* infant digestion experiments. If so, Maillard conjugation of α-la could be harnessed to intervene with the oxidative reactions occurring in the gut lumen. Nevertheless, further research is needed to ascertain this notion, mine digesta for antioxidant peptides and assess any possible ramifications downstream, e.g. on the colon microbiome composition and metabolism as well as challenging these postulates in *in vivo* models.

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Notes and References

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Figure captions

Figure 1. Changes in α-la physiochemical properties arising from Maillard processing with fructose or FOS under water restricted conditions. (A) UV absorbance at 305 nm of 0.2% (w/w) control and conjugate solutions ($p<0.05$). (B) SDS-PAGE stained with Coomassie Brilliant Blue R-250); M, Marker; 1, Native α-la; 2, α-la 12 h; 3, α-la 24 h; 4, α-la 36 h; 5, α-la-Fru 12 h; 6, α-la-Fru 36 h; 7, α-la-FOS 12 h; 8, α-la-FOS 24 h. (C) CIELAB color index (ΔE) plot of color development of control samples and conjugates ($p<0.05$).

Figure 2. Effect of Maillard reaction with fructose or FOS on α-la functionalities. (A) Colloid size responsiveness to pH (determined by dynamic light scattering); a, Native α-la; b, α-la 12 h; c, α-la 24 h; d, α-la 36 h; e, α-la-Fru 12 h; f, α-la-Fru 36 h; g, α-la-FOS 12 h; h, α-la-FOS 24 h. (B) Surface hydrophobicity (H0) values ($p<0.05$). (C) Antioxidant capacity measured by the FRAP assay ($p<0.05$).

Figure 3. SDS-PAGE of digesta following *in vitro* adult gastric digestion. (A) Native α-la. (B) α-la-Fru 12 h. (C) α-la-Fru 36 h. (D) α-la-FOS 12 h. (E) α-la-FOS 24 h; 1, G0; 2, G1; 3, G5; 4, G10; 5, G30; 6, G60; 7, D3; 8, D6; 9, D30; 10, D60.

Figure 4. SDS-PAGE of control samples digesta following *in vitro* infant gastro-duodenal digestion of (A) Native α-la. (B) α-la 12 h. (C) α-la 24 h. (D) α-la 36 h; 1, G0; 2, G5; 3, G10; 4, G60; 5, G120; 6, G180; 7, D3; 8, D6; 9, D30; 10, D60.

Figure 5. SDS-PAGE of α-la based conjugates digesta following *in vitro* infant gastro-duodenal digestion of (A) α-la-Fru 12 h. (B) α-la-Fru 36 h. (C) α-la-FOS 12 h. (D) α-la-FOS 24 h; 1, G0; 2, G5; 3, G10; 4, G60; 5, G120; 6, G180; 7, D3; 8, D6; 9, D30; 10, D60.

Figure 6. Impact of the Maillard reaction on the antioxidant capacity profile obtained through the DPPH assay of digesta samples following *in vitro* gastro-duodenal digestion of (A) Adult. (B) Infant.

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Figure 1.

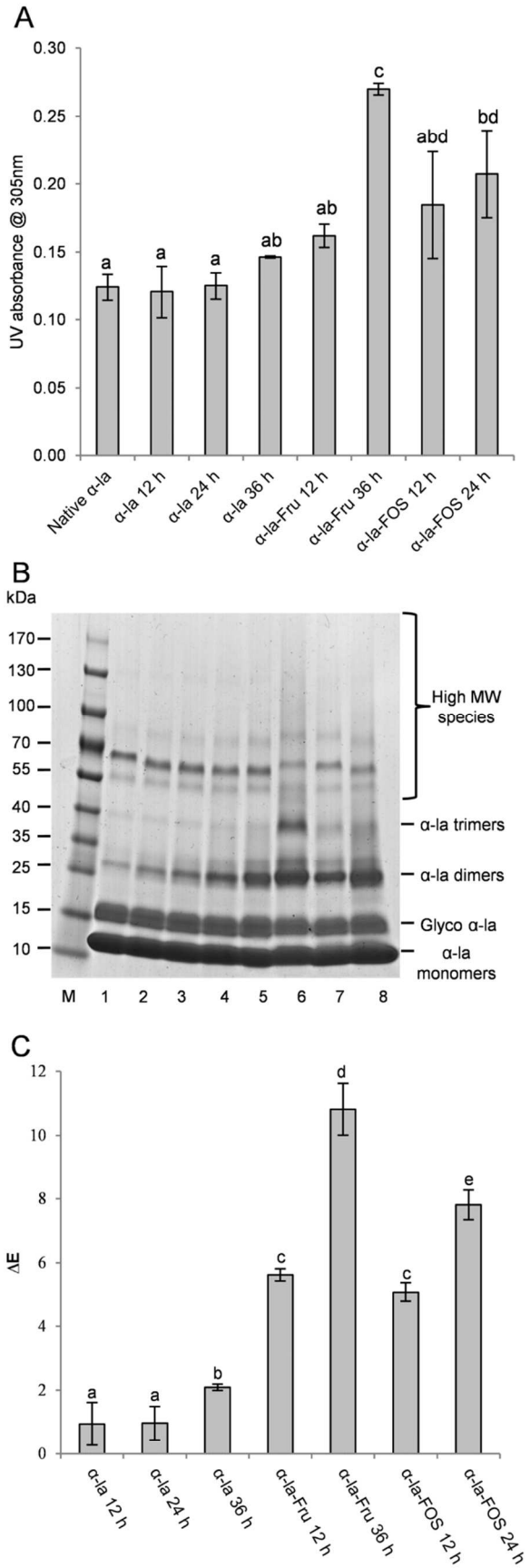

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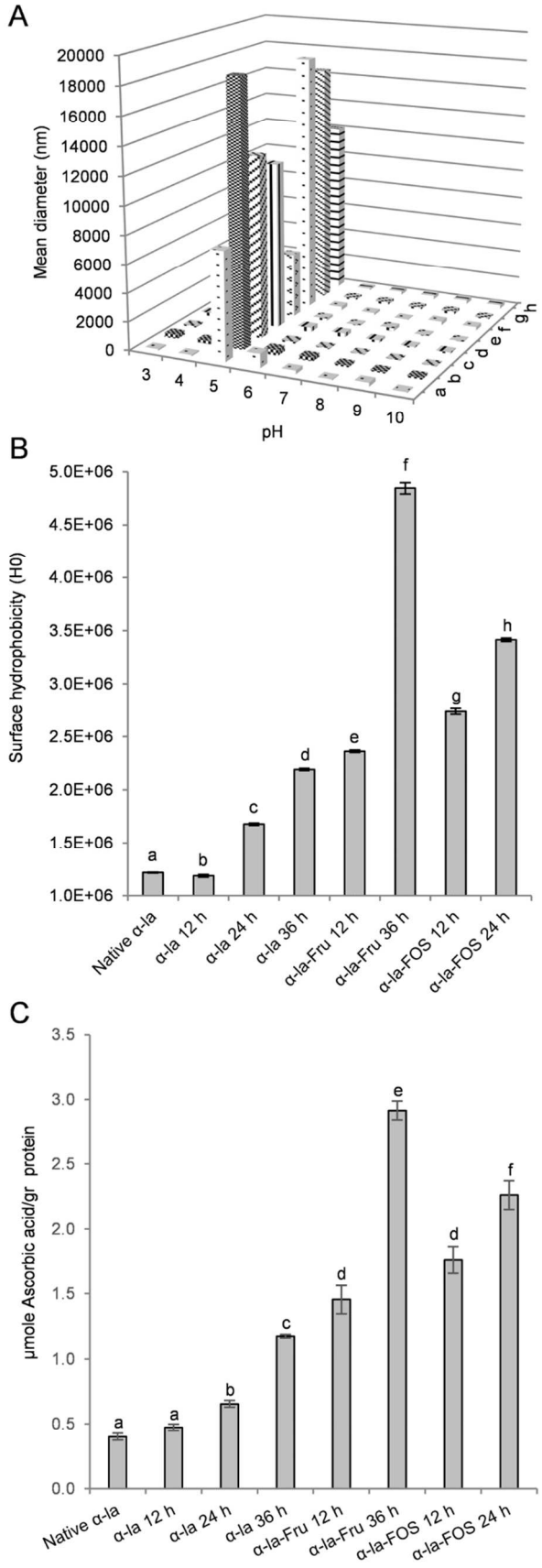
Figure 2.



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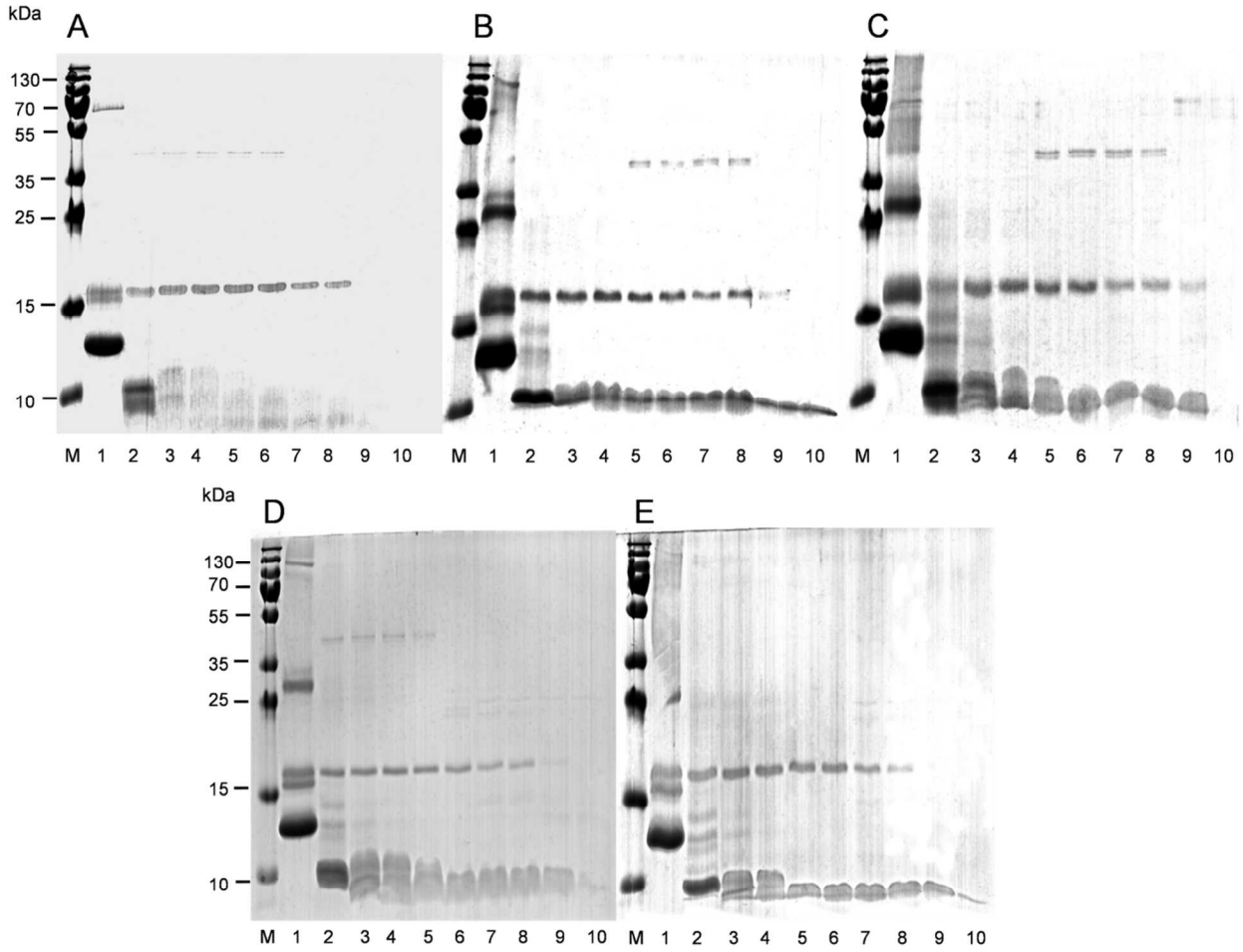

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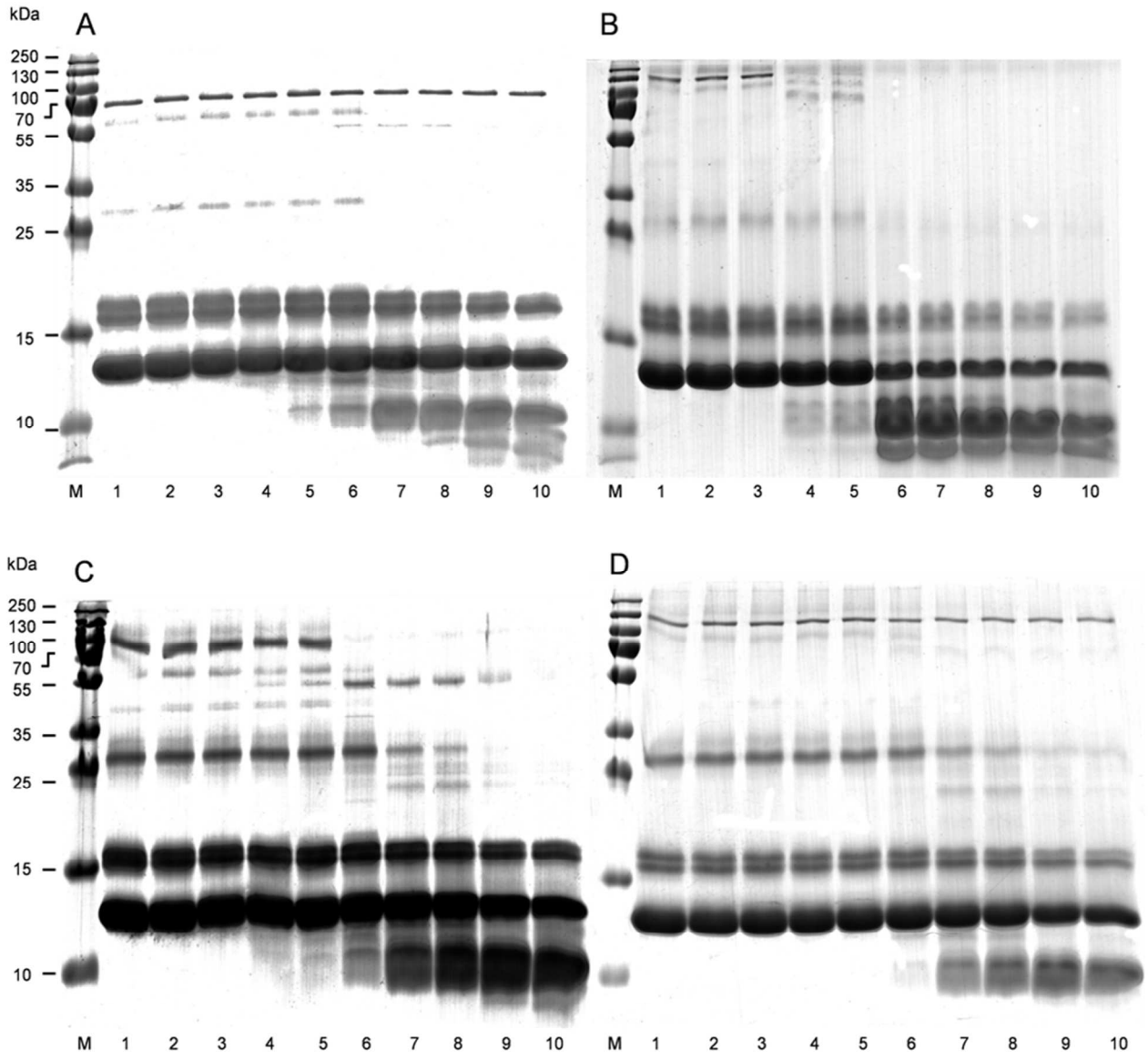

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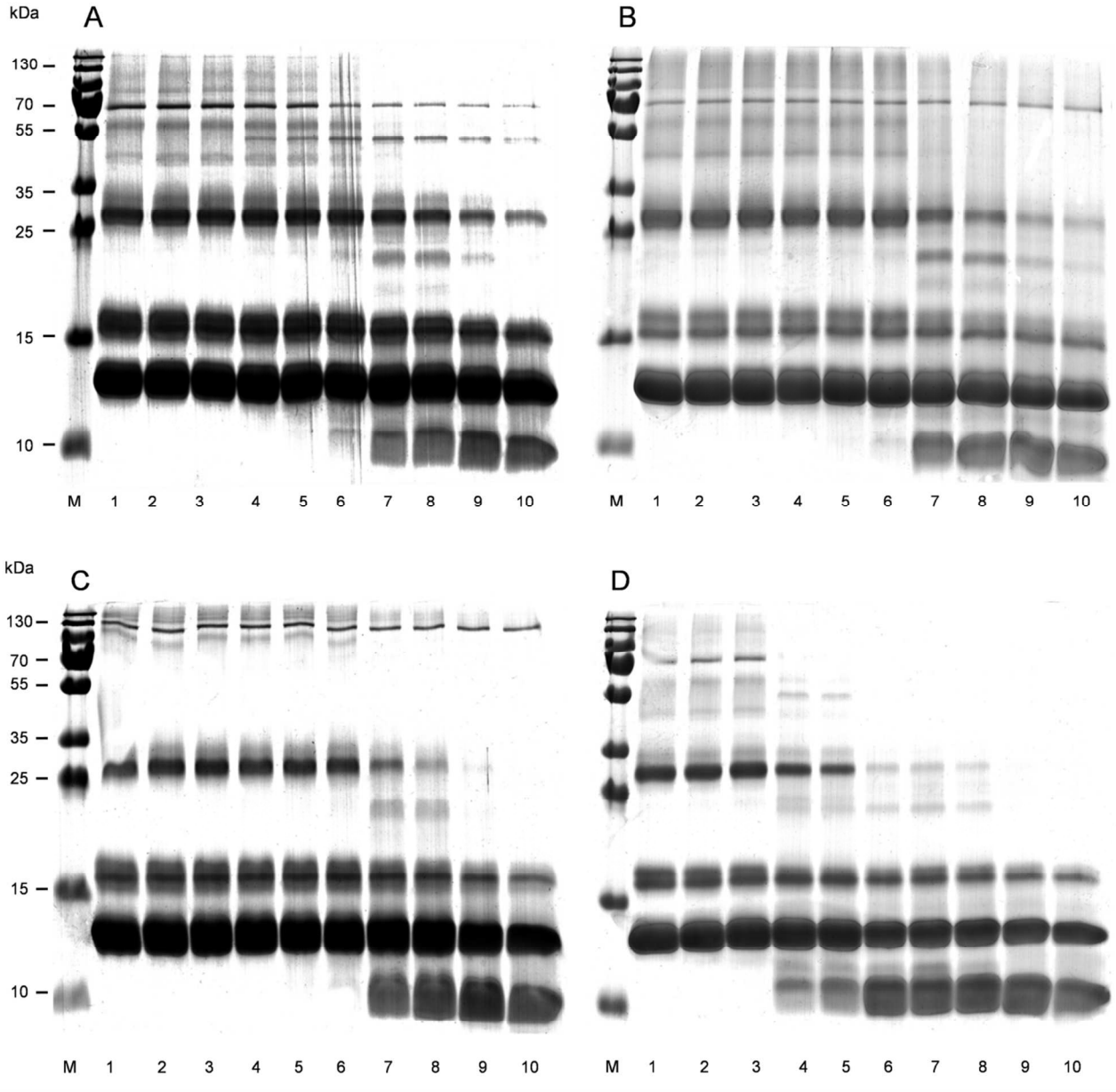

Figure 5.

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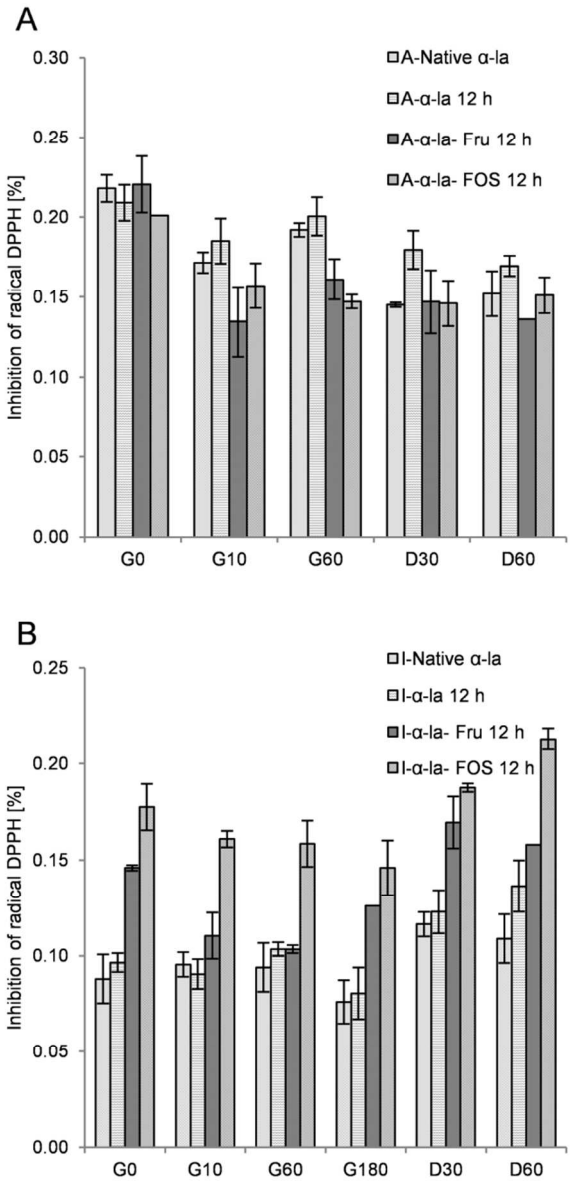

Figure 6.

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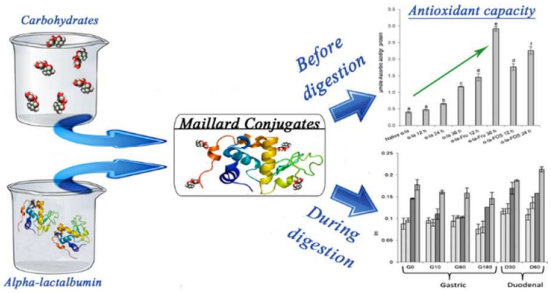
Graphical Abstract



Maillard conjugation alters alpha-lactalbumin antioxidant capacity including during in vitro digestion with different effects in adults versus infants

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