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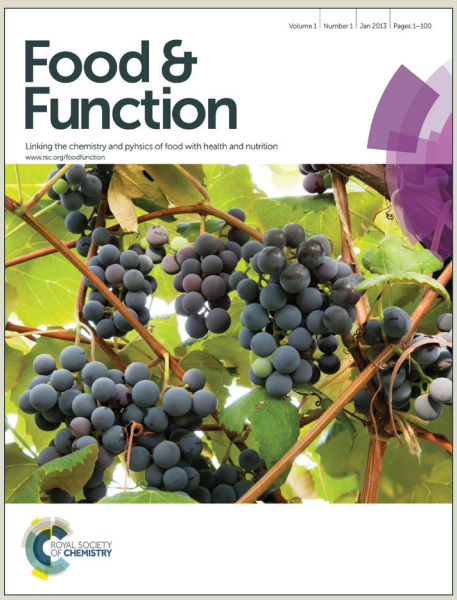

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ARTICLE

Impact of the Maillard Reaction on the *In Vitro* Proteolytic Breakdown of Bovine Lactoferrin in Adults and Infants

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Alice M. Moscovici,[a] Yousef Joubran, [a] Valerie Briard-Bion,[b] Alan Mackie, [c] Didier Dupont[b] and Uri Lesmes[*a]

The Maillard reaction has been proposed as a natural pathway to functionalize proteins and modulate their proteolysis. Nevertheless, gaps in understanding the digestive fate of Maillard reaction products (MRPs) still exist, especially regarding bioactive proteins as lactoferrin (LF). UV absorbance and SDS-PAGE were used to monitor reaction progression under mild thermal processing (60 °C, 79 %RH). Dynamic light scattering showed MRPs had increased colloidal size and turbidity at 3<pH<10. FRAP analysis and *in vitro* digestion experiments demonstrated MRPs possessed improved antioxidant capacity and higher susceptibility to proteolysis to varying extents under adult conditions compared to infant conditions. Proteomic analyses of MRP digesta revealed altered enzymatic cleavage patterns with no pronounced changes in the formation of known bioactive peptides. These also indicated that MRPs may breakdown in the gastro-intestinal tract to potentially form novel bioactive peptides. Overall, this work highlights the Maillard reaction could be harnessed to modify proteolysis extent and bioactivity of proteins.

1. Introduction

Nutrition exerts one of the strongest life-long environmental impacts on human health. This realization led to a demand for food products that promote and improve health and well-being as well as to various attempts to tailor products to consumer needs. In this respect, there has been an upsurge in research regarding the diverse health effects of alimentary proteins which surpass their mere nutritional value[1, 2]. Specifically, milk proteins such as β-lactoglobulin, α-lactalbumin and lactoferrin (LF) have been increasingly recognized as viable rich sources for bioactive peptides that may be liberated by human digestive enzymes. These bioactive peptides possess a myriad of beneficial biological properties, including antimicrobial, antioxidant, antihypertensive and immune-modulatory activities[1, 3-5]. Until recently, analysis of the digestive fate of proteinaceous compounds and the identification of peptides generated during digestion was a considerable scientific and technological challenge. Nevertheless, recent advances in proteomics and bioinformatics have been applied in the field of food science, offering opportunities to comprehensively tackle the challenges of elucidating the digestive fate of proteins[6-9]. Concomitantly, food professionals as well as consumers express tremendous interest in the implications of food processing on functionality, digestibility and ramifications to health. To this end, it is well-established that thermal processing of protein-carbohydrate mixtures may result even unintentionally in non-enzymatic browning, also known as the Maillard reaction[10, 11]. Maillard reaction products (MRPs) are responsible for the characteristic color and aroma of many cooked foods like bread, and are essential to the sensory properties of various food products. Studies have shown that MRPs may lead to both beneficial and deleterious implications, including higher solubility, surface activity and antioxidant capacity, but also to reduced nutritional value and formation of food contaminants such as furans[11-14]. For example, bovine LF was recently shown to be functionalized through the Maillard reaction using fructose and glucose culminating in enhanced antioxidant capacity of this bioactive protein[15]. The digestive

fate of MRPs has also been the focus of numerous studies concerning potential risks to consumer health, as reviewed by others[12, 16, 17]. In terms of digestibility, some studies indicate MRPs have reduced susceptibility to proteolysis attributed to steric hindrance caused by the carbohydrate moiety, allowing some fractions to evade digestion in the upper gastrointestinal tract (GIT) and rendering them available for bacterial fermentation in the colon[17-20]. Other studies suggest that modifications in the three-dimensional structure of proteins arising from Maillard conjugation, such as unfolding, expose cleavage sites and potentiate proteolysis[21, 22]. Thus, the Maillard reaction can either increase or decrease protein digestibility through changes in protein properties, as illustrated in **Figure 1**. Nevertheless, gaps in understanding the digestion of MRPs still exist, particularly as it pertains to bioactive proteins such as LF. This 80 kDa milk glycoprotein has lately been at the heart of various studies and industrial applications due to its diverse beneficial properties and biological functions[3, 23-26]. Furthermore, LF has attracted much attention due to its exceptionally high concentration in human milk and impact on the developing neonate, instigating its supplementation to infant formulas[1, 26-31]. This unique milk protein was also documented as a precursor for bioactive peptides which can form during digestion, including the antimicrobial peptides lactoferricin B and lactoferrampin, as well as bifidogenic peptides[1, 32-36]. In terms of LF susceptibility to digestion, contradicting results have been reported by *in vitro* and *in vivo* studies; some *in vivo* experiments have demonstrated partial LF degradation in the adult GIT, and even detected LF fragments containing lactoferricin in the feces of mice or gastric contents in men[33, 37, 38]. On the other hand, other *in vitro* and *in vivo* studies did not identify intact LF or lactoferricin to successfully evade gastric digestion[7, 39]. Moreover, LF and other milk proteins have been shown to differ in their digestive fate in adults versus infants[39-41]. To the best of our knowledge, age-dependent digestive variances have yet to be systematically applied to investigate the digestibility of MRPs with proteomic analyses, and specifically relating to bioactive peptides. Thus, the aim of this study was to examine the impact of Maillard reaction on the digestive fate of bovine LF and various LF-based MRPs in adults and infants. Particular emphasis was drawn to MRPs formed between bioactive LF and fructo-oligosacchrides (FOS) due to their high likelihood to be applicable to infant formulas, which are thermally processed and thus the Maillard reaction may occur even unintentionally during their production[13, 30, 42, 43]. Hence, the focus of the proteomic aspect of this study was on the digestive fate of LF-FOS MRPs in infants under the hypothesis that such MRPs would have modulated susceptibility to proteolysis and consequently altered peptide profiles.

2. Materials and Methods

2.1 Materials

Food grade bovine lactoferrin (Vivinal lactoferrin FD, 95.6% protein) (LF) was kindly donated by FrieslandCampina (Delhi, NY), and oligofructose (Orafti®P95, 95% oligofructose) (FOS) was kindly donated by BENEO (Universal Network LTD, Rosh HaAyin, Israel). All other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared with deionized water (DW) and all reagents were of analytical grade.

2.2 Preparation of Maillard reaction products (MRPs)

Maillard conjugates were prepared by mixing LF with glucose, fructose or FOS. Monosaccharide MRPs (glucose or fructose) were produced at a mole ratio of LF:monosaccharide 1:1 as previously described[15]. LF-FOS MRPs were produced in a similar manner, only they were dissolved in 10 mM pH 7.0 phosphate buffer (PB) at a practical 3:1 w/w ratio, respectively, to better simulate a food product formulation. After dissolution and freeze drying[15], LF-FOS MRPs were incubated at 60 °C under water restricted environment (79 %RH over saturated KBr) for periods of 12 or 24 h. Control samples were similarly produced but without being incubated (denoted t=0). Heated mixtures were termed as Maillard reaction products (MRPs) and abbreviated as LF-glucose, LF-fructose and LF-FOS with numerical indices denoting the heating duration. For example, a mixture of LF and FOS heated for 12 h was indexed as LF-FOS 12h.

2.3 Characterization of MRP physicochemical properties

It is well established that the Maillard reaction alters protein physicochemical and functional properties[11], thus MRPs were characterized in terms of ultraviolet (UV) absorbance, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), responsiveness to pH as it pertains to mean particle size and turbidity, as well as antioxidant capacity. The findings described in this part of the work are focused on LF-FOS MRPs, as the data for MRPs of LF-glucose or LF-fructose were described in detail in a recent publication[15].

2.3.1 UV absorbance

Initial evaluation of MRP formation was performed through a comparative analysis of UV absorbance. A full scan between 200-600 nm was performed, and the most significant differences were observed at 305 nm, similarly to previously described[15, 44]. The absorbance of 0.2% (w/w) solution samples loaded into quartz cuvettes was collected using a UV/visible spectrophotometer (OPTIZEN POP, MECASYS, Daejeon, Korea) with PB as a blank reference.

2.3.2 SDS-PAGE

MRP formation is known to affect the MW of proteins due to covalent binding of the carbohydrate moiety and/or possible cross-linking between protein molecules. Thus, SDS-PAGE was carried out with two different staining methods as previously described[15]. Briefly, electrophoresis was performed using a gradient gel (4-15% Mini-PROTEAN® TGX™ precast gel, Bio-Rad, Rishon LeZion, Israel) at 145 V for 1 h in Tris/glycine/SDS running buffer. Following electrophoresis, gels were stained for proteins and glycoproteins by Coomassie Brilliant Blue R-250 (Bio-Rad, Rishon

LeZion, Israel) and Pierce glycoprotein stain (Pierce Biotechnology, Rockford, IL), respectively. Gels were then scanned using a Microtek 9800XL Plus scanner (Microtek, Carson, CA).

2.3.3 Colloidal physicochemical responsiveness to pH

Mean particle size of LF-FOS MRPs as affected by pH (between 3.0 to 10.0) was monitored by dynamic light scattering (DLS) as previously described[15], only 0.2% (w/w) samples were dissolved in PB. Following size analysis, sample turbidity at 600 nm was also determined using a UV/visible spectrophotometer (OPTIZEN POP, MECASYS, Daejeon, Korea) and PB as a blank reference.

2.3.4 Antioxidant capacity in terms of ferric reducing power

The antioxidant capacity of samples was measured using the ferric reducing/antioxidant power (FRAP) assay, as previously described[15, 45]. Briefly, 200 µL of freshly prepared FRAP reagent (mixture of 1 mL of 10 mM 2,4,6-*tris*(2-pyridyl)-*s*-triazine (TPTZ), 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, 2.4 mL DW and 10 mL of 300 mM sodium acetate, pH 3.6) were mixed with 10 µL sample solution. Sample aliquots were transferred to a 96-well plate used to record the absorbance at 593 nm after 10 min by a microplate reader (OptiMax^{TM} microplate reader, Siloam Biosciences Inc., Cincinnati, OH). Data was compared to a calibration curve produced with 0-1000 µM ascorbic acid and results were expressed as triplicate means in µmol equivalents of ascorbic acid per gram protein.

2.4 Digestive fate of MRPs

In order to compare the potential digestive fate of LF and its MRPs, adult and infant *in vitro* digestion models were applied. Both models applied are simple and static yet are widely used as high-throughput tools designed to evaluate the digestibility of proteins[46].

2.4.1 In vitro adult gastric digestion

A 10 mg sample was dissolved in 9.8 mL of a simulated gastric fluid (SGF) (0.15 M NaCl, pH 2.5), pH was adjusted to 2.5 using 0.5 M HCl and volume was adjusted to 9.9 mL before starting a 60 min simulated gastric digestion, as described previously[40, 47, 48]. Briefly, gastric proteolysis was performed after incubation at 37 °C for 10 min, 100 µL of porcine gastric mucosa pepsin solution (pre-dissolved in SGF 0.15 M, pH 2.5) were added to reach a final concentration of 170 U pepsin per mg of protein in the digestion mix. Digesta aliquots were withdrawn before pepsin addition, immediately after pepsin addition, 1 min after pepsin addition and so on until 60 min after pepsin addition, and were denoted: $G0^-$, $G0^+$, G1, G2 … and G60, respectively. Samples were stored at -20 °C until further analysis.

2.4.2 In vitro infant gastro-duodenal digestion

Following an adapted protocol of Dupont et al.[49], a 10 mg sample was dissolved in 9.8 mL of a simulated duodenal fluid (SDF) (0.15 M NaCl, pH 6.5), pH was adjusted to 3.0 using 0.5 M HCl and volume was adjusted to 9.9 mL. Gastric proteolysis was performed after incubation at 37 °C for 10 min, 100 µL of porcine gastric mucosa pepsin solution (pre-dissolved in SGF 0.15 M, pH 2.5) were added to reach a final concentration of 22.75 U pepsin per mg of protein in the digestion mix. Similarly to the adult gastric model, digesta aliquots were denoted: $G0^-$, $G0^+$, G1, G2 … and G60. Chyme acquired after 60 min of *in vitro* gastric digestion (5 mL) was used as the starting material for duodenal digestion after an inactivation step in which sample pH was elevated to 7.5 (using 1 M and 0.1 M NaOH) and maintained for 10 min. Before duodenal proteolysis, the pH of inactivated gastric contents was adjusted to 6.5 through controlled addition of 0.1 M NaOH and 0.275 mL of pH 6.5 0.5 M Bis-Tris buffer and 0.125 mL of bile salts solution (sodium taurocholate and sodium glycodeoxycholate). After incubation at 37 °C for 10 min, 50 µL of porcine trypsin and 50 µL of porcine α-chymotrypsin solutions (pre-dissolved in SDF 0.15 M, pH 6.5) were added to reach final concentrations as follows: 1 mM sodium taurocholate, 1 mM sodium glycodeoxycholate, 3.45 U trypsin per mg of LF and 0.04 U α-chymotrypsin per mg of LF. Samples were placed in an orbital shaking incubator (37 °C, 170 rpm) and aliquots of 0.2 mL were withdrawn before the addition of enzymes, immediately after their addition, and 1, 2, 5, 10, 15 and 30 min after the addition of enzymes. Aliquots were mixed with 20 µL of 0.05 mg/mL Bowman-Birk inhibitor (BBI) and placed on ice to inactivate the enzymes. Duodenal digesta samples were denoted similarly to gastric samples, only with "D" instead of "G" (for example: D1 indicating a sample taken in the duodenal phase 1 min after addition of enzymes). All digestion samples were stored at -20 °C until further analysis.

2.4.3 Monitoring proteolysis products by SDS-PAGE

Comparison of peptide breakdown profiles in digesta samples was based on SDS-PAGE described herein and in previous work[47, 48, 50].

2.4.4 Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of infant digesta

Samples collected at the end of infant gastro-duodenal digestion were analyzed by a hybrid quadrupole time of flight (qTOF) MS QStar XL (MDS Sciex, Toronto, Canada) in order to identify the peptides remaining after digestion. The peptide fraction (10 µL) was trapped onto a micro-pre-column cartridge C18 PepMap 100 (300 µm i.d.x5 mm, Dionex) before separation of peptides onto a column C18 PepMap 100 (75 µm i.d.x150 mm, Dionex). The separation at a flow rate of 300 nL/min started with 5% solvent B (95% acetonitrile, 0.08% formic acid and 0.01% trifluoroacetic acid in LC-grade water) and 95% solvent A (2% acetonitrile, 0.08% formic acid and 0.01% trifluoroacetic acid in LC-grade water) for 5 min. A linear gradient from 5 to 50% of solvent B and from 95 to 50% of solvent A for 75 min was followed by a linear gradient from 50 to 85% solvent B and from 50 to 15% solvent A for 7 min, with end conditions maintained for 8 min. Another linear gradient from 85 to 3% solvent B and from 15 to 97% solvent A was performed for 1 min, with end conditions maintained for 4 min, followed by return to initial conditions which were maintained for another 35 min. The online separated peptides were analyzed by electrospray ionization qTOF in positive ion mode. An optimized voltage of 3.0 kV was applied to the nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). MS and MS/MS data were acquired in continuum mode. Data-direct analysis was employed to perform MS/MS analysis on 1+ to 4+ charged precursor ions. Precursor selection was based upon ion intensity,

charge state and if the precursors had been previously selected for fragmentation they were excluded for the rest of the analysis. Spectra were collected in the selected mass range 300-2000 m/z for MS spectra and 60-2000 m/z for MS/MS. The mass spectrometer was operated in data-dependant mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software (Applied Biosystems, Framingham, MA) when the intensity of the ions was above ten cps. To identify peptides, all data (MS and MS/MS) were submitted to MASCOT (v.2.2, Matrix Science, London, UK). The search was performed against a homemade database dealing with major milk proteins which represents a portion of the Swissprot database (http://www.expasy.org). No specific enzyme cleavage was used and the peptide mass tolerance was set to 0.2 Da for MS and MS/MS. For each peptide identified, a p-value<0.05 was considered as a prerequisite for peptide validation with a high degree of confidence.

The homology percentage between a peptide identified in the sample and a reported peptide was calculated as follows: complete identity was ranked as 100%; if the identified peptide sequence was overlapping or a proper subset of the reported peptide sequence, the number of overlapping amino acids was divided by the total length of the reported peptide; and if the reported peptide sequence was a proper subset of the identified peptide sequence, the number of overlapping amino acids was divided by the total length of the identified peptide.

2.5 Statistical analyses

Experiments were carried out in triplicates and results are presented as the calculated mean and standard deviation. Digestion experiments were performed in duplicates on two separate occasions. Statistical analyses were performed using Microsoft Excel 2010 data analysis toolpack and relied on t-tests assuming equal variances.

3. Results and Discussion

3.1 Fabrication and characterization of LF-based Maillard conjugates

This work focused on performing the Maillard reaction under water restricted conditions, and the resulting samples were subjected to UV, SDS-PAGE, size, solution turbidity and antioxidant capacity analyses in order to evaluate the extent of conjugation and some of its implications on protein functionality. The covalent binding of proteins and carbohydrates through the Maillard reaction has been documented to affect protein physicochemistry, namely expressed in parameters such as MW, charge, altered tertiary structure etc.[11]. Initial experiments of this study drew on past work[15, 44] showing that UV absorbance and SDS-PAGE can indicate the changes induced by thermal processing of protein-carbohydrate mixtures. Corresponding findings gathered for MRPs formed under controlled conditions (60 °C, 79 %RH) are given in **Figure 2**. These findings pointed out that the different processing of LF-FOS mixtures altered not only the UV absorbance at 305 nm of the protein (**Figure 2A**) but also its ability to migrate within an SDS-PAGE gel, as higher MW bands and smears were observed (marked in red at **Figure 2B**). The latter indicated the formation of species with MW exceeding that of the native protein (~80 kDa) and a mixture of products which were identified as glycated proteins using a specific glycoprotein stain (marked in red at **Figure 2C**). It is important to note that due to the relatively low MW of the FOS used (a mixture of degree of polymerization (DP) mainly between 2 and 8) and under the controlled conditions employed for MRP fabrication[15], one can assume that some LF was mildly glycated. In this case, the ability to resolve the mildly glycated LF from the unglycated fraction by SDS-PAGE is limited, thus the band at ~80 kDa in MRP samples may also contain conjugates. Therefore, analyses corroborated the occurrence of the Maillard reaction and were found to be in agreement with a recently published work on similarly produced LF-MRPs[15]. Application of advanced structural characterizations, i.e. Fourier Transform Infrared spectroscopy (FTIR) and circular dichroism (CD) (data not shown) like those performed recently[15] did not reveal any noteworthy changes, further suggesting Maillard reaction occurred to a limited extent. Physicochemical characterization of heated lactoferrin in the absence of carbohydrates did not reveal any noteworthy changes (data not shown), further supporting that physicochemical properties were modified as a consequence of the glycation process.

Additional characterization of MRPs was performed to evaluate their colloidal functionality, as this property is of applicative importance. Thus, pH responsiveness of MRPs and their antioxidant capacity were evaluated by DLS, spectrophotometer and FRAP assay, with results summarized in **Figure 3**. These experiments showed that thermal processing decreased the colloidal stability of the protein as expressed by the increased particle sizes (**Figure 3A**) and turbidity (**Figure 3B**) across the pH range tested. Particularly, extending heating duration from 12 h to 24 h led to more pronounced changes in the protein behavior, most notably around the apparent isoelectric point of native LF in the buffered system (pH=6)[51]; The characteristic isoelectric point of LF (pI~8.2) has shifted to pH=6 as a result of PB used as a solvent, an effect previously described in the literature[51]. Another important effect was the significant ($p<0.01$) enhancement of antioxidant capacity in MRPs compared to the inherent capacity of native LF in terms of ferric reducing power (**Figure 3C**). This beneficial outcome of LF functionalization through the Maillard reaction was recently described in detail with monosaccharides[15]. This result is also in agreement with a previous report regarding the increased antioxidant capacity of soy protein-FOS MRPs determined by oxygen radical absorbance capacity (ORAC) assay, based on hydrogen atom transfer[52]. Nevertheless, it is important to investigate other health-related effects of the Maillard reaction, such as protein digestibility, which was the focus of this paper.

3.2 Evaluation of MRP digestive fate in an adult gastric model

Protein absorption, distribution and bioactivity in the human body rely on gastro-intestinal proteolysis, with gastric proteolysis being a key step in protein digestion, affecting the generation of peptides that may modulate biological functions. In this part of the work LF and LF-based MRPs were subjected to an *in vitro* gastric model mimicking an adult stomach extensively used to study the digestive fate of proteins[40, 46, 53]. Proteolysis progression was monitored through SDS-PAGE of digesta aliquots collected at various time intervals. A previous study employing this *in vitro* adult gastric model has established the high susceptibility of bovine LF to gastric proteolysis in adults[48]. SDS-PAGE analysis of digesta further established this trend (data not shown) but also provided a look into the differential degradation of various LF-based MRPs (**Figure 4**). These findings demonstrate that Maillard-type conjugation modulated the susceptibility of LF to adult gastric conditions in certain cases. As can be seen in **Figures 4A** and **4C**, conjugation for 12 h with glucose or FOS resulted in rapid dissipation of the band at ~80 kDa, which was not observed immediately after pepsin addition. This indicated Maillard conjugation led to sensitization of the protein to proteolysis. One can speculate that such a phenomenon could arise from subtle structural changes such as partial unfolding induced by the conjugation of a carbohydrate moiety to the glycoprotein rendering it more susceptible to proteolysis, as illustrated in **Figure 1B** and suggested by others[21, 22]. In contrast, **Figures 4B** and **4D** demonstrate that in MRPs formed with fructose after 12 h or with FOS after 24 h, the LF band is observed for a longer period compared to the other MRPs (**Figures 4A** and **4C**) which concurs with the dissipation of native LF in control experiments and in previous reports[48]. Recent work has shown that under the restricted fabrication conditions, fructose advances the Maillard reaction to a lesser extent than glucose, possibly due to different electrophilicity[15]. This could be manifested in diminished impact on LF explaining the un-tempered digestibility. As for LF-FOS conjugates formed after 24 h, one can argue that the Maillard reaction may have two contradicting effects, elucidated in **Figure 1**. On one hand, conjugation can lead to structural changes exposing the polypeptide chain to enzymatic cleavage[21, 22] (**Figure 1B**). On the other hand, conjugation is accompanied by covalent binding of the carbohydrate moiety to the polypeptide backbone thereby limiting enzymatic accessibility through steric hindrance[18-20] (**Figure 1C**). The balance between these two counteracting effects could explain the differences between the digestibility of the various MRPs. The findings in **Figure 4** also provide evidence into the slightly different peptide breakdown patterns during the digestion of various LF-based MRPs, however, due to the relatively low variety of peptides observed, no significant differences could be concluded. The known bioactive peptides that may be generated during LF digestion[1, 33, 34], the potential use of LF in infant formula[25, 26, 30] and reports demonstrating the altered degradation of milk proteins in infants[39, 40], inspired the next section of this work.

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

In this section of the work the potential digestive fate of the LF-based MRPs was investigated using an *in vitro* gastro-duodenal model recreating the conditions of the infant gut[9, 40, 49]. In these experiments, digestion pH and enzymatic activity were adjusted to infant levels and SDS-PAGE of digesta aliquots was used to monitor the progression of proteolysis. Simulated infant digestion patterns of native LF and its monosacchride-based MRPs are presented in **Figure 5**. The findings in **Figure 5A** show native LF persisted longer in the infant gut compared to the adult counterpart[48] and also reveal a distinct breakdown pattern of peptides that endured longer periods of digestion. This delayed degradation in infants is in accordance with previous reports[40, 54], and is believed to arise from the unique physiological digestive conditions of the infant GIT. Interestingly, some peptide bands as those appearing at MW~14.4 kDa and ~3.5 kDa evaded even 30 min of duodenal digestion. Such fractions could persist into the small and perhaps even the large intestine where they may affect biological functions, e.g. antioxidant activity or antimicrobial activity. Furthermore, **Figures 5B** and **5C** provide evidence indicating that in infants, glucose did not markedly affect the digestive fate of LF in LF-glucose MRPs while fructose conjugation rendered the protein more liable to degradation. These effects arising from glucose and fructose conjugation were also observed to extend to the peptic breakdown patterns, in which peptides were found to be more readily degraded for MRPs formed using fructose compared to those formed with glucose. Additional experiments evaluated the gastro-duodenal breakdown of MRPs formed with FOS at different heating durations (**Figure 6**). MRPs formed after 12 h of thermal processing (**Figure 6A**) were found to have a protective effect on LF, as the ~80 kDa band evaded 10 min of gastric digestion compared to 1-5 min found for the corresponding LF-fructose MRPs or native form of LF. Differences were also noticed in the peptic breakdown patterns in comparison with digesta of LF-fructose MRPs or the native protein, which further supported this conclusion. Digestion of LF-FOS MRPs formed following extended thermal processing to 24 h (**Figure 6B**) revealed an increased susceptibility of LF in MRPs and increased proteolysis of its breakdown peptides. These results demonstrate that in LF-FOS MRPs, different heating duration led to an opposite effect on proteolytic breakdown, thus offering the possibility to tailor the desired digestibility by controlling production parameters. Overall, these experiments highlight that LF MRPs were differentially degraded under infant conditions yielding altered breakdown patterns in comparison with the results obtained for their digestibility in adults. These findings concur with the observation made by Dupont and co-workers[40], showing protein resistance to digestion and the products of proteolytic degradation can markedly differ between adults and infants owing to age-related physiological differences in GIT function.

Due to the bioactive nature of LF, LF-derived peptides and FOS, and the variability in peptide profiles during infant digestion of MRPs, advanced proteomic analyses were performed on digesta samples of LF-FOS collected at the end

of the duodenal phase. **Figure 7** shows a diagrammatic representation of the peptides remaining after gastro-duodenal infant digestion of native LF, LF-FOS 12h and LF-FOS 24h. Comparing the LC-MS/MS results revealed that samples differed in their enzymatic cleavage patterns, leading to altered peptide compositions. This is in accordance with the SDS-PAGE results (**Figures 5A** and **6**), and may arise from mild structural changes following Maillard conjugation, which blocked or exposed enzymatic cleavage sites[18-22]. Interestingly, mapping the peptides allowed once again demonstrating these two contradicting effects Maillard reaction may have on proteolysis, as explained earlier and illustrated in **Figure 1**. For example, the peptide (f501-520) was found after gastro-duodenal digestion of native LF and LF-FOS 12h, but was not identified in digested sample of LF-FOS 24h. Based on the known cleavage sites for pepsin, trypsin and chymotrypsin and the other peptides identified in this sample, it may be that prolonged heating for 24 h resulted in further conjugation which blocked a cleavage site. Arg is one of the major reactive amino acids in the Maillard reaction[11], thus conjugation in this position could have limited the enzymatic access to the cleavage site between Arg and Leu, resulting in the formation of a large fragment which was not detectable under the conditions used. In a different area, the peptide (f378-383) was identified in digesta of LF-FOS 24h, but not in digesta of native LF or LF-FOS 12h. In this case, one can postulate that further conjugation in LF-FOS 24h led to a structural modification which exposed a previously hidden enzymatic cleavage site. Nevertheless, other reasons can also explain the absence of certain peptides and should be considered, including a concentration of peptides lower than detection threshold as well as the use of a database which is based on LF and therefore limits the ability to identify conjugated fragments.

Peptide sequences in the various digesta samples were further compared to previously described LF-derived peptides (**Table 1**), based on a database of LF-derived bioactive peptides (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php) and a recent study which identified LF-derived peptides in aspirates following *in vivo* digestion of LF[7], and a homology percentage was calculated (as earlier described in section 2.4.4). All digesta samples contained peptides derived from the extensively studied antimicrobial peptides lactoferrampin and lactoferricin[32, 35], with homology of up to 71% and 52%, respectively (**Table 1**). As these peptide fragments were present in all samples, this result indicates that the Maillard reaction, in both heating durations examined, did not affect the cleavage patterns of these antimicrobial peptides. Although previous studies reported that both lactoferrampin and lactoferricin can be liberated by pepsin in the gastric phase[33, 36], the complete peptide sequences were not found in all samples. This is in accordance with a recent research which did not identify these peptides following *in vitro* and *in vivo* digestions of LF[7]. Other peptides overlapping with previously reported antiviral[55] and immuno-modulating[56] peptides were identified in all samples with homology as high as 89%. Moreover, all digesta samples consisted of six peptides with 100% homology with peptides identified in aspirates following *in vivo* digestion of LF[7]. These findings highlight the bio-relevance of the *in vitro* model applied in this study; however, it is important to note that the samples collected in the *in vivo* experiment were aspirated from adults, while digesta samples in this study were obtained from an infant gastro-duodenal digestion. Thus, in spite of the numerous identical peptides, most were different, in line with previous studies regarding the altered digestion of proteins in adults compared with infants[39, 40] and the results discussed earlier. It is important to note that overlapping peptides with previously described antimicrobial peptides CIRA and LECIRA[36, 57] were identified only in digesta of native LF, with homology of 57% and 86%, respectively. Differences between the various samples regarding the presence of bioactive peptides probably arise from the distinct enzymatic cleavage patterns affected by the Maillard reaction, as discussed earlier. Nevertheless, there may be other bioactive peptides present in all samples or even unique to MRPs digesta, which were not previously characterized and may impact the specific or overall bioactivity. Therefore, identified sequences in digesta samples were inserted to a bioinformatics server named PeptideRanker which evaluates the probability of peptide sequences to be bioactive[58] (**Table 2**). This revealed all samples contained various peptide sequences with a bioactivity likelihood of up to 0.89, which were not previously described. Interestingly, two of these peptides, WIIPMGILRPYL and FGSPPGQRDLL (with p=0.77 and 0.8, respectively), were also identified in aspirates following *in vivo* digestion of LF[7]. In addition, PeptideRanker enabled to identify peptides derived from LF digestion, e.g. TAGWNIPMGL, which are likely bioactive and were not reported in the literature to this end. These peptides may exhibit biological functions that were not previously characterized and may contribute to understanding the bioactivity of LF and its digesta. Furthermore, some potential bioactive peptides were identified in the digesta of both MRPs but not in the digesta of native LF such as FSASCVPCIDRQAYPNL, while others were unique to digesta of LF-FOS 12h or 24h as QAYPNLCQLCK and WIIPMGIL, respectively. These results demonstrate that processing may lead to the production of potentially health-promoting products, generating bioactive peptides during digestion which wouldn't have been otherwise derived from the native protein. However, the results obtained using PeptideRanker should be addressed with caution until verified experimentally and other parameters including intestinal absorption should be taken into account to ultimately demonstrate in *vivo* efficacy.

Conclusions

Overall, this study has shown that Maillard conjugation with FOS alters LF's colloidal stability to pH and significantly improves its inherent antioxidant capacity in terms of ferric reducing power, in line with a recent work[15]. In addition, LF's susceptibility to proteolysis was found to be affected by the Maillard reaction, differing between the reacting carbohydrate

moiety as well as the model used to evaluate the digestive fate, either adult or infant. In infant digestion of LF-FOS MRPs there seems to be an optimization challenge, as processing for 12 h resulted in a decrease in LF susceptibility to proteolysis, while prolonged heating for 24 h led to an enhanced sensitivity to proteolysis. Proteomic analyses further elucidated the differences in enzymatic cleavage patterns and peptide formation. Numerous peptides reported in an *in vivo* study[7] were also identified in digesta samples, demonstrating the bio-relevance of the *in vitro* model used in this study. Proteomic data mining revealed that the Maillard reaction did not have a marked adverse effect on the formation of known LF-derived bioactive peptides. Moreover, these analyses pointed out that LF MRPs may serve as precursors to potentially bioactive peptides not produced during digestion of native LF. Nevertheless, further research is needed to ascertain the potential biological ramifications of the Maillard reaction on LF's digestive fate including under more realistic and bio-relevant conditions, for example applying more advanced digestion models or even human feeding trials.

Acknowledgements

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Notes and References

[a] Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Haifa 32000, Israel.

[b] UMR1253 STLO, INRA, 65 Rue de Saint Brieuc, F-35042 Rennes, France.

[c] Food and Health Group, Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK.

* Corresponding author: Asst. Prof. Uri Lesmes, E-mail: lesmesu@tx.technion.ac.il; Tel: +972-77-8871869; Fax: +972-4-8293399.

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Figure captions

Figure 1. Illustration of the impact of the Maillard reaction on protein digestibility. (A) Native protein with natural enzymatic access. (B) MRPs with increased access following exposure of cleavage site. (C) MRPs with limited access due to steric hindrance.

Figure 2. Changes in LF properties arising from Maillard processing with 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, red marks indicate higher MW formation. (C) Corresponding SDS-PAGE obtained using a glycoprotein stain, red marks indicate higher MW formation; 1, LF native; 2, LF-FOS t=0; 3, LF-FOS 12h; 4, LF-FOS 24h.

Figure 3. Effect of Maillard reaction with FOS on LF functionalities. (A) Colloid size responsiveness to pH (determined by dynamic light scattering). (B) Solution turbidity responsiveness to pH. (C) Antioxidant capacity measured by the FRAP assay ($p<0.01$). (LF native; LF-FOS t=0; LF-FOS 12h; LF-FOS 24h)

Figure 4. SDS-PAGE of MRPs digesta following *in vitro* adult gastric digestion. (A) LF-glucose 12h. (B) LF-fructose 12h. (C) LF-FOS 12h. (D) LF-FOS 24h; 1, $G0^-$; 2, $G0^+$; 3, G1; 4, G2; 5, G5; 6, G10; 7, G20; 8, G40; 9, G60.

Figure 5. SDS-PAGE following *in vitro* infant gastro-duodenal digestion of (A) LF native. (B) LF-glucose 12h. (C) LF-fructose 12h; 1, $G0^-$; 2, $G0^+$; 3, G1; 4, G2; 5, G5; 6, G10; 7, G20; 8, G60; 9, $D0^-$; 10, $D0^+$; 11, D1; 12, D5; 13, D15; 14, D30.

Figure 6. Impact of processing duration of LF-FOS on proteolysis under infant conditions (determined by SDS-PAGE). (A) 12h. (B) 24h; 1, $G0^-$; 2, $G0^+$; 3, G1; 4, G2; 5, G5; 6, G10; 7, G20; 8, G60; 9, $D0^-$; 10, $D0^+$; 11, D1; 12, D5; 13, D15; 14, D30.

Figure 7. Diagrammatic representation of the peptides remaining after gastro-duodenal infant digestion of LF native, LF-FOS 12h and LF-FOS 24h.

Tables

Table 1: LC-MS/MS results comparison with known LF- derived peptides

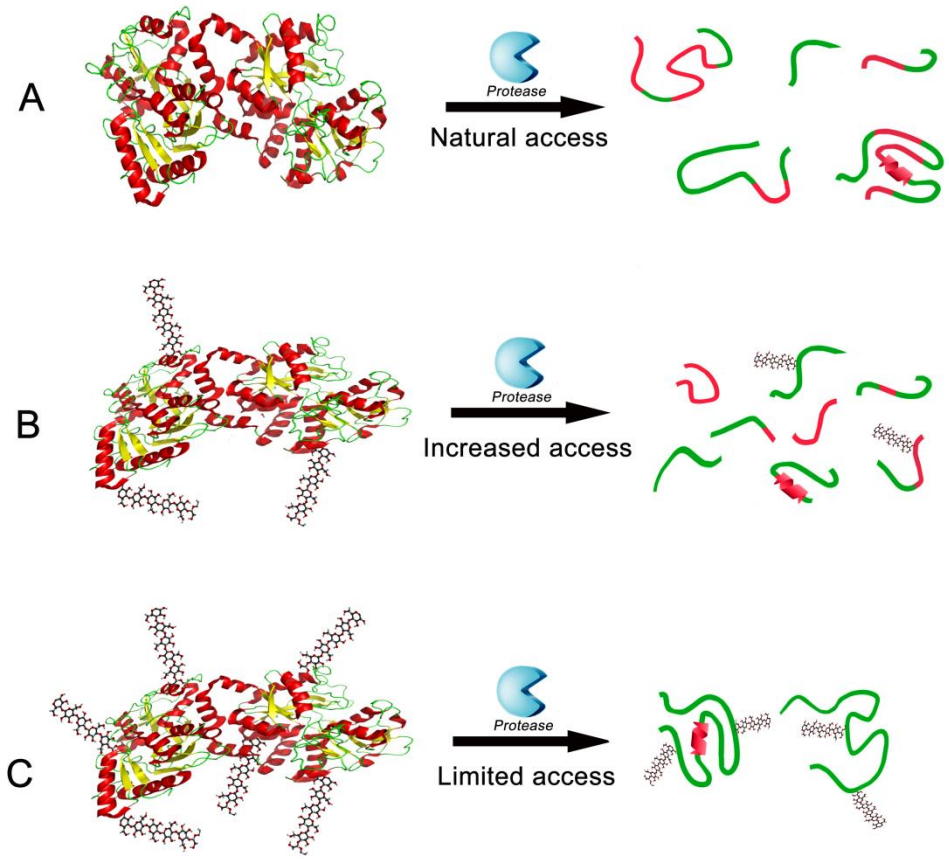
Sample	Identified peptide	Literature peptide	Homology (%)	Reported relevance; Ref
All	**KLLSKAQEK**	WKLLSKAQEKFGKNKSR	53	Antibacterial[32]
	LSKAQEKFGK		59	
	LSKAQEKFGKNK		71	
	MKKLGAPSITCVR	FKCRRWQWRMKKLGAPSITCVR RAF	52	Antibacterial[36]
	ENLPEK**ADRDQY**	ADRDQYELL	67	Antiviral[55]
	ENLPEK**ADRDQYE**		78	
	ENLPEK**ADRDQYEL**		89	
	IYGTKESPQTHY	AGIYGTKESPQTHYY	80	Immuno-modulating[56]
	E**IYGTKESPQTHY**		80	
	AEIYGTKESPQTHY		87	
	EIYGTKESPQTHY	EIYGTKESPQTHY	100	Found *in vivo*, unknown activity[7]
	AEIYGTKESPQTHY	AEIYGTKESPQTHY	100	
	WIIPMGILRPYL	WIIPMGILRPYL	100	
	ENLPEKADRDQYE	ENLPEKADRDQYE	100	
	FGSPPGQRDLL	FGSPPGQRDLL	100	
	LRIPSKVDSAL	LRIPSKVDSAL	100	
LF	ALE**CIRA**	CIRA	57	Antibacterial[57]
	A**LECIRA**	LECIRA	86	Antibacterial[36]
	LCLDGTRKPVTEAQSCHL	LCLDGTRKPVTEAQSCHL	100	Found *in vivo*, unknown activity[7]
LF and LF-FOS 12h	**WCTISQPEW**	APRKNVRWCTISQPEW	56	Antibacterial[57]
LF and LF-FOS 24h	**CTISQPEW**		50	

Table 2: Peptides with probability>0.5 of bioactivity

Sample	Identified peptide	Bioactivity (probability)
All	FGKNGKNCPDKFCL	0.89
	QAYPNLCQLCKGEGENQCACSSREPY	0.85
	QAYPNLCQLCKGEGENQCACSSREPYF	0.83
	FGSPPGQRDLL	0.80
	WIIPMGILRPYL	0.77
	QAYPNLCQLCKGEGENQCACSSREPYFG	0.77
	FSASCVPCIDR	0.73
	GSPPGQRDLL	0.71
	GAPSITCVRR	0.71
	FGKNGKNCPDKF	0.70
	KDSALGF	0.66
	LGAPSITCVRR	0.62
	IANLKKCSTSPLL	0.62
	FKCLQDGAGDVAF	0.55
	LGAPSITCVR	0.52
	KLGAPSITCVR	0.52
LF and LF-FOS 12h	WCTISQPEW	0.58
	FQLDQLQGRK	0.54
	LCALCAGDDQGLDKCVPNSK	0.52
	APVDAFQECHLAQVPSHA	0.52
LF and LF-FOS 24h	FSASCVPCIDRQAYPNLCQL	0.83
	VWCAVGPEEQKKCQQW	0.57
LF-FOS 12h and 24h	FSASCVPCIDRQAYPNL	0.69
	KLGAPSITCVRR	0.60
LF	FSASCVPCIDRQAYPNLCQLCKGEGENQCACSSREPY	0.91
	TAGWNIPMGL	0.88
	QAYPNLCQLCKGEGENQCAC	0.83
	SSREPYFG	0.72
LF-FOS 12h	QAYPNLCQLCK	0.80
	SRLCALCAGDDQGLDKCVPNSK	0.75
	FQLFGSPPGQRDLL	0.69
	SGAFKCLQDGAGDVAF	0.63
	APVDAFQECHLAQVPSHAVV	0.57
LF-FOS 24h	WIIPMGIL	0.88
	DGGMVF	0.82
	FKDSALGF	0.73

List of Figures

Figure 1.



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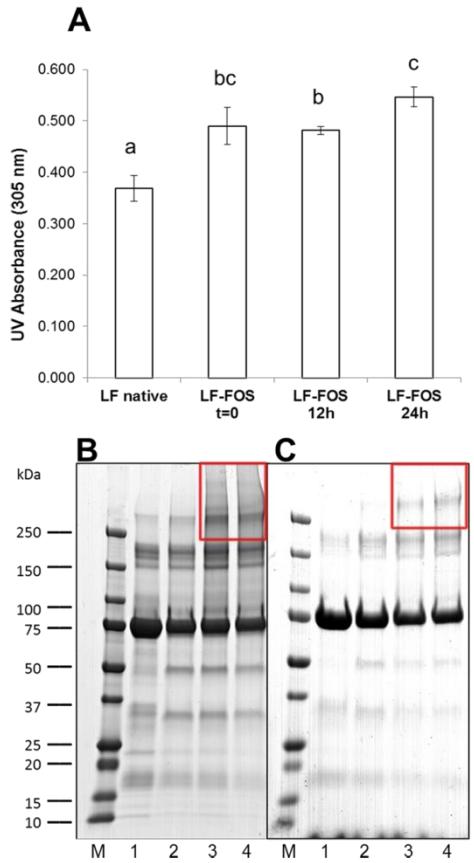
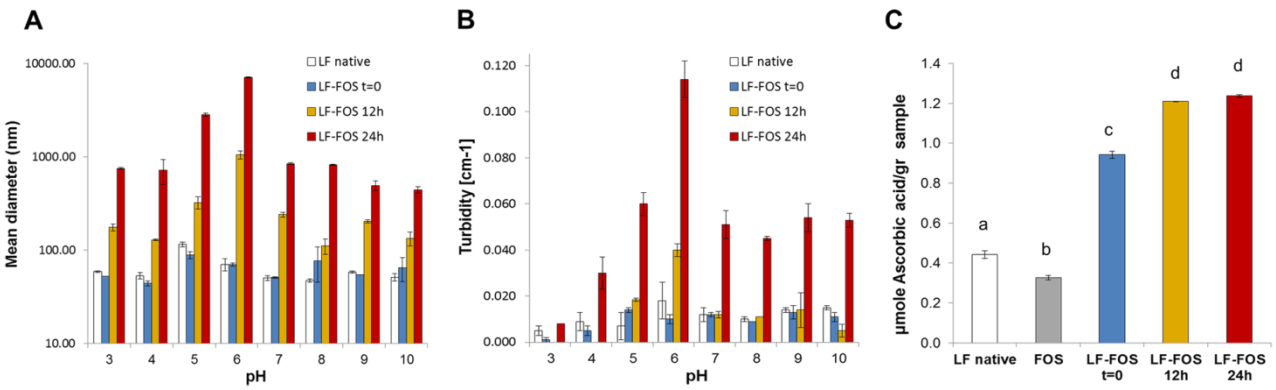


Figure 3.



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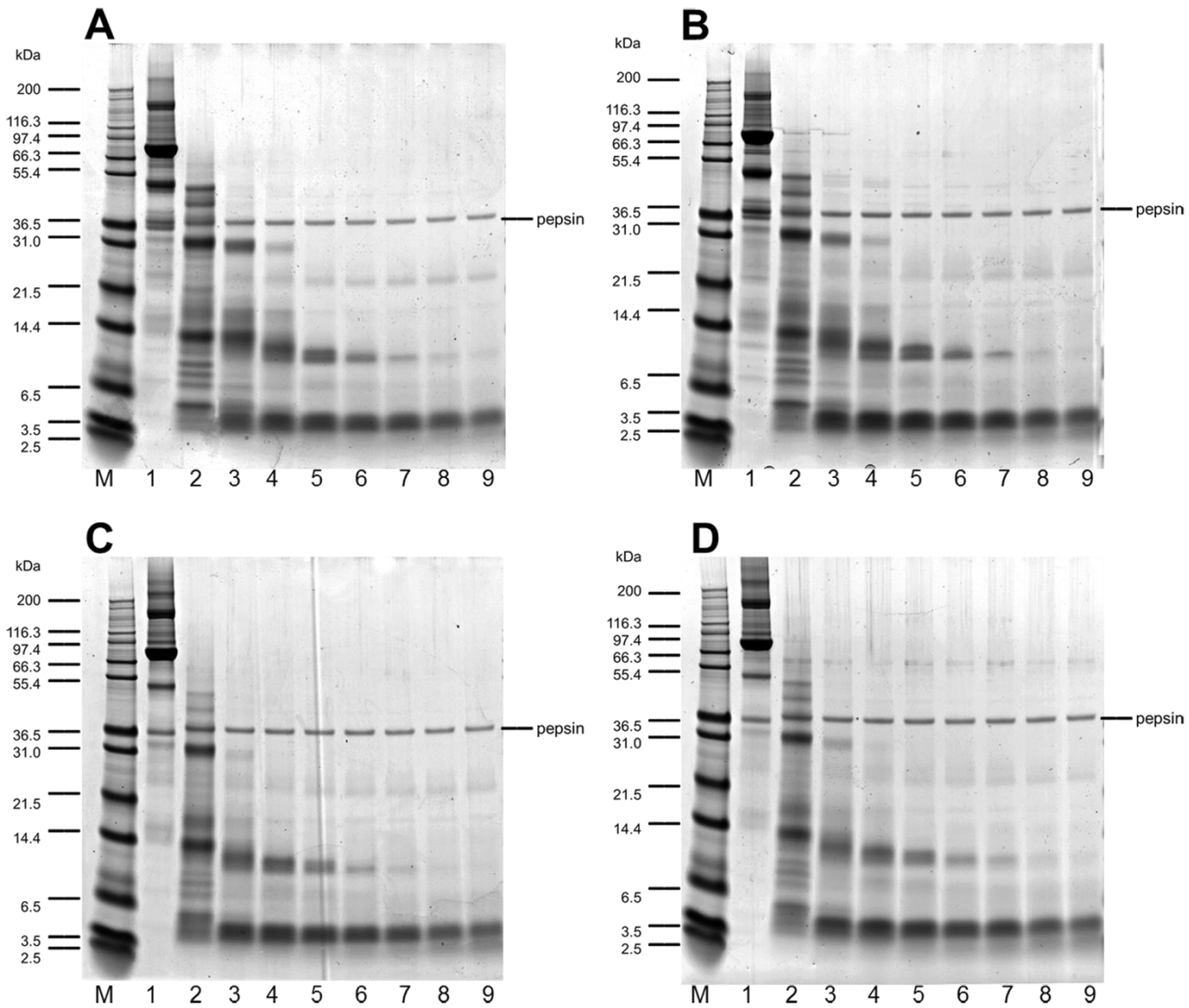

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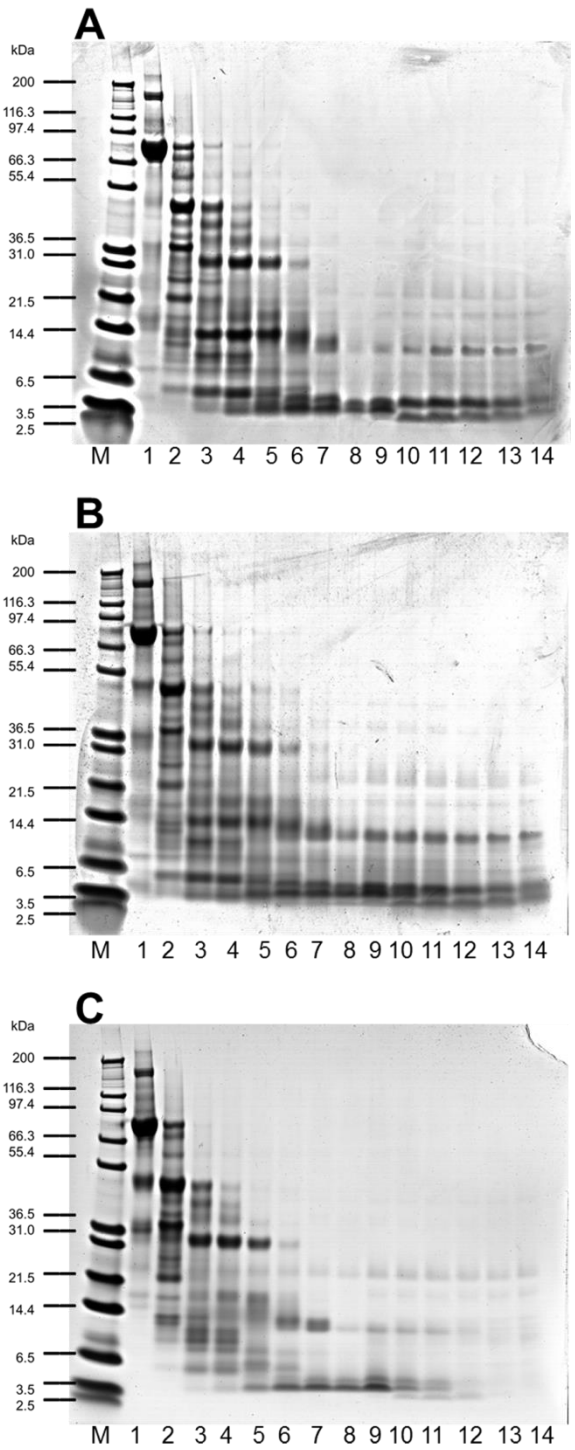

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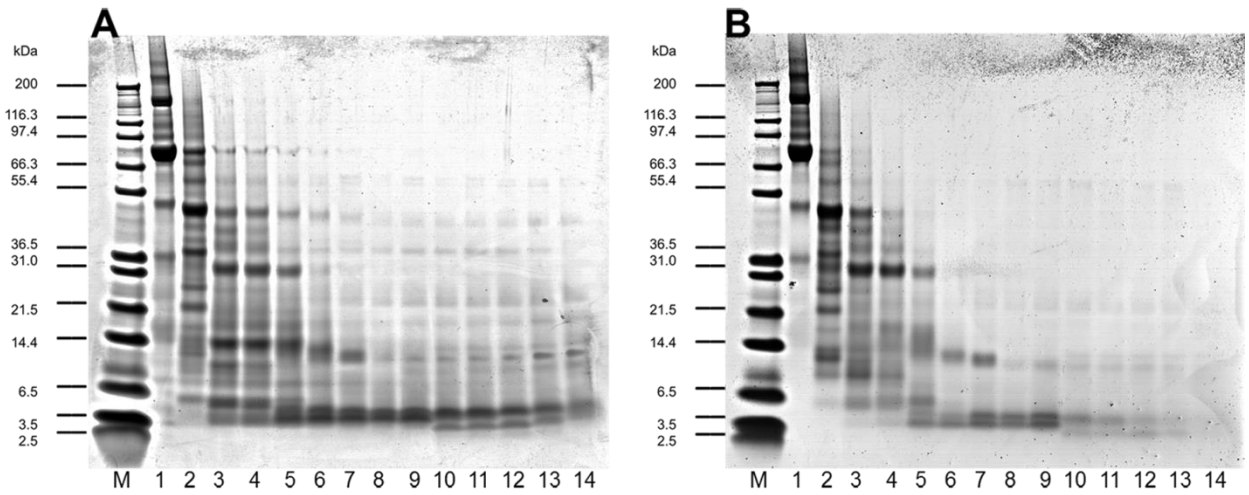

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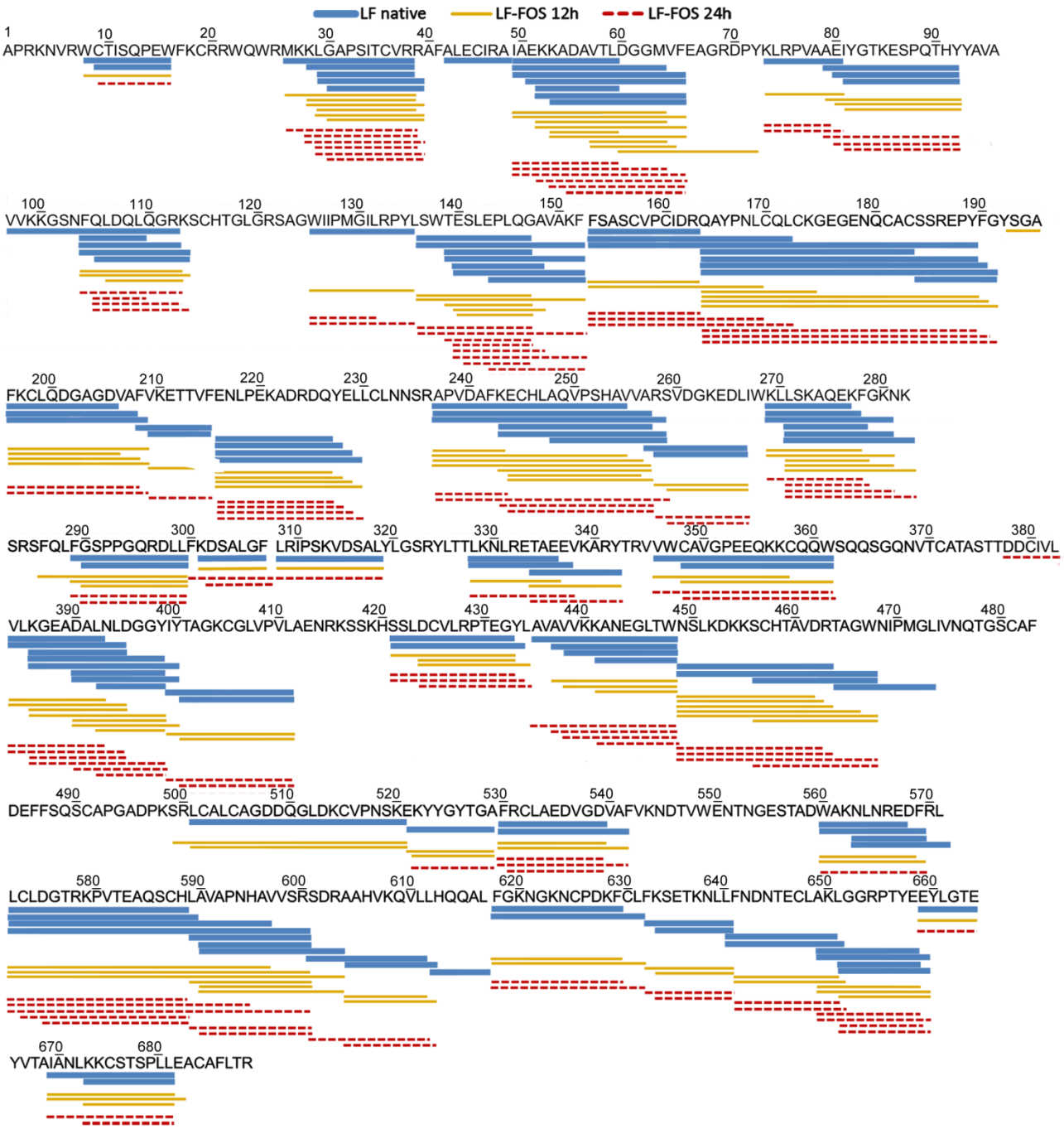
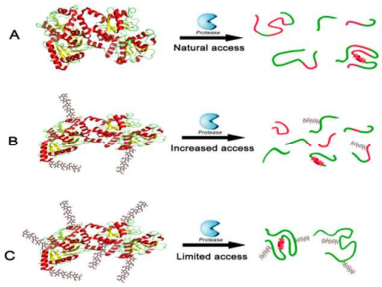


Graphical abstract



Maillard reaction impact on proteolysis of the bioactive bovine LF is compared *in vitro* between adults and infants for the first time, coupling proteomics to elucidate bioactive peptide formation.