

Protecting lactoferrin for iron delivery: Effects of matrix composition and iron loading on gastric stability

Ana Blanco-Doval^a, Younas Dadmohammadi^{b,1}, Alireza Abbaspourrad^b,
 Claire E. Noack^b, Yunan Huang^b, Tiantian Lin^b, Lotti Egger^a, Michael B. Zimmermann^c,
 Reto Portmann^{a,*}

^a Competence Division Methods Development and Analytics, Agroscope, Liebefeld, 3097, Bern, Switzerland

^b Department of Food Science, College of Agricultural and Life Sciences, Cornell University, 14853, Ithaca, NY, USA

^c Medical Research Council (MRC) Translational Immune Discovery Unit, MRC Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, OX3 9DS, Oxford, United Kingdom

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ABSTRACT

Oral iron salts are commonly used as supplements and fortificants to treat iron deficiency and anemia, but they are often poorly absorbed and cause gastrointestinal side effects, highlighting the need for more efficient and better-tolerated alternatives. Bovine lactoferrin (LF), a natural iron-binding glycoprotein, offers a promising option for iron supplementation, as it can deliver iron via receptor-mediated uptake in the small intestine. However, its efficacy may be compromised by proteolytic degradation during gastrointestinal digestion. In this study, we systematically evaluated novel LF-based formulations designed to resist gastric conditions. We focused on complexes with succinylated sodium caseinate, low-methoxyl pectin, and whey protein hydrolysate, each combined with varying iron loadings. Static *in vitro* digestion was performed using the INFOGEST 2.0 protocol, and LF integrity was assessed by SDS-PAGE and LC-MS/MS. Unprotected LF was extensively hydrolyzed under these conditions. By contrast, ternary complexes of LF with sodium caseinate and pectin at high iron saturation, as well as binary LF-caseinate complexes with high levels of extrinsic iron, showed the highest stability. During intestinal digestion, highly iron-saturated ternary complexes of LF with sodium caseinate released the greatest amount of potential iron-binding peptides. These results indicated that specific LF-protein-polysaccharide-iron combinations are promising next-generation iron supplements and fortificants with improved tolerability and bioavailability.

1. Introduction

Iron is an essential trace element required for oxygen transport, energy metabolism, enzymatic reactions, and gene regulation (Pasricha et al., 2021). Despite this, iron deficiency affects 16.7 % of the global population, with much higher rates among young children and women of reproductive age (Lee et al., 2025). In pregnancy, anemia heightens maternal and neonatal risks, while early-life deficiency impairs cognitive and motor development and increases mortality (Pasricha et al., 2021). The World Health Organization (WHO) aims to halve anemia in women of reproductive age by 2025 (WHO, 2014), a goal requiring

improved diets and more effective, tolerable supplementation and improved fortification strategies (Helmyati et al., 2024).

Oral iron supplementation with salts such as ferrous sulfate is the standard treatment, but its benefits are often offset by gastrointestinal discomfort and altered gut microbiota (Pasricha et al., 2021). The fortification of foods with mineral iron presents additional technological challenges (Hurrell, 2022). Consequently, there is a pressing need for alternative delivery strategies that combine efficacy with good tolerability.

Lactoferrin (LF), a glycoprotein of the transferrin family naturally present in milk and mucosal secretions, is an attractive candidate. LF

* Corresponding author.

E-mail addresses: ana.blancodova@agroscope.admin.ch (A. Blanco-Doval), younas@cornell.edu (Y. Dadmohammadi), alireza@cornell.edu (A. Abbaspourrad), cen44@cornell.edu (C.E. Noack), yh974@cornell.edu (Y. Huang), tl475@cornell.edu (T. Lin), charlotte.egger@agroscope.admin.ch (L. Egger), michael.zimmermann@rdm.ox.ac.uk (M.B. Zimmermann), reto.portmann@agroscope.admin.ch (R. Portmann).

¹ Present address: Food Innovation Center, Oregon State University, 97209 Portland, OR, USA.

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reversibly binds ferric iron (Fe^{3+}) with high affinity at two specific sites, one on each lobe (N-lobe and C-lobe), both formed by two highly homologous domains (I and II) (Piacentini et al., 2024). Beyond its enzymatic role, LF exhibits a broad range of bioactivities, including immunomodulatory, anticarcinogenic, anti-inflammatory and antimicrobial capacities (Valenti et al., 2025; Vogel, 2012). Its primary biological function, however, is iron binding. LF has a high affinity for iron with an equilibrium association constant value (K_a) of about 10^{-22} (Baker & Baker, 2005). LF can interact with specific receptors in the small intestine and may enter enterocytes via receptor-mediated endocytosis (Suzuki et al., 2005). In addition, the anti-inflammatory activity of LF can downregulate the expression of hepcidin (El Amrousy et al., 2022; Lepanto et al., 2018), a hormone stimulated by inflammation that controls plasma iron levels by impairing enterocyte iron absorption, release from hepatic stores, and recycling from senescent erythrocytes (Ianiro et al., 2023; McCarthy & O'Callaghan, 2024). Thus, the effect of LF likely relies on both direct iron delivery and anti-inflammatory action. Previous studies, primarily in pregnant women and children, have shown that bovine LF supplementation can be effective, and in some cases more effective than ferrous sulfate, in treating iron deficiency and anemia, with the added advantage of avoiding gastrointestinal side effects (Christofi et al., 2024; Hashim et al., 2017; June et al., 2023; Paesano et al., 2010; Zhao et al., 2022).

For optimal efficacy, lactoferrin must retain its native structure to bind specific receptors in the small intestine. Thus, it must survive passage through the stomach and reach the enterocytes largely intact. While several studies indicate that LF is relatively resistant to gastric digestion (Fu et al., 2023; Sutantawong et al., 2025; Troost et al., 2001), it remains susceptible to proteolytic cleavage in the gastrointestinal tract and can be partially degraded before absorption (Troost et al., 2001). One promising strategy for enhancing LF stability is to embed it in protective complexes with proteins, polysaccharides, or bioactive compounds. Previous work has shown that LF can be shielded from gastric digestion by complexing it with tannic acid (Jing et al., 2021), pterostilbene (Ma et al., 2025), and lactose (Chen et al., 2022), among other compounds.

Complexes of LF with low-methoxyl pectin have also been shown to protect LF from degradation during adult gastric digestion (Niu et al., 2019). This is particularly relevant given the prebiotic and anti-inflammatory properties of pectin, as well as its inherent resistance to digestion (Blanco-Pérez et al., 2021; Popov et al., 2013). In addition, low-methoxyl pectin can bind iron and serve as an iron carrier on its own, being effective in the treatment of iron deficiency anemia (Jing et al., 2024). Thus, LF-pectin complexes may act synergistically in iron delivery. In infant digestion models, complexes formulated with other milk proteins and peptides likewise improved LF survival during gastric digestion (Jiang et al., 2024). However, few studies on such complexes have considered LF's iron saturation as a factor to optimize its resistance, despite its well-known impact on protein structure and susceptibility to proteolysis (Baker & Baker, 2005).

Succinylated sodium caseinate was selected for this study due to its increased negative charge density, which promotes electrostatic interactions with positively charged lactoferrin domains and enhances complex stability. Whey protein hydrolysate was included as a contrasting stabilizer composed of low-molecular-weight peptides, enabling steric and electrostatic shielding of lactoferrin while representing a mechanistically distinct stabilization strategy compared to polysaccharide-based systems.

The stabilized formulations investigated in this study were intentionally restricted to native bovine lactoferrin, which had been pre-selected for subsequent nutritional and clinical applications. Recombinant bovine and human lactoferrins were therefore included exclusively for benchmarking purposes and not subjected to further stabilization or complexation.

Although previous studies have highlighted the potential of LF in human nutrition, its susceptibility to gastrointestinal degradation

remains a major limitation. This *in vitro* study therefore evaluated novel LF formulations engineered to maintain structural integrity during gastric transit and in the proximal small intestine, with the goal of enhancing receptor-mediated intestinal iron absorption.

2. Materials and methods

2.1. Reagents and sample materials

2.1.1. Reagents

The following reagents were purchased from Merck (Darmstadt, Germany): Potassium chloride (KCl), monopotassium phosphate (KH_2PO_4), sodium chloride (NaCl), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$), hydrochloric acid (HCl), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 4-(2-aminoethyl)-benzene-sulfonyl fluoride (Pefabloc), acetonitrile (LiChrosolv hyper-grade for LC-MS), pepsin (from porcine gastric mucosa), bile salts (porcine extract), and pancreatin (from porcine pancreas). Sodium bicarbonate (NaHCO_3) and iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were obtained from VWR Chemicals (Dietikon, Switzerland), and formic acid (ultrapure for HPLC) from Honeywell (Charlotte, NC, USA). All reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA, USA).

Simulated salivary, gastric and intestinal fluids were prepared with KCl, KH_2PO_4 , NaHCO_3 , NaCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{CO}_3$, HCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at concentrations specified in the INFOGEST 2.0 protocol (Brodtkorb et al., 2019).

2.1.2. Sample materials: LF and stabilizing compounds

Native bovine lactoferrin (nb) was kindly provided by Glanbia Nutritional (Bioferrin® 2000, Kilkenny, Ireland) and recombinant bovine lactoferrin (rb) by TurtleTree (LF^+ , Singapore). Partially iron-saturated (rh) and fully iron-saturated (rh-Fe) recombinant human LF were purchased from Merck. Succinylated sodium caseinate was prepared in-house with casein sodium salt from bovine milk and succinic anhydride (Sigma-Aldrich, St. Louis, MO, USA). Low-methoxyl pectin (non-amidated, 35 % degree of esterification, extracted from citrus peel) was obtained from CP Kelco (San Diego, CA, USA), and whey protein hydrolysate was purchased from Agropur Ingredients (BioZate® 9, Appleton, WI, USA).

2.2. Sample preparation

All LF complexes were formulated with nb. Three stabilizing compounds were tested: succinylated sodium caseinate, low-methoxyl pectin, and whey protein hydrolysate. Two types of complexes were prepared: (1) binary complexes consisting of nb and a stabilizing compound without added iron (with succinylated sodium caseinate: BC, with low-methoxyl pectin: BP, and with whey protein hydrolysate: BW), and (2) ternary complexes containing nb, a stabilizing compound, and bound iron (succinylated sodium caseinate: TC, with low-methoxyl pectin: TP, and with whey protein hydrolysate: TW). The ternary complexes were prepared either with high (TC_H, TP_H, and TW_H) or low (TC_L and TP_L) iron content. No ternary complex with whey protein hydrolysate and low iron loading was included in the study.

All complexes were prepared using the pH-first method. Binary and ternary complexes with succinylated sodium caseinate (BC and TC) and whey protein hydrolysate (BW and TW) were prepared according to Huang et al. (2024). Briefly, LF and succinylated sodium caseinate or whey protein hydrolysate solutions (10 mg/mL, in Milli-Q water) were prepared separately and adjusted to the target pH values. The solutions were mixed at the ratios shown in Table 1 to create the binary complexes. For the ternary complexes, a solution of FeSO_4 (1 mol/L, in Milli-Q water) was added to the protein mixtures to achieve the target iron concentrations (Table 1). Ferrous sulfate was selected as iron source due to its higher solubility and milder impact on protein structure

Table 1

Description of the lactoferrin samples included in the study.

Sample	LF type	Complex type	SC	LF:SC ratio	LF loading (mg LF/g complex)	Fe loading (mg Fe/g LF)*
Non-stabilized samples						
rb	Recombinant bovine	–	–	–	–	0.977–1.40
nb-Fe	Native bovine	–	–	–	–	0.267
nb	Native bovine	–	–	–	–	0.191
rh-Fe	Recombinant human	–	–	–	–	1.26
rh	Recombinant human	–	–	–	–	1.26
Stabilized samples						
TC_H	Native bovine	Ternary	S.Na.C	1:2	296	40.9
TC_L	Native bovine	Ternary	S.Na.C	1:1	521	13.2
BC-Fe	Native bovine	Binary	S.Na.C	1:2	296	40.9
BC	Native bovine	Binary	S.Na.C	2:1	645	–
TP_H	Native bovine	Ternary	LMP	1:1	542	69.8
TP_L	Native bovine	Ternary	LMP	2:1	690	36.0
BP-Fe	Native bovine	Binary	LMP	1:1	542	69.8
BP	Native bovine	Binary	LMP	1:1	850	–
TW_H	Native bovine	Ternary	WPH	1:2	413	13.3
BW	Native bovine	Binary	WPH	1:2	402	–

Fe: iron; LF: lactoferrin; LMP: low-methoxyl pectin; SC: stabilization compound; S.Na.C: succinylated sodium caseinate; WPH: whey protein hydrolysate. *100 % iron saturation is equal to 1.4 mg Fe/g lactoferrin.

compared to ferric salts, which readily hydrolyse and may induce protein precipitation or denaturation during complex formation. All mixtures were incubated at 25 °C for 30 min with constant shaking (200 rpm). Pellets separated by centrifugation (15,000 g, 15 min, 4 °C) were collected and subsequently freeze-dried. Binary and ternary complexes with low-methoxyl pectin were formed as described by Noack et al. (2025). Briefly, LF and low-methoxyl pectin solutions were prepared at a concentration of 0.2 % (w/v), mixed for 1 h at room temperature, and centrifuged (10,000 g, 10 min, 20 °C).

The stabilized lactoferrin complexes investigated in this work are identical to formulations previously reported (Huang et al., 2024; Noack et al., 2025), for which detailed microstructural characterisation by scanning electron microscopy has already been performed. These analyses demonstrated well-defined protein–matrix architectures underlying the stabilizing effects and are therefore not repeated here.

Furthermore, BC and BP, identified in preliminary tests as the most stable binary complexes, were tested in the presence of extrinsic iron, maintaining the same LF-to-stabilizer-to-iron ratio as in the corresponding high-iron ternary complexes. Similarly, an iron-saturated nb (nb-Fe) was produced by dissolving 40 mg of nb in 960 mL FeSO₄ aqueous solution (final Fe:LF molar ratio 3:1) and incubating at 22 °C for 1 h with agitation (800 rpm; ThermoMixer C, Eppendorf, Hamburg, Germany).

As controls, non-stabilized nb, rb, rh, and rh-Fe were digested without extrinsic iron, containing only their naturally present iron. Table 1 provides a complete list and description of the samples included in the study.

2.3. Static *in vitro* simulation of human gastrointestinal digestion

Stabilized and non-stabilized lactoferrin samples were digested using the static *in vitro* digestion model developed by the INFOGEST Cost Action network (Brodkorb et al., 2019; Minekus et al., 2014). In all cases, the amount of sample used corresponded to 40 mg of lactoferrin. All samples, except nb-Fe, were dissolved in ultrapure water to a final mass of 1 g; 40 mg of nb-Fe was already prepared as a 1 g aqueous solution during the iron-saturation process. As the stabilized LF formulations are intended for future incorporation into porridge, they must withstand a cooking step. To simulate this process, all samples were heated in a thermomixer at 95 °C and 800 rpm for 5 min (ThermoMixer C, Eppendorf), then cooled to room temperature for 5 min prior to digestion.

In vitro digestion consisted of three sequential phases. (1) For the oral phase, samples were mixed with simulated salivary fluid (pH 7.0) without amylase. (2) For the gastric phase, oral digesta were combined

with simulated gastric fluid (pH 3.0) and pepsin (2000 U/mL final activity) and then incubated at 37 °C under continuous rotation (SB3 rotator, Stuart, VWR Chemicals, Dietikon, Switzerland). (3) Finally, for the intestinal phase, gastric digesta were mixed with simulated intestinal fluid (pH 7.0), bile salts (10 mM final concentration), and pancreatin (100 U/mL final trypsin activity), and incubated under the same conditions as in the gastric digestion. Amylase was omitted from the oral phase, as the tested samples contained no starch or other amylase-digestible substrates, in accordance with INFOGEST recommendations.

Gastric digestion was terminated after 5, 30, or 120 min (G5, G30, G120), and intestinal digestion was terminated after 10 or 120 min (I10, I120). For recombinant human LF (rh and rh-Fe), gastric digestion was stopped only at 30 or 120 min due to limited sample availability. Gastric digestions were stopped by adding 2 mol/L NaOH to adjust the pH to 7.0; intestinal digestions were stopped by adding 16 µL 500 mmol/L Pefabloc. Immediately after termination, the samples were snap-frozen in liquid nitrogen, stored at –20 °C, and thawed at 4 °C immediately before analysis. A separate digestion was performed for each time point for all samples, resulting in four to five independent digestions *per* sample.

2.4. Characterization of proteolysis during gastrointestinal digestion

2.4.1. SDS-PAGE

The survival of intact LF after digestion was studied using SDS-PAGE. Aqueous solutions of undigested nb or rb at 5 mg/mL served as reference standards for band identification. Standards, gastric digesta, and intestinal digesta were diluted 1:20, 1:10, and 1:5, respectively, with a 1x sample buffer final concentration. Proteins were denatured at 95 °C for 5 min (Stuart SBH130D block heater, Huberlab, Aesch, Switzerland), and 10 µL of each sample was loaded onto 15 % acrylamide gels. A Benchmark protein ladder (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used as the molecular weight standard. Electrophoresis was performed with a 0.2 M glycine buffer at 40 mA *per* gel (PowerPac 3000, Bio-Rad). Gels were stained with Coomassie Brilliant Blue G-250, destained in 10 % ethanol and 2 % ortho-phosphoric acid aqueous solution (v/v), and scanned using a calibrated densitometer (GS-900, Bio-Rad).

2.4.2. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was employed to characterize the LF-derived peptides generated during digestion. Aliquots (500 µL) of gastric and intestinal digesta were filtered through 30 kDa molecular weight cut-off centrifugal filters (Amicon Ultra 0.5 mL, Merck) by centrifugation at 18,000 g and 4 °C for 10 min. The filtrates were transferred to 0.5 mL glass vials

and analyzed by ultra-high-performance liquid chromatography (Vanquish Flex, Thermo Fisher Scientific) coupled to hybrid quadrupole-orbitrap mass spectrometry (Q Exactive Plus, Thermo Fisher Scientific).

The mobile phases consisted of (A) 0.1 % (v/v) formic acid in ultrapure water and (B) 0.1 % (v/v) formic acid in acetonitrile. A linear gradient from 5 % to 95 % B was applied over 2–20 min, followed by re-equilibration to 5 % B. The flow rate was 0.3 mL/min, the autosampler was maintained at 8 °C, the column was maintained at 40 °C, and the injection volume was 20 µL.

Mass spectrometry was performed in positive ionization mode with a scan range of m/z 150–2,000, using data-dependent acquisition with repetitive fragmentation of the 10 most intense precursor ions. MS/MS spectra were processed using Mascot Daemon Search Engine (version 2.8.0, Matrix Science, London, UK) against an in-house database of milk proteins compiled from UniProtKB (<https://www.uniprot.org/>). The precursor mass tolerance was set to ± 20 ppm. Considered variable modifications included lysine acetylation, asparagine and glutamine deamidation, N-terminal glutamate cyclization to pyroglutamate, methionine oxidation, and phosphorylation and sulfonation of serine, threonine and tyrosine residues. For peptide identification, an ion score cut-off (significance level) of 20 was applied in Mascot to ensure confident assignments.

2.5. Data treatment and visualization

MS/MS analysis provided the sequence and intensity of peptides released during digestion for each sample. Peptide abundance was quantified using the peptide pattern (amino acid count) method (Portmann et al., 2023), in which the number of times each amino acid in the bovine LF sequence was identified by MS/MS within a sample was recorded. These data were visualized as clustered heatmaps generated in RStudio (version 2023.09.1, Posit, Boston, MA, USA). Hierarchical clustering was performed using Ward's minimum variance method with Euclidean distance as the similarity metric.

The protein sequences of bovine and human LF were retrieved from the UniProtKB/Swiss-Prot database (<https://www.uniprot.org/>; primary accession numbers: P24627 and P02788, respectively). Bioactive proteins were identified by comparison with entries in the Milk Bioactive Peptide Database (MBPDB; Nielsen et al., 2017, 2024).

3. Results

3.1. SDS-PAGE

Representative SDS-PAGE scans (Fig. 1) illustrate the profile across

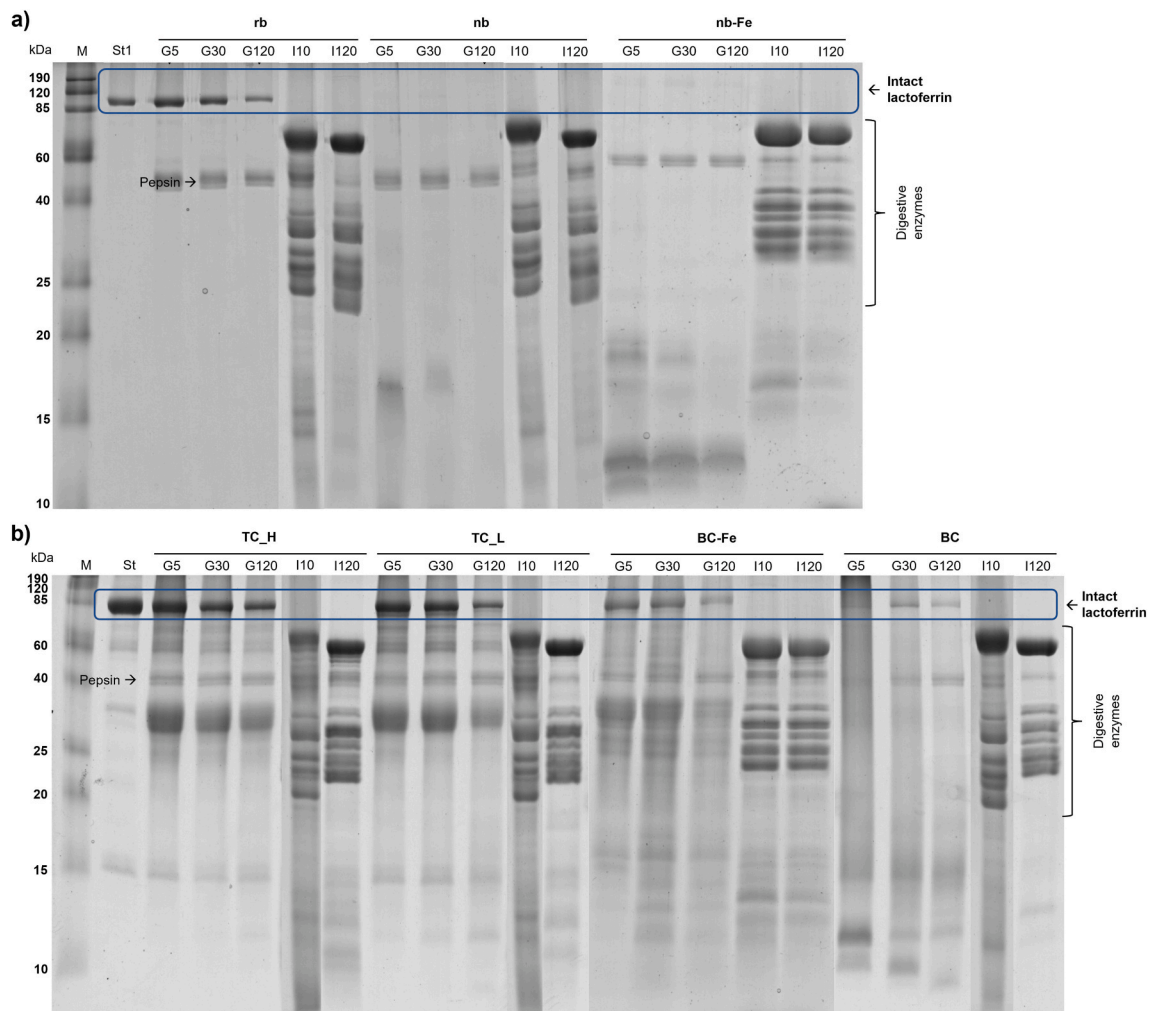


Fig. 1. Scans of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels run with a) non-stabilized recombinant bovine (rb), native bovine (nb) and iron-saturated native bovine (nb-Fe) lactoferrins; and b) complexes of lactoferrin stabilized with sodium caseinate (TC_H, TC_L, BC-Fe and BC; see Table 1 for sample description), at different digestion points (G5: gastric 5 min; G30: gastric 30 min; G120: gastric 120 min; I10: intestinal 10 min; I120: intestinal 120 min). M: molecular weight marker; St1: undigested recombinant bovine lactoferrin; St: undigested native bovine lactoferrin. Blue rectangles indicate the location of protein bands corresponding to intact lactoferrin; bands corresponding to digestive enzymes have also been indicated. Original SDS-PAGE scans are provided in Supplementary material 2.

all time points for the digestion of (a) non-stabilized bovine LF (nb, nb-Fe) and (b) sodium caseinate complexes (shown as an example of a stable formulation; detailed below). Gel scans of all other samples are provided in [Supplementary Material 1, Figs. S1–S3](#), and [Supplementary Material 2](#). The band corresponding to intact LF was identified by comparison with undigested protein standards.

As shown in [Fig. 1a](#), non-stabilized nb was extensively degraded at the earliest gastric time point (G5), regardless of its iron loading. Recombinant bovine LF (rb) exhibited greater stability, persisting throughout gastric digestion, although progressive degradation from G5 to G120 was evident, and no intact rb remained at I10. Recombinant human LF (rh and rh-Fe) also resisted complete gastric digestion and appeared more stable than rb at G120 and I10. However, in all cases, intact human LF was absent by the end of the intestinal phase (I120) ([Supplementary Material 1, Fig. S1](#)).

All stabilized complexes outperformed nb in resisting gastrointestinal digestion, although to varying degrees. For sodium caseinate ([Fig. 1b](#)) and low-methoxyl pectin ([Supplementary Material 1, Fig. S2](#)), the ternary complexes (TC_H, TP_H, TC_L and TP_L) withstood the entire gastric phase to a greater or lesser extent and were degraded only under intestinal conditions. By contrast, binary complexes (BC and BP) underwent notable degradation during gastric digestion, with only trace amounts of intact LF present at G120. Saturation of BC and BP with extrinsic iron (BC-Fe and BP-Fe) markedly improved protection, resulting in more intense LF bands after G120.

Complexes with whey protein hydrolysate behaved slightly differently ([Supplementary Material 1, Fig. S3](#)). In both the ternary and binary forms, intact LF bands were almost completely absent by G120. Similar to non-stabilized nb, none of the whey protein hydrolysate complexes remained intact beyond 10 min of intestinal digestion.

3.2. LC-MS/MS

3.2.1. Total peptide abundance

LC-MS/MS analysis enabled a comprehensive characterization of LF-derived peptides (≥ 7 amino acids long; analytical limit) formed during gastrointestinal digestion, providing a measure of protein degradation. The degradation of LF in the different digestion steps was assessed in three ways, based on the same dataset. During MS acquisition, an exclusion time of 10 s was applied, preventing the re-measurement of a sequenced peptide within that period. This ensured that a wide variety of peptides could be detected while still allowing highly abundant peptides to be measured multiple times, so their relative abundance contributed to the dataset. The total peptide count ([Fig. 2](#)) refers to the summed number of all identified peptides in a sample. The total peptide

intensity ([Supplementary Material 1, Fig. S4](#)) was calculated from the same measurements, but instead of counting each identified peptide once, the fragment ion intensities from all MS/MS spectra of the identified peptides were summed, thereby reflecting both peptide diversity and relative abundance. The coverage of peptides ([Supplementary Material 1, Fig. S5](#)) indicates which regions of the LF sequence were represented by the identified peptides.

In general, substantially more peptides were released during gastric digestion than during intestinal digestion. In most samples, peptide count and intensity increased progressively from G5 to G30 or G120, indicating gradual degradation during gastric digestion. Upon transition to intestinal conditions, peptide abundance dropped sharply within 10 min (I10). Since peptides shorter than 5 or longer than approximately 40 amino acids were not identifiable by Mascot, this decrease likely reflects further hydrolysis into smaller peptides or free amino acids.

Across all stages, the digestion of nb and nb-Fe released more peptides than rb. Iron saturation of nb (nb-Fe) provided slight protection but did not achieve the stability observed for most stabilized formulations. Recombinant human LF (rh, rh-Fe) showed degradation patterns similar to nb, particularly by the end of gastric digestion and throughout the intestinal phase, releasing high peptide counts and intensities. At G30, discrepancies emerged between peptide count (closer to nb) and total peptide intensity (closer to rb), likely reflecting sequence differences between human and bovine LF and resulting variations in MS/MS response factors.

Complex formation generally reduced gastric peptide release in terms of both count and intensity. Binary complexes released more peptides than their corresponding ternary complexes, and lower iron loading in the ternary complexes was associated with greater peptide release. Among the high-iron ternary complexes, TC_H and TP_H were the most stable by the end of gastric digestion, whereas BC, TP_L, BP, and BP-Fe were the least stable. Notably, the sodium caseinate binary complex with extrinsic iron (BC-Fe) released a peptide abundance comparable to TC_H.

Peptide coverage was consistently higher in non-stabilized samples than in stabilized ones, although the differences were small ([Supplementary Material 1, Fig. S5](#)).

3.2.2. Length of the peptides

In this study, “oligopeptides” are defined as sequences containing 5–20 amino acids, and longer chains are referred to as “polypeptides” ([Zamyatnin, 2018](#)), following the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB) recommendation ([IUPAC-IUB, 1984](#)).

During the gastric phase, rb, TC_H, TP_H and TP_L released the

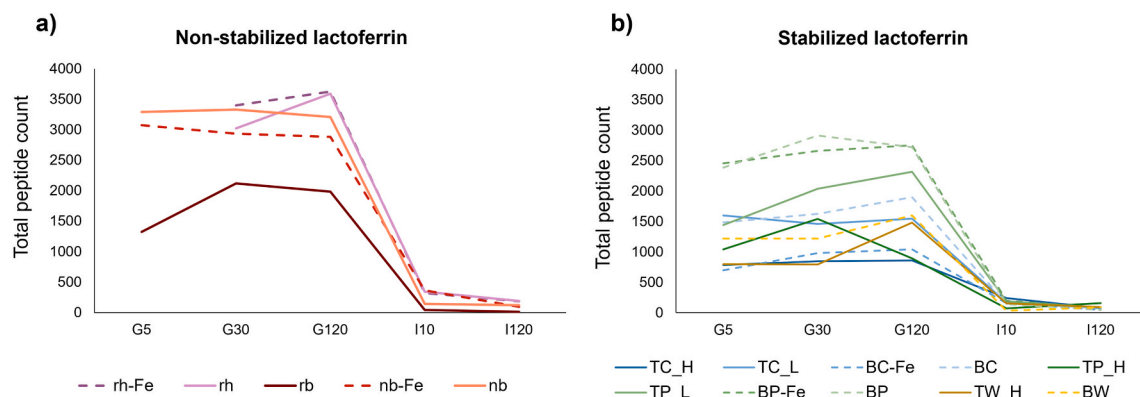


Fig. 2. Total MS/MS count of lactoferrin peptides released from a) non-stabilized lactoferrin; and b) stabilized lactoferrin samples, at different digestion points (G5: gastric 5 min; G30: gastric 30 min; G120: gastric 120 min; I10: intestinal 10 min; I120: intestinal 120 min). Non-stabilized lactoferrin: native bovine (nb), iron-saturated native bovine (nb-Fe), recombinant bovine (rb), partially iron saturated recombinant human (rh) and iron saturated recombinant human (rh-Fe) lactoferrins. Stabilized lactoferrin: complexes with sodium caseinate (TC_H, TC_L, BC-Fe and BC), pectin (TP_H, TP_L, BP-Fe and BP), and whey protein hydrolysate (TW_H and BW) (see [Table 1](#) for sample description).

shortest peptides (Fig. 3; Supplementary Material 1, Figs. S6 and S7), with polypeptides accounting for less than 1 % of all peptides. At G5, the maximum peptide lengths were 18 amino acids for rb, 22 for TC_H and TP_H, and 23 for TP_L. By G30, all peptides were ≤ 16 amino acids. TC_L, BP-Fe, and BP produced slightly larger peptides at G5 (1.3–1.8 % polypeptides up to 30 amino acids) that shortened as gastric digestion progressed. BC-Fe displayed a length distribution similar to TC_L, but with a higher polypeptide content at G30 (2.3 % vs. 1.8 %).

By contrast, BC and BW were degraded into relatively large peptides throughout gastric digestion, with polypeptides representing ~ 5 % at G5, ~ 4 % at G30 and 2–3 % at G120. TW_H also yielded larger peptides, although its degradation was moderate at G5 (maximum 18 amino acids) and more pronounced after G30 (maximum 30 amino acids; 6.2 % and 3.1 % of polypeptides at G30 and G120, respectively). Among all bovine LF samples, non-stabilized nb and nb-Fe had the longest peptides at G5 (4.3 % and 3.9 % of polypeptides, up to 32 and 38 amino acids, respectively). These were progressively shortened, and by the end of the gastric phase, no peptides exceeded 19 amino acids. Following gastric digestion, all bovine LF samples were reduced to oligopeptides ≤ 12 amino acids at I10, and to 7–8 amino acids at I120. The only exception was TP_L, which still contained two polypeptides (28 amino acids) at I10 (Supplementary Material 1, Figs. S8 and S9).

However, rh and rh-Fe released long peptides throughout digestion, including during the intestinal phase, and these were never fully degraded to short oligopeptides. At I10, maximum lengths were 31–32

amino acids, compared with ≤ 44 amino acids during gastric digestion, and polypeptides accounted for ~ 9 % (rh-Fe) and 10–12 % (rh) of the peptide population. By I120, peptides as long as 32 amino acids were still present, although more than 98 % of all peptides were ≤ 10 amino acids.

3.2.3. Amino acid counts and hierarchical clustering

The heatmaps in Figs. 4 and 5, Supplementary Material 1, and Figs. S10–S13 show the coverage and abundance of the LF-derived peptides, color-coded as the number of times each amino acid in the bovine LF sequence was identified by MS/MS. Human LF samples were excluded from this analysis due to sequence dissimilarity.

Fig. 4 illustrates the protective effect of complex formation compared to non-stabilized nb. During gastric digestion, degradation was substantially greater in nb than in TC_H, whereas TC_H released slightly more peptides during the intestinal phase (a pattern consistent with stronger protection in the gastric phase). Nonetheless, the very low peptide abundance at I10 and I120 suggests extensive hydrolysis to peptides shorter than 5 amino acids or to free amino acids.

Hierarchical clustering based on amino acid counts (Fig. 5 for G120; Supplementary Material 1, Figs. S10–S13 for other time points) revealed consistent grouping patterns. Across all gastric time points, nb and nb-Fe clustered together as the least stable samples, and at G30 and G120, they were joined by BP and BP-Fe. The sample rb consistently clustered with TP_L during gastric digestion, and both were grouped with TP_H at G5

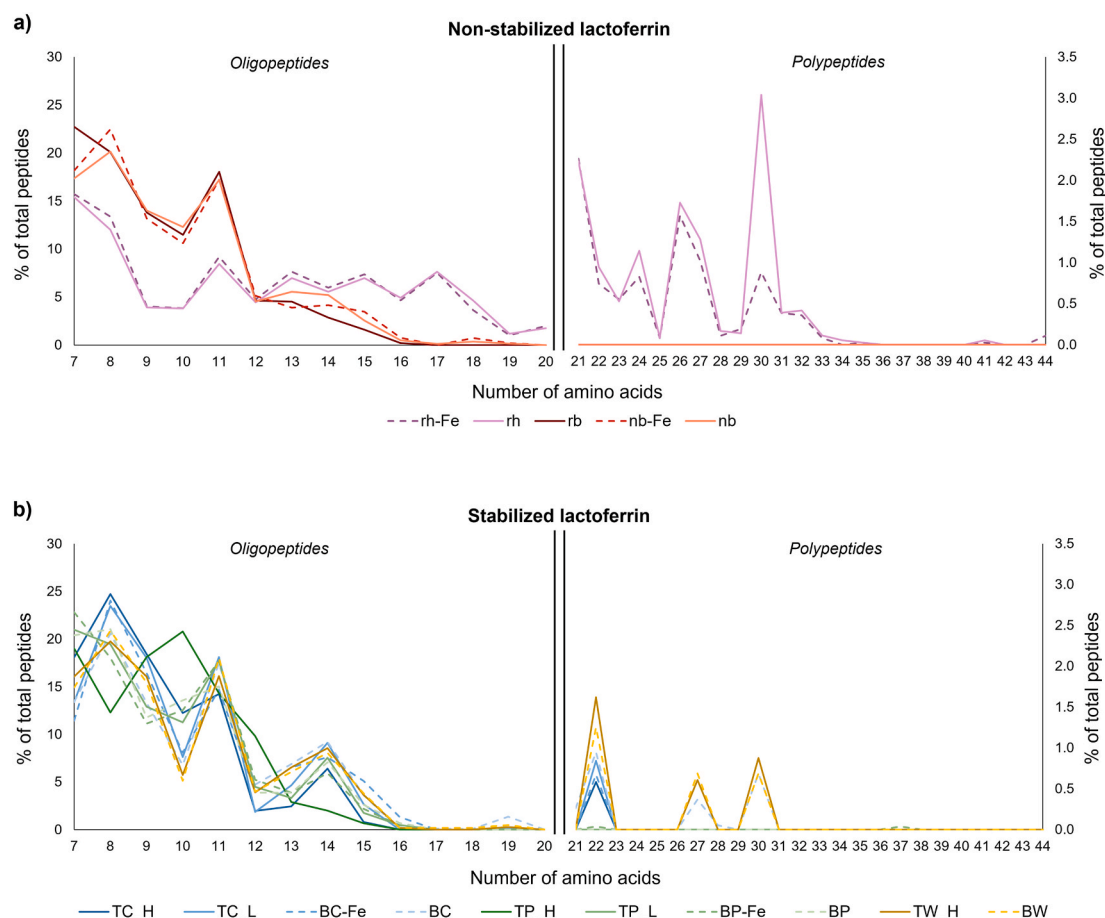


Fig. 3. Length (number of amino acids) distribution of the peptides released from a) non-stabilized lactoferrin; and b) stabilized lactoferrin samples, after 120 min gastric digestion, expressed as percentage of total peptides. The left y-axis refers to oligopeptides (7–20 amino acids) and the right y-axis refers to polypeptides (21–44 amino acids); the x-axis refers to the number of amino acids in individual peptides. Non-stabilized lactoferrin: native bovine (nb), iron-saturated native bovine (nb-Fe), recombinant bovine (rb), partially iron saturated recombinant human (rh) and iron saturated recombinant human (rh-Fe) lactoferrins. Stabilized lactoferrin: complexes with sodium caseinate (TC_H, TC_L, BC-Fe and BC), pectin (TP_H, TP_L, BP-Fe and BP), and whey protein hydrolysate (TW_H and BW) (see Table 1 for sample description).

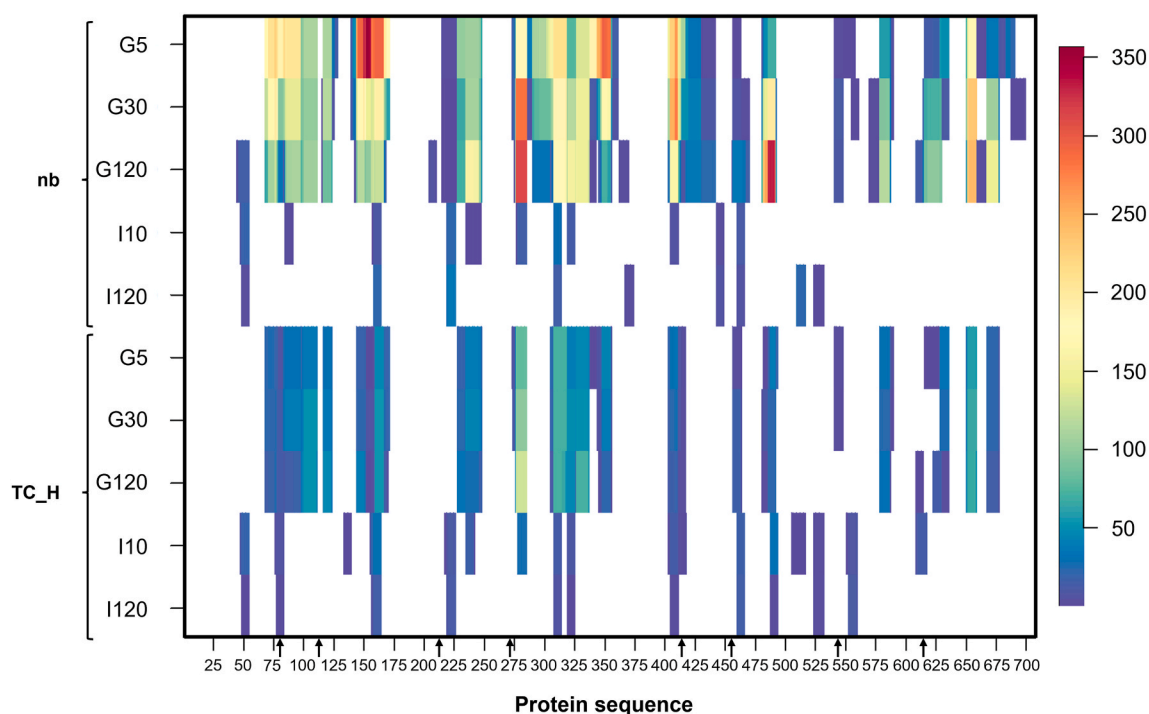


Fig. 4. Heatmap showing the peptide patterns of non-stabilized native bovine lactoferrin (nb) and a ternary complex of bovine lactoferrin stabilized with sodium caseinate (TC_H) at different digestion points (G5: gastric 5 min; G30: gastric 30 min; G120: gastric 120 min; I10: intestinal 10 min; I120: intestinal 120 min). Color-coded peptide intensities represent the number of MS/MS identifications of each individual amino acid in the bovine lactoferrin sequence and range from red (maximum intensity) to blue (minimum intensity). Columns correspond to individual amino acids in the lactoferrin sequence, from the N terminal (left) to the C terminal (right), and numbers on the x-axis refer to the amino acid position in the lactoferrin sequence. Black arrows on the x-axis indicate the approximate location of the iron-binding amino acids in lactoferrin. The rows correspond to individual samples, which have been identified in the y-axis; see [Table 1](#) for sample identification and description.

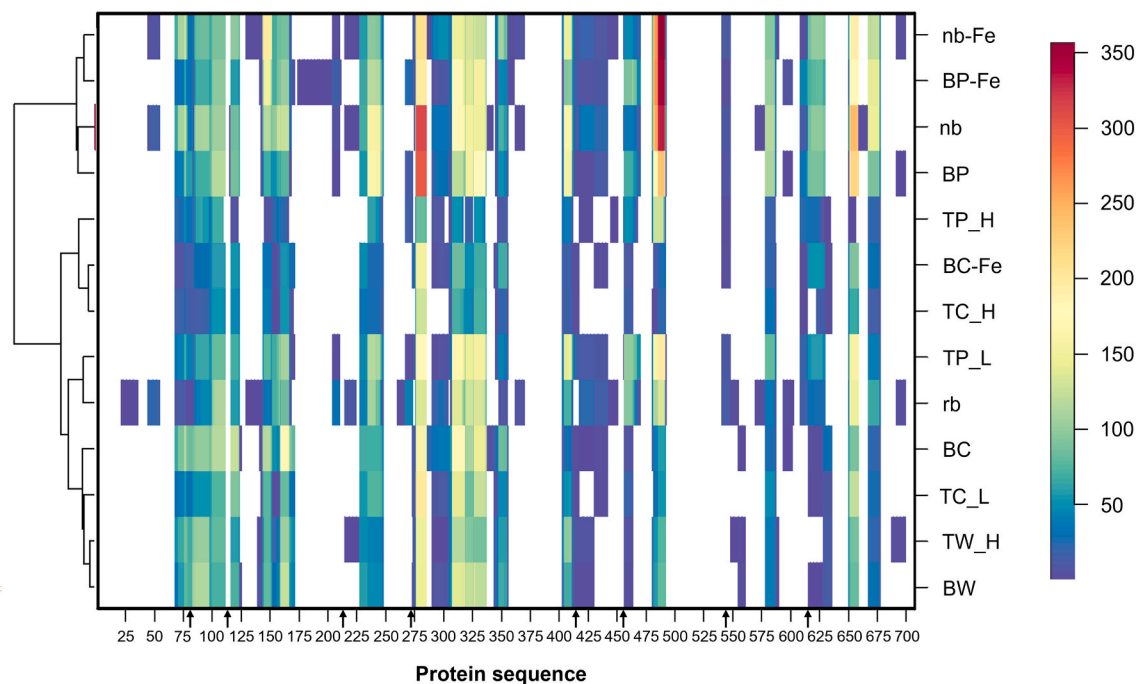


Fig. 5. Clustered heatmap classifying all individual samples after 120 min gastric digestion according to the peptide patterns. Color-coded peptide intensities represent the number of MS/MS identifications of each individual amino acid in the bovine lactoferrin sequence and range from red (maximum intensity) to blue (minimum intensity). Columns correspond to individual amino acids in the lactoferrin sequence, from the N terminal (left) to the C terminal (right), and numbers on the x-axis refer to the amino acid position in the lactoferrin sequence. Black arrows on the x-axis indicate the approximate location of the iron-binding amino acids in lactoferrin. The rows correspond to individual samples, which have been identified in the right y-axis; see [Table 1](#) for sample identification and description. The dendrogram on the left arranges the clusters produced by the hierarchical clustering, with Euclidean distance between data points.

and G30. TC_H and BC-Fe formed a pair at all gastric time points, while BW and BC often clustered with TC_L and/or TW_H. Other associations varied between time points.

In the transition to intestine conditions (G120), TC_H, BC-Fe and TP_H clustered together as the most stable complex formulations. Lower-stability groupings included TP_L with rb, and BC, TC_L, BW and TW_H. The least stable samples formed two subclusters: one containing nb-Fe and BP-Fe, and the other containing nb and BP.

3.3. Bioactive and potential iron-binding peptides

Across all peptides identified in this study (full list in [Supplementary Material 3](#)), three sequences previously reported as bioactive were detected: TRVVWCAVG, FKSETKNLL and KYLGPQY. The peptide TRVVWCAVG, corresponding to residues 362–370 of bovine LF, has documented antibacterial activity against *Escherichia coli* and *Bacillus subtilis* (Elbarbary et al., 2010). In this study, it was detected at G120 in all non-stabilized bovine LF samples (rb, nb-Fe and nb) and at G30 in rb and BP-Fe. FKSETKNLL (residues 651–659, bovine sequence), which has been shown to exhibit osteogenic activity (Shi et al., 2020), was released from all non-stabilized and stabilized bovine lactoferrin samples throughout gastric digestion, except in TP_H at G5 and G120, and TW_H at G5. In early gastric digestion (G5 and G30), this peptide was more abundant in the least stable samples (nb, nb-Fe and BP-Fe), whereas by G120, higher levels were observed in nb-Fe, BC, TC_L, TW_H and BW. KYLGPQY (residues 680–686, human LF), a known opioid antagonist peptide (Tani et al., 1990), was detected only in rh-Fe and rh samples appearing at G30 and G120.

Potential iron-binding peptides were identified by the presence of at least one of the eight known iron-binding sites in bovine LF (Asp79, Tyr111, Tyr211, His272 in the N-lobe; Asp414, Tyr452, Tyr545, His614 in the C-lobe) or in human LF (Asp79, Tyr111, Tyr211, His272 in the N-lobe; Asp414, Tyr454, Tyr547, His616 in the C-lobe) as annotated in UniProt (<https://www.uniprot.org/>). As shown in Fig. 6, the release of these peptides during gastric digestion was greater (in terms of intensity) in ternary complexes with a lower iron loading and highest in binary complexes. Loading BC and nb with extrinsic iron (BC-Fe and nb-Fe) reduced the formation of iron-binding peptides during gastric digestion, consistent with the increased LF stability described above. During intestinal digestion, TC_H released the highest amount of potential iron-binding peptides.

4. Discussion

Oral iron is absorbed primarily in the proximal duodenum

(Zimmermann & Hurrell, 2007). Thus, our work focused on the integrity of various stabilized LF formulations at the end of gastric digestion (G120) while also monitoring earlier and subsequent time points to capture the overall degradation profile. Although LF is relatively resistant to gastric digestion (Fu et al., 2023; Sutantawong et al., 2025; Troost et al., 2001), it remains susceptible to proteolysis by digestive enzymes, which can impair its functional role in iron delivery. The formulations tested in this study included binary (without iron) and ternary (with bound iron) complexes of LF with succinylated sodium caseinate, low-methoxyl pectin, or whey protein hydrolysate, with varying iron loadings. Previous research has shown that such complexes do not impair LF receptor binding in intestinal epithelial cells (Jiang et al., 2024), making them promising candidates for iron supplementation.

Non-stabilized native bovine LF (nb) was highly susceptible to gastric digestion, being almost completely degraded within 5 min, regardless of iron loading, and absent at G120. This highlights both its vulnerability to proteases and the limited protective effect of extrinsic iron alone. By contrast, recombinant bovine LF (rb) was markedly more resistant, with stability comparable to that of some ternary complexes, particularly pectin-based complexes with low iron loading. Recombinant human LF (rh, rh-Fe) was even more stable persisting through gastric digestion and yielding large polypeptides even at the early intestinal stage, indicating slow degradation. These findings align with previous work comparing native bovine and recombinant human LF (Kim et al., 2024). This increased resistance of recombinant lactoferrin may partly originate from structural features introduced during recombinant production, including differences in post-translational modifications such as glycosylation. N-linked glycosylation has previously been shown to protect lactoferrin from proteolytic cleavage at specific sites. Although detailed glycosylation profiling was beyond the scope of this study, recombinant lactoferrins were included here as benchmarking references to contextualise the performance of the stabilized native bovine lactoferrin formulations.

All stabilizing formulations conferred some protection, though with clear differences. Whey protein hydrolysate complexes were less stable than sodium caseinate or pectin complexes. Pectin- and caseinate-based ternary complexes resisted gastric digestion more effectively, whereas the whey protein hydrolysate ternary complex was protective only in early digestion (G5). This may relate to the structural resilience of pectin, which is unaffected by proteases, versus the greater protease accessibility of peptide-rich whey hydrolysates. For caseinate complexes, prior studies have shown mutual protection between LF and β -casein, likely due to shielding of protease-binding sites (van der Schaaf et al., 2024).

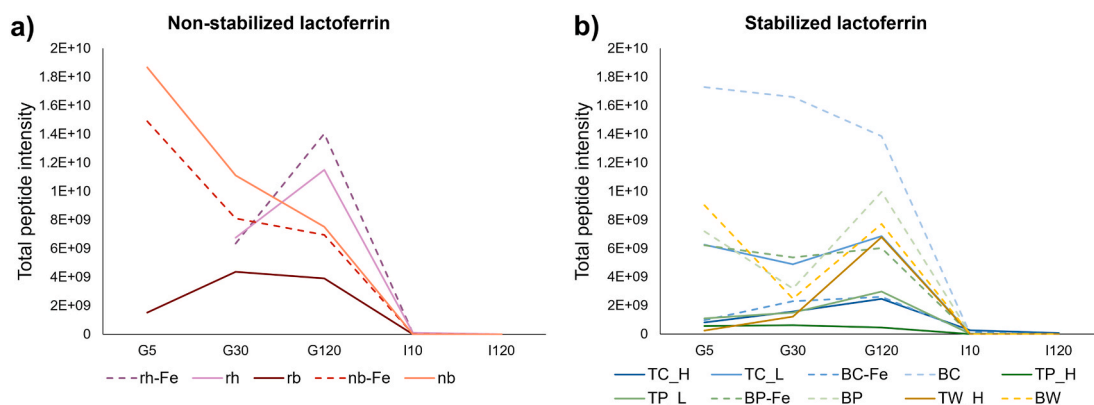


Fig. 6. Total MS/MS intensity of potential iron-binding peptides (peptides containing one of the iron-binding sites in lactoferrin) released from a) non-stabilized lactoferrin; and b) stabilized lactoferrin samples, at different digestion points (G5: gastric 5 min; G30: gastric 30 min; G120: gastric 120 min; I10: intestinal 10 min; I120: intestinal 120 min). Non-stabilized lactoferrin: native bovine (nb), iron-saturated native bovine (nb-Fe), recombinant bovine (rb), partially iron saturated recombinant human (rh) and iron saturated recombinant human (rh-Fe) lactoferrins. Stabilized lactoferrin: complexes with sodium caseinate (TC_H, TC_L, BC-Fe and BC), pectin (TP_H, TP_L, BP-Fe and BP), and whey protein hydrolysate (TW_H and BW) (see [Table 1](#) for sample description).

Ternary complexes generally outperformed binary ones, with higher iron loadings enhancing stability, except in the binary pectin complex, where this trend was less evident. Notably, the sodium caseinate binary complex with extrinsic iron performed comparably to its ternary equivalent, likely because pepsin's action is divided between caseinate and LF, reducing LF degradation. By contrast, pectin, a carbohydrate, does not compete for pepsin cleavage sites.

Although extrinsic iron improved nb stability, it did not match the protection conferred by complex formation, indicating that both iron binding and matrix encapsulation contribute to stability. Iron binding alters LF's conformation: apo-LF adopts a more open, flexible form where the rigid body domains separate to expose the iron-binding sites of the molecule, while holo-LF is more compact, with the two domains of each lobe crosslinked via Fe^{3+} covalent binding (Baker & Baker, 2005), improving resistance to thermal denaturation (Bokkhim et al., 2013; Fu et al., 2023; Sánchez et al., 1992) and proteolysis (Brines & Brock, 1983; Brock et al., 1976). Although *in vivo* gastric digestion studies found holo-LF to be less susceptible to pepsin than apo-LF, the differences were not statistically significant (Troost et al., 2001).

From a functional perspective, we identified three bioactive peptides. Stabilization prevented the formation of the antibacterial peptide TRVVWCAVG but not the formation of the osteogenic peptide FKSETKNLL, which stimulates osteoblast proliferation and differentiation (Shi et al., 2020). FKSETKNLL was most abundant in less stable samples at early digestion and in moderately stable samples at G120, while the most stable complexes produced fewer bioactive peptides overall. Similarly, iron-binding peptide release during gastric digestion was the lowest in the most stable complexes but the highest during intestinal digestion, which might be potentially advantageous for iron uptake in the small intestine.

It remains unclear whether the stabilized complexes prevent Fe^{3+} release until intestinal absorption. At low pH (≤ 4), LF partially unfolds, which can trigger iron release (Baker & Baker, 2005; Sreedhara et al., 2010). Structural comparisons show that apo- and holo-LF have similar solvent-accessible surface areas, despite apo-LF's more open conformation (Jameson et al., 1998). In our study, despite the gastric pH being set at 3.0, formulations with higher iron loadings were less susceptible to degradation, suggesting that complexation may help maintain the closed conformation and retain bound iron. All formulations, however, underwent some degree of hydrolysis. Notably, partially hydrolyzed LF can retain iron-binding capacity (Brock et al., 1976; Troost et al., 2001), underscoring the importance of future work assessing the iron-binding activity of stabilized complexes during and after digestion. Iron concentrations were not quantified during digestion, as the focus of this study was structural integrity rather than iron release kinetics. Therefore, it remains unclear whether the stabilized complexes prevent Fe^{3+} release until intestinal absorption. Structural comparisons show that apo- and holo-LF have similar solvent-accessible surface areas, despite apo-LF's more open conformation (Jameson et al., 1998), and at low pH (≤ 4), LF partially unfolds, which can trigger iron release (Baker & Baker, 2005; Sreedhara et al., 2010). In our study, despite the gastric pH being set at 3.0, formulations with higher iron loadings were less susceptible to degradation, suggesting that complexation may help maintain the closed conformation and retain bound iron. All formulations, however, underwent some degree of hydrolysis. Hence, the reduced proteolysis observed for highly iron-loaded and matrix-stabilized formulations suggests delayed structural opening rather than complete iron loss. Notably, partially hydrolyzed LF can retain iron-binding capacity (Brock et al., 1976; Troost et al., 2001), underscoring the importance of future work assessing the iron-binding activity of stabilized complexes during and after digestion.

Although no formal cost analysis was conducted, it is well established that several clinically used iron compounds are substantially more expensive than ferrous sulfate. While lactoferrin contains less elemental iron, its improved tolerability, reduced gastrointestinal side effects, and additional bioactivities may justify its use in specific vulnerable

populations.

5. Conclusion

Complex formation with succinylated sodium caseinate, low-methoxyl pectin or whey protein hydrolysate enhanced the resistance of native bovine LF to gastric proteases and acidic pH in most formulations tested. Higher iron loadings generally improved stability, but iron saturating alone did not sufficiently protect non-stabilized LF, underscoring the importance of matrix-based stabilization. The strongest protection was achieved with ternary complexes of sodium caseinate or pectin containing high iron levels, as well as with binary sodium caseinate complexes supplemented with high concentrations of extrinsic iron. By contrast, the ternary whey protein hydrolysate complex with high iron loading was less effective, showing marked degradation after only 5 min of gastric digestion. These results highlight promising candidates for use in iron deficiency and anemia management. However clinical trials are essential to confirm their efficacy in humans.

CRediT authorship contribution statement

Ana Blanco-Doval: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Younas Dadmohammadi:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Alireza Abbaspourrad:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Claire E. Noack:** Writing – review & editing, Validation, Methodology, Investigation. **Yunan Huang:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Tiantian Lin:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Lotti Egger:** Writing – review & editing, Supervision, Resources, Methodology. **Michael B. Zimmermann:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Reto Portmann:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2025.112368>.

Data availability

Data will be made available on request.

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