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Ling Xiong[1], Sjef Boeren[2], Jacques Vervoort[2], Kasper Hettinga[1*]

[1] Dairy Science and Technology, Food Quality and Design, Wageningen University and Research, P.O. Box 17, 6700 AA, Wageningen, The Netherlands

[2] Laboratory of Biochemistry, Wageningen University and Research, P.O. Box 8128, 6700 ET, Wageningen, The Netherlands

* Corresponding author

Abstract: To establish the effect of the presence of milk serum proteins on heat-induced changes to lactoferrin, lactoferrin alone, and lactoferrin mixed with either milk serum or β-lactoglobulin was heated at 65 °C, 70 °C and 75 °C for 30 min. After heating, the effect of milk serum proteins on aggregation of lactoferrin was characterized, after which the effect of such aggregation on digestion and bacteriostatic capacity of lactoferrin were determined. The presence of milk serum proteins accelerated the aggregation of lactoferrin during heating through thiol/disulphide interchange. Lactoferrin also formed disulphide-linked aggregates when it was heated with β-lactoglobulin. Protein aggregates formed at 75 °C were much more resistant to infant digestion, causing decreased peptide release from lactoferrin. Heating lactoferrin and milk serum proteins together accelerated the loss of bacteriostatic activity upon heating. In conclusion, heat-induced aggregation of lactoferrin with milk serum proteins affected both its digestion and its bacteriostatic activity.

Key words: Lactoferrin, milk serum proteins, thermal aggregation, bacteriostatic activity, *in vitro* digestion

1. Introduction

Lactoferrin is a multifunctional iron-binding glycoprotein with bacteriostatic and immunomodulatory activities, which protects neonates against infection. Therefore, it is added to infant formula and other functional food products. Heat treatment is extensively applied for the processing of such products to inactivate foodborne pathogen and food spoilage bacteria, which may induce denaturation and aggregation of lactoferrin, leading to a decrease or loss of its bacteriostatic activity. Lactoferrin is a heat labile protein, of which the denaturation and aggregation are affected by multiple factors, including iron saturation (Bokkhim, Bansal, GrØndahl, & Bhandari, 2013), pH (Abe et al., 1991; Sreedhara et al., 2010), ionic strength of the solution (Bokkhim et al., 2013; Kawakami, Tanaka, Tatsumi, & Dosako, 1992) or the presence of polysaccharides (Li, Lan, & Zhao, 2019; Xu, Zhao, Guo, & Du, 2019). The protein composition could modify the denaturation kinetics of whey protein. In a recent study, β-lactoglobulin (β-LG) enhanced the denaturation of α-lactalbumin (α-LA), without any effect on denaturation of lactoferrin (Halabi, Deglaire, Hamon, Bouhallab, Dupont, & Croguennec, 2020), a result similar to an earlier study (Sánchez, Peiro, Castillo, Perez, Ena, & Calvo, 1992).

Most of the research discussed above is on the denaturation of lactoferrin. However, in complex systems (biological mixtures) not only denaturation is important but perhaps even more aggregation of proteins concomitant with denaturation. Heat-induced aggregation as occurs on milk processing may have important implications for the properties of lactoferrin-containing milk products. Bovine lactoferrin contains 17 intramolecular disulphide bonds (Moore, Anderson, Groom, Haridas, & Baker, 1997), which could influence its interaction with other proteins through disulphide interchange. Studies showed that lactoferrin formed large insoluble aggregates through non-covalent interactions and intermolecular thiol/disulphide interchange after being heated at 60 °C-70 °C for 5 min in the absence of other proteins (Brisson, Britten, & Pouliot, 2007b). However, the effects will probably be different when lactoferrin is heated

48 in a complex milk-like system that includes multiple milk proteins. These milk proteins are not
49 only present in milk products, but also are extensively utilized as ingredient in food products.
50 Most of them are thermo-labile, tending to interact with each other and other proteins after heat
51 treatment. For example, interactions and association of β-LG, α_{s2}-casein, and κ-casein (κ-CN)
52 via disulphide interchange in heated milk was previously reported (Chevalier & Kelly, 2010).
53 Aggregation among β-LG, α-LA, and bovine serum albumin (BSA), and the consequences of
54 this aggregation, were extensively explored in the past years (Havea, Singh, & Creamer, 2001;
55 Krämer, Torreggiani, & Davies, 2017; Oldfield, Singh, & Taylor, 2005; Peram, Loveday, Ye,
56 & Singh, 2013). Due to its high concentration in milk, and the presence of free thiol group
57 within its structure, β-LG is more reactive than other whey proteins in disulphide interchange
58 and often is recognized as the initiating protein in the thermal aggregation of whey protein
59 (Wagner, Biliaderis, & Moschakis, 2020; Wijayanti, Bansal, & Deeth, 2014). It was reported
60 that α-LA aggregated slowly when heated alone, while it was readily involved in disulphide-
61 bonded and hydrophobically associated aggregates when heated with β-LG, indicating that
62 aggregation of these two proteins is governed by β-LG (Schokker, Singh, & Creamer, 2000).
63 In addition, interaction between β-LG and κ-CN was recognized as the main force involved in
64 the formation of the casein-whey protein complexes (Cho, Singh, & Creamer, 2003; Corredig
65 & Dalgleish, 1999). However, little is known about interaction of lactoferrin specifically with
66 other milk proteins.

67 Although a few studies reported the formation of lactoferrin/whey protein complexes after heat
68 treatment (Brisson, Britten, & Pouliot, 2007a; Li & Zhao, 2018), detailed information is lacking
69 about the aggregation of lactoferrin and whey proteins, including the microstructure of the
70 resulting protein aggregates, the mechanisms of aggregate formation, and the involvement of
71 β-LG. More in particular, the effect of such aggregation on *in vitro* digestion and bacteriostatic
72 activity of lactoferrin has not been investigated. Therefore, the aim of this study was to explore

the aggregation of lactoferrin with milk serum proteins after different heating intensities, and its effect on *in vitro* digestion and bacteriostatic activity.

2. Material and methods

2.1 Material

Bovine lactoferrin was obtained from FrieslandCampina Ingredients (Vivinal Lactoferrin; estimated purity: 95%; iron saturation: 9%; ash: 0.5%; Veghel, the Netherlands) and was used without further purification. Bovine raw tank milk was obtained from the Wageningen University farm (Wageningen, Netherlands) from clinically healthy cows. Raw milk was centrifuged at 1500×g for 20 min at 4 °C (with rotor 16.250, Avanti Centrifuge J-26 XP, Beckman Coulter, USA) to remove the fat. Skim milk was ultracentrifuged at 100,000×g for 90 min at 30 °C (with rotor 70 Ti, Beckman L-60, Beckman Coulter, USA), to precipitate casein micelles. The supernatant was milk serum, containing whey proteins and non-micellar caseins, named "milk serum proteins (MS)" further on. For the preparation of milk ultrafiltrate, milk serum was filtered through a 0.2 µm membrane (Minisart® syringe filters, Sartorius AG, Göttingen, Germany), and was then ultrafiltered with a 10 kDa filter tube (Amicon® Ultra Centrifugal Filters, Sigma-Aldrich, St. Louis, Missouri, United States). The milk ultrafiltrate was loaded onto an SDS-PAGE gel to check for the absence of the main milk proteins. The ultrafiltrate was collected and used to dissolve the lactoferrin and β-LG to simulate the milk environment.

2.2 Sample preparation and heat treatment

To study the interaction of lactoferrin with β-LG, 1 mg/ml lactoferrin was mixed with 3 mg/ml β-LG in milk ultrafiltrate (pH 6.6). For the interaction of lactoferrin with whey proteins, 1 mg/ml lactoferrin was added into milk serum, named "LFMS" further on. Lactoferrin at 1 mg/ml was heated, freeze-dried and mixed with heated milk serum before analysis as a control,

named “LF+MS” further on. The concentration of lactoferrin was set at 1 mg/ml according to the maximum use levels of bovine LF in infant formula, as defined by Novel Food and GRAS regulations in the EU https://eur-lex.europa.eu/eli/dec_impl/2012/727/ (European Commission, 2012). All samples were prepared in duplicate and were heated in 15 mL plastic tubes by immersion in a temperature-controlled water bath at 65 °C, 70 °C, 75 °C, respectively, for 30 min. The corresponding samples were named as “LFMS-65”, “LFMS-70”, “LFMS-75”, “LF+MS-65”, “LF+MS-70”, and “LF+MS-75”. After heat treatment, all samples were cooled with ice water and stored at 4 °C for further analysis.

2.3 Measurement of turbidity and particle size

The turbidity of protein samples was measured according to the absorbance at 600 nm with a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, California, US). The particle size distribution was determined using a Zeta-sizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). All the measurements were performed in triplicate at 25 ˚C. The refractive index was set to 1.38 for all samples according to the refractive index used for a whey protein concentrate solution in a previous study (Westerik, Scholten, & Corredig, 2015).

2.4 SDS-PAGE

Milk serum and lactoferrin samples were loaded onto an SDS-PAGE and proteins were visualized using Coomassie Brilliant Blue R-250 (1610436, Biorad, California, USA) to determine the presence of the most abundant milk proteins in the samples. The reducing SDS-PAGE was carried out as described previously (Xiong, Li, Boeren, Vervoort, & Hettinga, 2020). For the non-reducing SDS-PAGE, all samples were prepared without dithiothreitol, and running buffer was prepared without antioxidant. The rest of the procedure was performed as described for the reducing SDS-PAGE. After destaining, the gels were scanned using ChemiDoc XRS+ Imaging System with Image Lab Software (Bio-Rad, California, USA).

121 2.5 Bacteriostatic assay

122 *Enterobacter cloacae subsp. cloacae* (ATCC® 13047™, American Type Culture Collection,
123 Manassas, USA) and *Staphylococcus epidermidis* (ATCC® 14990™, American Type Culture
124 Collection, Manassas, USA) were selected, to have one Gram-negative and one Gram-positive
125 species that are known to occur in milk and clinically relevant. The two stains were activated
126 in nutrient broth (CM0001, Thermo Fisher Scientific, Massachusetts, USA) from frozen stock
127 for 16-24 hours at their optimum growth temperature (*Enterobacter cloacae* 30 °C;
128 *Staphylococcus epidermidis*, 37 °C). Reactivated bacterial strains were centrifuged for 5 min at
129 4,000 ×g (with Rotor FA-45-30-11, Microcentrifuge 5430R, Eppendorf, Hamburg, Germany),
130 after which the bacterial pellets were dissolved in PFZ (peptone physiological salt solution;
131 Tritium Microbiology, The Netherlands). After the optical density (OD) had been measured
132 with a spectrophotometer (Cary 50 UV-Visible Spectrophotometer, Agilent Technologies, USA)
133 to estimate bacterial numbers, dilutions of bacteria were made with PFZ for inoculation. All
134 milk serum samples were micro-filtrated by RC membrane (Ø 0.2 µm with 26 mm syringe
135 filters; Phenomenex, Torrance, Canada) to eliminate bacteria in the starting material (<10
136 CFU/ml). Bacterial solutions in PFZ (0.1 ml) were inoculated into 0.9 ml milk serum samples,
137 to reach a final bacterial concentration of around 3×10^3 CFU/ml. After incubation at their
138 optimum growth temperatures for 2 hours, 0.1 ml of the sample was taken for plating on
139 tryptone soya agar (CM0131, Thermo Fisher Scientific, Massachusetts, USA) and then
140 incubated at 30 °C for 24 hours for *Enterobacter cloacae*, or on mannitol salt agar (CM0085B,
141 Thermo Fisher Scientific, Massachusetts, USA) and then incubated at 37 °C for 48 hours for
142 *Staphylococcus epidermidis*. Only the plates with between 20 and 300 colonies were used for
143 determining bacterial levels (CFU/ml). Multiplication rate per hour $= \frac{Nt}{N0}/t$, N_0= number of
144 bacteria immediately after inoculation, N_t= number of bacteria after 2 hours or 4 hours of
145 incubation, t= incubation time (2 hours or 4 hours).

2.6 *In vitro* digestion

After heat treatment, the sample was digested with a simulated *in vitro* infant digestion model according to Menard et al. (Ménard et al., 2018), with some modifications. Lactoferrin samples were mixed with gastric simulated fluid including 268 U/ml pepsin (P6887, Sigma, St. Louis, Missouri, USA), with a ratio (v/v) of 63 to 37 (sample to simulated gastric fluid), the pH of the mixture was adjusted to 5.3, and the samples were incubated at 37 °C for 1h. After 1h gastric digestion, the pH was adjusted to 6.6 to stop the pepsin activity. For gastrointestinal digestion, gastric digested samples were mixed with simulated intestinal fluid including 16 U/ml trypsin (P7550, 4×USP, Sigma, St. Louis, Missouri, USA), with a ratio (v/v) of 39 to 61 (samples to total simulated fluid). pH of the mixture was adjusted to 6.6 and was incubated at 37 °C for 1h. After 1h, 50 µl of Pefabloc (0.1 M) per ml of intestinal digesta was added to the mixture to stop the intestinal digestion. The digested samples were collected at 0, 15, 30 and 60 min in the gastric phase and at the end of the intestinal phase. All samples were frozen at -20 °C for further analysis.

2.7 Liquid chromatography with tandem mass spectrometry

2.7.1 Proteomics

To determine the composition of aggregates, the bands of aggregates in the SDS-PAGE gel were cut into small pieces of < 1 mm^2 with a sharp clean scalpel. The gel pieces were transferred into a clean 0.5 ml low binding micro centrifuge tube and were reduced by 20 mM dithiothreitol, followed by alkylation with 20 mM acrylamide. After that, the gel pieces were thoroughly washed with water and digested with 50 µl (5 ng/µl) cold freshly prepared trypsin solution at 45 °C for 2 hours. The peptide fraction was cleaned up with a C18 µColumn according to a previous study (Dingess et al., 2017).

169 The LC-MS/MS parameters are described as followed. One microliter of the peptide samples
170 were loaded directly onto a 0.10 * 250 mm ReproSil-Pur 120 C18-AQ 1.9 µm beads analytical
171 column (prepared in-house) at a constant pressure of 800 bar with 1 ml/L HCOOH in water
172 and eluted at a flow of 0.5 µl/min with a 50 min linear gradient from 9% to 34% acetonitrile in
173 water with 1 ml/L formic acid with a Thermo EASY nanoLC1000. An electrospray potential
174 of 3.5 kV was applied directly to the eluent via a stainless steel needle fitted into the waste line
175 of the micro cross that was connected between the pump and the analytical column. Full scan
176 positive mode FTMS spectra were measured between m/z 380 and 1400 on a Q-Exactive HFX
177 (Thermo electron, San Jose, CA, USA) in the Orbitrap at high resolution (60000). MS and
178 MSMS AGC targets were set to 3×10^6, 5×10^4 respectively or maximum ion injection times of
179 50 ms (MS) and 25 ms (MSMS) were used. HCD fragmented (Isolation width 1.2 m/z, 24%
180 normalized collision energy) MSMS scans of the 20 most abundant 2-5+ charged peaks in the
181 MS scan were recorded in data dependent mode (Threshold 1.2×10^5, 15 s exclusion duration
182 for the selected m/z +/- 10 ppm). LCMS runs with all MSMS spectra obtained were analysed
183 with MaxQuant 1.6.3.4 (Cox & Mann, 2008) using the "specific" digestion mode and further
184 default settings for the Andromeda search engine (First search 20 ppm peptide tolerance, main
185 search 4.5 ppm tolerance, MSMS fragment match tolerance of 20 ppm. Variable modifications
186 were set for Protein N-terminal Acetylation and M oxidation which were completed by non-
187 default settings for de-amidation of N and Q the maximum number of modifications per peptide
188 was 5 (Cox, Neuhauser, Michalski, Scheltema, Olsen, & Mann, 2011). The UP000009136
189 bovine database containing 23965 protein sequences (Boggs, Hine, Smolenski, Hettinga,
190 Zhang, & Wheeler, 2016) was used together with a contaminants database that contains
191 sequences of common contaminants like Trypsins (P00760, bovine and P00761, porcine) and
192 human keratins (Keratin K22E (P35908), Keratin K1C9 (P35527), Keratin K2C1 (P04264) and
193 Keratin K1CI (P35527)). The "label-free quantification" as well as the "match between runs"

options were enabled. De-amidated peptides were allowed to be used for protein quantification and all other quantification settings were kept default. nLC-MSMS system quality was checked with PTXQC (Bielow, Mastrobuoni, & Kempa, 2016) using the MaxQuant result files.

2.7.2 Peptidomics

To determine the peptides profile from lactoferrin, the isolation and purification of peptides from digested mixture were carried out based on previously described methods (Dingess et al., 2017). The LC-MS/MS parameters was the same as mentioned above except that the digestion mode was set as "Unspecific" and that instead of the complete bovine database a partial bovine database was used with all proteins observed in bovine milk serum as found by Boggs et al. (Boggs et al., 2016).

2.8 Data analysis

Analysis of proteomics data was performed as described previously (Xiong et al., 2020). Data analysis was performed using R 3.6.1. The package "ggplot2" was used for graphical representation of the data. Multiple comparisons of means was performed with SPSS statistics 25. ANOVA and Tukey's HSD for post-hoc testing, were used to identify which treatments were significantly different, where $p < 0.05$ was considered to indicate significant differences among groups. Data were presented as mean ± standard deviation of duplicates.

3. Results

3.1 Changes of turbidity and particle size in differently heated lactoferrin samples

The turbidity of differently heated samples was measured according to their absorbance at 600 nm (**Fig. 1**). When lactoferrin was heated alone, the turbidity of the solution increased after being heated at 65 °C for 30 min, followed by a further increase after being heated at 70 °C or 75 °C for 30 min (**Fig. 1A**, $p < 0.05$). For all MS samples, the turbidity of the samples increased

slowly from the unheated to the 75 °C heated samples. A statistically significant difference in turbidity was observed between the 65 °C and 70 °C heated samples (**Fig. 1B**, $p < 0.05$). A further increase of turbidity in the 75 °C heated samples was observed, probably due to an increased number of small aggregates or formation of larger aggregates (**Fig. 1B**). Compared to MS samples, turbidity of LFMS samples showed a larger increase from unheated to 75 °C. Statistically significant increases in turbidity were found among all samples (**Fig. 1B**, $p < 0.05$), with a rapid increase of turbidity from 65 °C to 75 °C. The changes in the size distribution of all samples showed a similar trend to the changes of turbidity (**Fig. 1C**). The size of particles in unheated MS and LFMS samples are in the range of 9-37 nm, with a dominant particle size of 15-17 nm (**Fig. 1C**). After heating, the size distribution became broader, with the size of the dominant particles becoming above 50 nm in all heated samples. For heated MS samples, the peak gradually changed from 68 nm to 92 nm with increasing temperatures, whereas for heated LFMS samples, the peak gradually changed from 79 nm to 125 nm.

3.2 Aggregation of lactoferrin with milk serum proteins after heat treatment

Disulphide-linked aggregates can be identified through differences between reducing (**Fig. 2C& 2D**) and non-reducing SDS-PAGE (**Fig. 2A& 2B**). The rate of decrease of the monomeric lactoferrin band was rapid when heated in the presence of whey protein, compared with it being heated alone. Comparing reducing (**Fig. 2C**) and non-reducing gels (**Fig. 2A**), lactoferrin heated alone started to form disulphide-linked aggregates from 65 °C. The intensity and number of bands representing aggregates increased from 65 °C to 75 °C (**Fig. 2A**). Lactoferrin formed different aggregates when heated in the presence of milk serum proteins, of which the size and protein composition changed with the heating temperatures (**Fig. 2B**), when compared with the reducing gel (**Fig. 2D**). When the LFMS sample was heated at 65 °C, the intensity of the band of monomeric lactoferrin decreased, while new bands appeared between lactoferrin (80 kDa)

241 and immunoglobulin (150 kDa) that were not visible in the similarly heated MS samples (**Fig.**
242 **2B**). When the LFMS sample was heated at 70 °C, monomeric lactoferrin and the aggregates
243 between 80 kDa and 150 kDa decreased, while larger aggregate were formed, as shown in **Fig.**
244 **2B**. Monomeric lactoferrin almost disappeared after heating at 75 °C, accompanied by a sharp
245 decrease of monomeric BSA and immunoglobulin, suggesting that these proteins were also
246 involved in the formation of large aggregates **(Fig. 2B)**. Bands of monomeric β-LG and α-LA
247 were also decreasing in 70 °C and 75 °C heated milk serum to which lactoferrin was added,
248 although this decrease was not as obvious as for BSA and immunoglobulin. From the SDS-
249 PAGE gel, the decrease of the monomeric lactoferrin and the decrease of the main whey
250 proteins like BSA can be observed. It is assumed that aggregates were formed during heat
251 treatment. Therefore, LC-MS/MS based proteomics was used to identify the protein
252 composition of the aggregates. In total, 36 proteins were quantified, for which the detailed
253 information was shown in **Supplementary table 1**. A clustering heatmap of these proteins was
254 made to find differences in the protein profile between the differently heated samples. In MS
255 samples, the protein profile of aggregates in 75 °C heated samples was different from unheated
256 samples and lower temperature heated samples (**Fig. 3A**). In LFMS samples, 70 °C and 75 °C
257 heated samples showed a similar protein profile of the aggregates, differing from unheated
258 samples and 65 °C heated samples (**Fig. 3B**). Comparing MS and LFMS samples, it could be
259 observed that the presence of lactoferrin appeared to accelerate the aggregation of milk serum
260 proteins. This thus indicates that thiol/disulphide interchanges occurred between lactoferrin and
261 other milk serum proteins.

262 To get more insights about how lactoferrin quantitatively affected the main milk proteins, the
263 levels of the main milk proteins in the aggregates were shown in **Fig. 3C** and **Fig. 3D**. In the
264 MS samples, β-LG, α-LA, BSA, κ-CN, and α_{s1}-casein (α_{s1}-CN) were present at similar levels
265 in the aggregates of all differently heated samples. Lactoferrin and lactoperoxidase (LPO), on

266 the other hand, significantly increased in the 75 °C heated samples (**Fig. 3C**). After adding
267 lactoferrin into the milk serum, obvious changes were observed for these proteins, except for
268 α-LA and α_{s1}-CN. The levels of β-LG, LF and LPO in the aggregates increased after heating at
269 70 °C, whereas the level of BSA increased in 75 °C heated LFMS samples. κ-CN showed a
270 gradually increase in the aggregates in the 65 °C and 70 °C heated samples, which further
271 increased in 75 °C heated samples (**Fig. 3D**). The changes of those proteins among samples
272 indicate that aggregation of β-LG, BSA, LPO, and κ-CN were all affected by the presence of
273 lactoferrin.

274 β-LG is the predominant whey protein in milk and is highly reactive due to the free thiol group
275 buried inside its folded structure (Hoffmann & van Mil, 1997). Proteomics data also showed
276 that the amount of β-LG in proteins aggregates increased after adding lactoferrin. Therefore, a
277 more simple lactoferrin/β-LG model system was used to investigate the role of β-LG in the
278 aggregation of lactoferrin. When heating lactoferrin with purified β-LG, monomeric lactoferrin
279 showed a sharp decrease from 65 °C, almost disappearing from 70 °C onwards.
280 Correspondingly, larger aggregates were observed from 65 °C, which further increased after
281 heating at 70 °C and 75 °C (**Fig. 4**). After adding a reducing agent, larger aggregates
282 disappeared, but still some small aggregates, corresponding to the bands between 80 kDa and
283 150 kDa in the LFMS samples (**Fig. 2B)**, remained visible. This is probably due to difficulties
284 in achieving full reduction of disulphide bonds for all protein aggregates. A decrease in the
285 level of monomeric β-LG was not clearly visible on the SDS-PAGE gel after heating with
286 lactoferrin, which may have been caused by overloading of the sample (**Fig. 4A**). After
287 decreasing the concentration of β-LG to 1 mg/ml, a decrease of monomeric β-LG was observed
288 from 65 °C when it was heated with lactoferrin (**Supplementary fig. 1**). When β-LG was heated
289 alone, no larger aggregates were observed until 75 °C. Taken together, this data confirmed that
290 β-LG and lactoferrin influenced each other's response to the heat treatment.

3.3 Bacteriostatic activity of differently heated lactoferrin and milk serum samples

Both lactoferrin and milk serum have been shown to have bacteriostatic activity (Wang, Timilsena, Blanch, & Adhikari, 2019; Xiong et al., 2020). Inhibition on bacterial growth may change after different heat treatments, due to the denaturation and/or aggregation of antibacterial proteins (Xiong et al., 2020). Bacteriostatic activity of LFMS samples and LF+MS samples was tested to investigate the effect of aggregation of lactoferrin with milk serum proteins on the bacteriostatic capacity. Unheated LFMS showed the highest inhibition capacity among all samples. For heated LFMS samples, a rapid decrease of bacteriostatic activity against *Enterobacter cloacae* occurred from LFMS-65 to LFMS-70 (**Fig. 5A**). Bacteriostatic capacity already started to decrease in the LFMS-65 samples (**Fig. 5A**), although not statistically significant, whereas its capacity decreased significantly in the LFMS-70 and LFMS-75 samples. LF+MS samples showed a different trend compared to LFMS samples (**Fig. 5A**). Compared to unheated LFMS, LF+MS-65 and LF+MS-70 showed a similar, but reduced, bacteriostatic activity, while LF+MS-75 showed the lowest activity. The largest change of bacteriostatic activity against *Enterobacter cloacae* occurred between LF+MS-70 and LF+MS-75 (**Fig. 5A**, $p < 0.05$). A similar pattern was observed for the activity against *Staphylococcus epidermidis*, where the bacteriostatic activity was similar in LFMS, LFMS-65, LF+MS-65 and LF+MS-70 samples (**Fig. 5B**). However, it significantly decreased in LFMS-70 samples compared with LFMS-65 ($p < 0.05$). The bacteriostatic activity further decreased in LFMS-75 samples, as well as the LF+MS-75 samples. In general, the largest reduction of bacteriostatic activity occurred between 70 °C and 75 °C, when lactoferrin and milk serum were heated separately, while the largest reduction of bacteriostatic activity occurred from 70 °C onwards when they were heated together. Although the bacteriostatic activity of all samples decreased to the same level after being heated at 75 °C, the decrease was accelerated by heating lactoferrin and milk serum proteins together during 65 °C-75 °C.

316 3.4 Protein degradation and peptide release of heated lactoferrin after *in vitro* infant digestion

317 Different heat treatments resulted in differences in the degradation pattern of lactoferrin upon
318 *in vitro* digestion. The protein degradation of LFMS samples was displayed in **Fig.6**. The
319 identification of these bands according to protein marker was shown in **Supplementary Fig 2**.
320 For the unheated LFMS sample (**Fig. 6A**), no significant degradation of lactoferrin was
321 observed in the gastric phase, which differed from the intestinal digestion, after which complete
322 disappearance of monomeric lactoferrin was observed. For LFMS heated at 65 °C (**Fig. 6B**),
323 monomeric lactoferrin decreased after 15 min gastric digestion, and then remained similar in
324 the later gastric digestion. The bands >150 kDa became lighter during gastric digestion and
325 almost disappeared after intestinal digestion, possibly due to partial digestion of the aggregates.
326 For LFMS heated at 70 °C (**Fig. 6C**), more aggregates >150 kDa were present in the sample,
327 which decreased after gastric digestion for 60 min, although some aggregates remained. All
328 aggregates almost completely disappeared after intestinal digestion. For the LFMS heated at
329 75 °C (**Fig. 6D**), a decrease of aggregates after gastric digestion was not found, but the
330 aggregates disappeared completely after intestinal digestion.

331 The non-reducing gel showed the changes of the aggregates during digestion. Because multiple
332 proteins were involved in the aggregates, it was hard to see the change of individual proteins,
333 like lactoferrin. Therefore, the reducing gel was used as well to achieve more detailed insights.
334 Combining the reducing and non-reducing gel, the effect of aggregation on individual proteins
335 can be better observed. For the unheated LFMS in the reducing gel (**Fig. 6E**), the result was the
336 same as for the non-reducing gel, because of the absence of disulphide related aggregation. For
337 the LFMS heated at 65 °C (**Fig. 6F**), partial digestion of lactoferrin was found, which is similar
338 to the results of the non-reducing gel. For the LFMS heated at 70 °C (**Fig. 6G**), no monomeric
339 lactoferrin was observed in the non-reducing gel due to aggregation, while some monomeric

lactoferrin appeared in the reducing gel, even after gastric digestion for 60 min, indicating that lactoferrin in these aggregates wasn't digested completely. Similar result occurred in LFMS heated at 75 °C (**Fig. 6H**). When the digestion of the differently heated LFMS samples was compared, lactoferrin was shown to be digested better in 65 °C and 70 °C heated samples than in unheated samples. Remarkably, lactoferrin appeared to be slightly less digested in the 75 °C heated sample than the 70 °C heated sample.

Differential release of peptides from lactoferrin due to differences in its degradation may occur. Our data showed a similar cleavage pattern for all samples (**Supplementary Fig. 3)**, although the intensity profile of peptides originating from lactoferrin showed differences **(Supplementary Fig. 4**). Corresponding to the least degradation of intact lactoferrin, the unheated LFMS sample released the lowest levels of peptides from lactoferrin after digestion. From **Supplementary Fig. 4**, peptide profiles of LF+MS sample was different from that of LFMS sample, with a lower signal in all LF+MS samples. No significant trend was observed in differently heated LF+MS samples. When it comes to LFMS, peptides profile showed an obvious difference between differently heated samples, with LFMS being heated at 70 °C showing the highest peptide signals, followed by the samples heated at 65 °C. The samples heated at 75 °C showed the lowest peptide signals **(Supplementary Fig. 4**).

4. Discussion

4.1 Whey proteins accelerate disulphide linked aggregation involving lactoferrin

In the present study, a combination of turbidity and particle size determination, SDS-PAGE, and proteomics was used to investigate the thermal aggregation of lactoferrin with milk serum proteins and its consequences. When lactoferrin was heated alone, an increase in turbidity was observed at 65 °C (**Fig. 1A**), indicating that insoluble aggregates started to form from 65 °C. The turbidity further increased in the 70 °C and 75 °C heated samples (**Fig. 1A**), which may

have been due to the formation of either larger or more aggregates in those samples. Disulphide-linked aggregates of lactoferrin were observed in the 70 °C and 75 °C heated samples (**Fig. 2A**). From this data, it can be concluded that non-covalent interactions are underlying the protein aggregation in the 65 °C heated samples. Intermolecular disulphide interchange reactions occur upon heating, leading to aggregation of proteins as also suggested in a previous study (Brisson et al., 2007b). This formation of aggregates indicates that lactoferrin has undergone significant structural changes.

The results also showed that the presence of lactoferrin altered the thermal aggregation of milk serum proteins (**Fig. 3 & Supplementary table 1**). For the MS samples, the sharp increase of turbidity from 70 °C onward indicated the formation of aggregates (**Fig. 1B**). With a further increase in temperature, the aggregates increased in size, as indicated by the increase of turbidity and particle size (**Fig. 1C**). When MS and LFMS samples heated at the same temperature were compared, the turbidity and the average size of all heated LFMS samples was higher than that of the respective MS samples (**Fig. 1B & 1C**). Therefore, the differences between these samples seem to result from the interaction between lactoferrin and whey proteins during heating. Combining data on the changes in particle size distribution and turbidity shows that this is induced by the formation of larger aggregates rather than an increase of the number of small aggregates. The aggregates with different size, as formed at various heating temperatures, may behave differently during the digestion.

The aggregation of lactoferrin was faster when heated in the presence of milk serum proteins (**Fig. 2B**), due to intermolecular thiol/disulphide interchange reactions, as shown by the disappearance of the aggregates under reducing conditions (**Fig. 2D**). The proteomics data (**Fig. 3C& 3D**) highlighted the involvement of several high abundant whey proteins in the aggregation during heating. Lactoferrin contains 17 intramolecular disulphide bonds without a

388 free thiol group (Pierce et al., 1991). The presence of proteins containing free thiol groups, such
389 as β-LG or BSA, facilitates aggregation via intermolecular thiol/disulphide interchanges. This
390 was also demonstrated by previous studies, which showed no rapid self-aggregation of α-LA
391 upon heating, whereas the concomitant presence of β-LG or BSA was shown to considerably
392 enhance the heat-induced aggregation of α-LA (Dalgleish, Senaratne, & Francois, 1997; Havea
393 et al., 2001; Schokker et al., 2000). The presence of β-LG and/or BSA may thus increase the
394 tendency of other proteins, like lactoferrin, to aggregate upon heating.

395 Our data confirms the direct thiol/disulphide interchange between lactoferrin and β-LG (**Fig.**
396 **4**), in accordance with another study that also reported the aggregation of lactoferrin with β-LG
397 in lactoferrin-enriched milk (Brisson et al., 2007a). Our data indicate that upon heating,
398 lactoferrin will have exposed S-S bridges that can be reduced by for instance free SH-groups
399 of β-LG or BSA. This was further confirmed by the bands between 80 kDa-150 kDa on the
400 SDS-PAGE gel (**Fig. 2B)** that were not visible in the similarly heated MS samples, indicating
401 the formation of aggregates consisting of lactoferrin and other milk proteins in the LFMS
402 samples. Interestingly, the above-mentioned bands in the LFMS samples were similar in
403 appearance to those on the SDS-PAGE gel of lactoferrin with β-LG (**Fig. 4B).** When β-LG was
404 heated alone, no change of turbidity (**Supplementary Fig. 5**) and no formation of disulphide
405 linked aggregates was observed (**Fig. 4**), indicating that it is stable in the temperature range of
406 65 °C-75 °C. Our data also showed that lactoferrin affected the response of β-LG to the heat
407 treatment, which confirms the recent study (Halabi et al., 2020) that reported a more extensive
408 loss of native β-LG in the presence of lactoferrin upon heating. Due to the heat sensitivity of
409 lactoferrin, its disulphide bridges may have been more exposed when β-LG started unfolding
410 to expose its free thiol group in the temperature range of 65 °C-75 °C. The free thiol group from
411 β-LG may subsequently have undergone disulphide interchange reactions with the exposed S-
412 S bridges of lactoferrin, which explains the enhanced aggregation of β-LG in the presence of

lactoferrin. When heated in milk serum, lactoferrin probably mainly interact with β-LG, making β-LG more available for other proteins to aggregate at lower temperature, resulting in the change of protein aggregate composition (**Fig. 3B**). In summary, the data shows that acceleration of the aggregation of lactoferrin in the presence of milk serum proteins can be partly ascribed to β-LG. Whether aggregation of lactoferrin with other milk serum proteins is mainly mediated by β-LG, or that other proteins like BSA also play a role, is not clear. Further studies should be done to confirm the contribution of individual whey proteins to the thiol/disulphide induced aggregation of lactoferrin.

4.2 Aggregation caused loss of bacteriostatic activity of lactoferrin and whey protein

The bacteriostatic activity of whey proteins is highly dependent on their native structure and is negatively affected by denaturation and aggregation (Xiong et al., 2020). The observed changes in bacteriostatic activity after different heat processing were generally consistent with the changes due to aggregation in these samples. When lactoferrin and whey proteins were heated separately, a rapid decrease of bacteriostatic activity was observed after heating at 75 °C (**Fig. 5**), which corresponds to the extensive aggregation of lactoferrin and milk serum proteins when heated separately (**Fig. 2A& 2B**). Similar changes of the bacteriostatic capacity of milk serum after heat treatment were reported in our previous study (Xiong et al., 2020). When lactoferrin in milk serum was heated at 70 °C and above, a rapid decrease of bacteriostatic activity was observed (**Fig. 5)**, which coincides with the observed aggregation of LFMS samples (**Fig. 2B**). The bacteriostatic mechanisms of lactoferrin have been ascribed to its ability in iron scavenging and LPS binding (Wang et al., 2019). After heating at 85 °C for 20 min, the ability to bind iron of soluble lactoferrin remained at 50%, while aggregates of lactoferrin lost their ability to bind iron (Mata, Sánchez, Headon, & Calvo, 1998). It was reported that aggregated lactoferrin had lost its bacteriostatic activity against *E. coli* O157:H7 and *S. enteritidis* (Conesa, Rota, Castillo,

Perez, Calvo, & Sanchez, 2010). These studies together with our data thus suggest that aggregation diminishes the bacteriostatic activity of lactoferrin. Aggregation between lactoferrin and milk serum proteins may change the iron binding domain of lactoferrin during heat treatment, thus causing a more extensive loss of its bacteriostatic activity.

4.3 Aggregation decreased peptic degradation of lactoferrin and release of peptides

In the present study, most of the lactoferrin was degraded after the complete gastrointestinal digestion in all samples, without much differences between different heat treatments (**Fig. 6**). Nevertheless, large differences in first phase of gastric digestion of lactoferrin were observed, indicating that unfolding and aggregation of lactoferrin affected gastric digestion more than intestinal digestion. Native lactoferrin was hardly digested after the infant gastric digestion (**Fig. 6A & 6E**), which is consistent with an early study, showing that native lactoferrin is not extensively digested by infant's gastric secretions (Spik, Brunet, Mazurier‐Dehaine, Fontaine, & Montreuil, 1982). Even for *in vivo* and *in vitro* adult studies, it was shown that still some lactoferrin molecules remained intact after gastric digestion (Bokkhim, Bansal, Grøndahl, & Bhandari, 2016). Heating induced structural changes of lactoferrin to a different extent, depending on the intensity of the heat treatment, which may affect the digestion pattern of lactoferrin. Grosvenor et al. (Grosvenor, Haigh, & Dyer, 2014) reported that pasteurization (72 °C/15 s) altered the kinetics of release of specific peptides during gastric digestion of lactoferrin. On the other hand, Wang et al. reported that no difference in the degradation of lactoferrin was observed between samples heated at 70 °C for 10 min and its unheated counterparts based on an *in vitro* adult digestion model (Wang, Timilsena, Blanch, & Adhikari, 2017). We observed a more extensive decrease of the intact lactoferrin band on SDS-PAGE and a higher peptide intensity in all heated samples compared to their unheated counterparts

460 (**Fig. 6 & Supplementary Fig. 4**), indicating that conformational changes induced by heat
461 treatment enhanced the susceptibility of lactoferrin to gastric hydrolysis.

462 When lactoferrin was heated in the presence of milk serum proteins, the digestion pattern
463 showed a difference among the three differently heated samples (**Fig. 6**). This difference was
464 probably associated with the extent of aggregation. The lowest retention of intact lactoferrin
465 after gastric digestion in the LFMS-70 samples in the reducing gel suggests that most of the
466 lactoferrin that was present in these aggregates was hydrolysed by pepsin. The increased
467 retention of intact lactoferrin and the lowest level of peptides in the LFMS-75 samples indicates
468 that at this heat load lactoferrin present in larger aggregates can't be digested as well as the
469 samples treated at lower temperature. It was previously demonstrated that heat-induced
470 increases in protein digestibility due to unfolding of protein may be overwhelmed by formation
471 of proteolytic-resistant aggregates (Wada & Loennerdal, 2014), and physicochemical properties
472 of protein aggregates significantly affected their digestion behaviour, with a higher degradation
473 rate for smaller aggregates (Zhang & Vardhanabhuti, 2014). Combining the data from our study
474 and existing studies, it can be concluded that aggregation not only affected the overall
475 digestibility of milk serum proteins but also played a critical role in the kinetics of digestion of
476 specific proteins, in this case lactoferrin. The rate of digestion of lactoferrin is thus a balance of
477 an increased digestion rate due to unfolding and a decreased digestion rate due to aggregation.

478 In summary, the data show how heating of lactoferrin in a milk matrix may change its digestion
479 kinetics and cause a loss of its activity. It thereby corroborates that heating lactoferrin and other
480 protein ingredients in food products separately may decrease the disulphide linked aggregation
481 of lactoferrin with other proteins, which may ultimately help to retain its bioactivity. This can
482 be of important relevance for producers of lactoferrin-containing functional foods.

5. Conclusion

Milk serum proteins accelerated the aggregation of lactoferrin via thiol/disulphide interchange reactions during heat treatment, which was partly mediated by β-LG. Interaction of lactoferrin with milk serum proteins accelerated the loss of its bacteriostatic activity. Moderate heat treatment could enhance hydrolysis of lactoferrin during gastric digestion. However, extensive aggregation of lactoferrin with milk serum proteins into large aggregates counteracted the improved digestion induced by unfolding, and subsequently changes the peptide profile after *in vitro* infant gastric digestion.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Figure captions

Figure 1 The effect of heating of lactoferrin in the absence and presence of milk serum proteins on its aggregation. Change of turbidity of lactoferrin (A) and milk serum sample (B), and number based particle size distribution of different samples (C) after heating. LF, lactoferrin; MS, milk serum; LFMS, milk serum with addition of 1 mg/ml lactoferrin. N, sample without heat treatment; 65/70/75 refer to samples being heated at 65/70/75 °C for 30 min, respectively. Statistical significance for (A) and (B) was analyzed using ANOVA and Tukey's HSD test. Means were compared and marked as "a, b, c, d, e" from small to large. Means followed by the same letter were not significantly different ($p > 0.05$). Different letters indicate statistically significant difference among different heat treatments ($p < 0.05$).

Figure 2 The effect of heating of lactoferrin in the absence and presence of milk serum proteins on its disulphide linked aggregation. Non-reducing SDS-PAGE gel of differently heated lactoferrin (A) and milk serum samples (B), and reducing SDS-PAGE gel of differently heated lactoferrin (C) and milk serum samples (D), with the main proteins indicated. Lane 1, protein marker; Lane 2, unheated lactoferrin; Lane 3, 4, 5, lactoferrin being heated at 65/70/75 °C for 30 min, respectively. Lane 6, milk serum without heat treatment; Lane 7, 8, 9, milk serum being heated at 65/70/75 °C for 30 min, respectively. Lane 10, milk serum with addition of 1 mg/ml lactoferrin without heat treatment; Lane 11, 12, 13, milk serum with addition of 1 mg/ml lactoferrin being heated at 65/70/75 °C for 30 min, respectively. Abbreviations: IgG, immunoglobulin G; LF, lactoferrin; BSA, bovine serum albumin; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

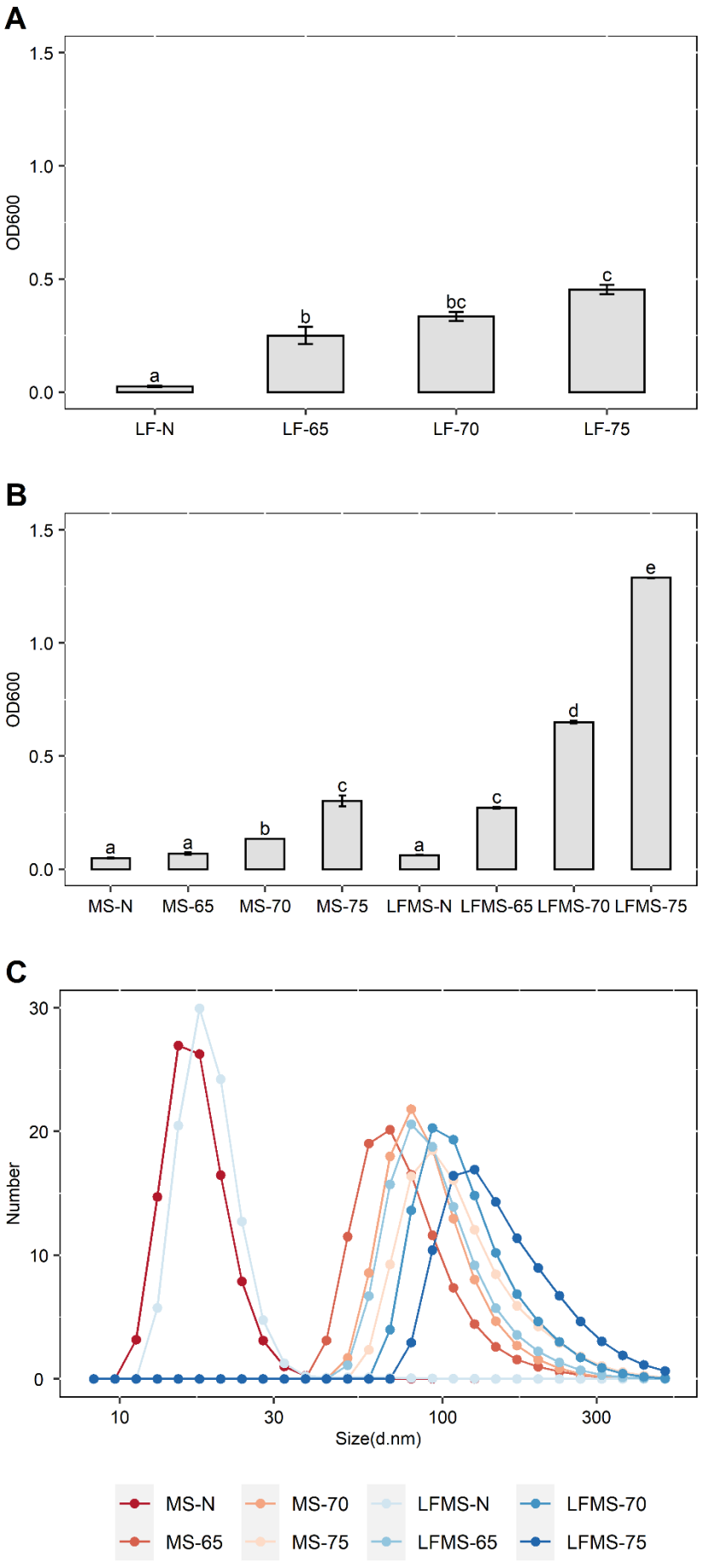
Figure 3 The effect of lactoferrin on protein composition of disulphide linked aggregates in the presence of milk serum proteins after heat treatment. Heatmap of protein aggregates in MS samples (A) and LFMS samples (B), and changes of individual milk protein in aggregates from MS samples (C) and LFMS samples (D). The number indicates the heating temperature, while the number after the underscore indicates the replicate. MS, milk serum; LFMS, milk serum with addition of 1 mg/ml lactoferrin; N, sample without heat treatment; 65/70/75 refer to samples being heated at 65/70/75 °C for 30 min, respectively. The number behind underscore indicates the heating temperature, while the number after the underscore indicates the duplicate Abbreviations: β-LG, β-lactoglobulin; LF, lactoferrin; α-LA, α-lactalbumin; BSA, bovine serum albumin; LPO, lactoperoxidase; κ-CN, kappa casein; α-s1-CN, alpha-s1 casein. Statistical significance for (C) and (D) was analyzed using ANOVA and Tukey's HSD test. Means were compared and marked as "a, b" from small to large. Means followed by the same letter were not significantly different ($p > 0.05$). Different letters indicate statistically significant difference among different heat treatments ($p < 0.05$).

Figure 4 The effect of heating of lactoferrin in the absence and presence of β-lactoglobulin on its disulphide linked aggregation. Non-reducing (A) and reducing (B) SDS-PAGE gel of different heated mixture of lactoferrin and β-lactoglobulin. Lane 1, β-LG without heat treatment; Lane 2, 3, 4, β-LG being heated at 65/70/75 °C for 30 min,

respectively. Lane 5, mixture of lactoferrin and β-LG without heat treatment; Lane 6, 7, 8, mixture of lactoferrin and β-LG being heated at 65/70/75 °C for 30 min. Abbreviations: LF, lactoferrin; β-LG, β-lactoglobulin.

Figure 5 The effect of heating of lactoferrin and milk serum proteins on bacteriostatic activity. Multiplication rates of *Enterobacter cloacae* (A) and *Staphylococcus epidermidis* (B) in differently heated lactoferrin and milk serum samples. LFMS, milk serum with addition of 1 mg/ml lactoferrin; LF+MS, lactoferrin and milk serum samples were heated at same temperature separately, and then mixed before the bacteriostatic activity assay. 65/70/75 refer to samples being heated at 65/70/75 °C for 30 min, respectively. Statistical significance was analyzed using ANOVA and Tukey's HSD test. Means were compared and marked as "a, b, c, d" from small to large. Means followed by the same letter were not significantly different ($p > 0.05$). Different letters indicate statistically significant difference among different heat treatments ($p < 0.05$).

Figure 6 The effect of heating of lactoferrin in the absence and presence of milk serum proteins on its degradation after infant gastric digestion. A, B, C and D refer to LFMS sample without heat treatment, being heated at 65/70/75 °C for 30 min under non-reducing conditions, respectively. E, F, G, H refer to LFMS sample without heat treatment, being heated at 65/70/75 °C for 30 min under reducing condition, respectively. G0/G15/G30/G60 are digested samples collected after 0/15/30/60 min of gastric digestion, respectively; GI means samples collected after complete gastrointestinal digestion. Abbreviations: IgG, immunoglobulin G; LF, lactoferrin; BSA, bovine serum albumin; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.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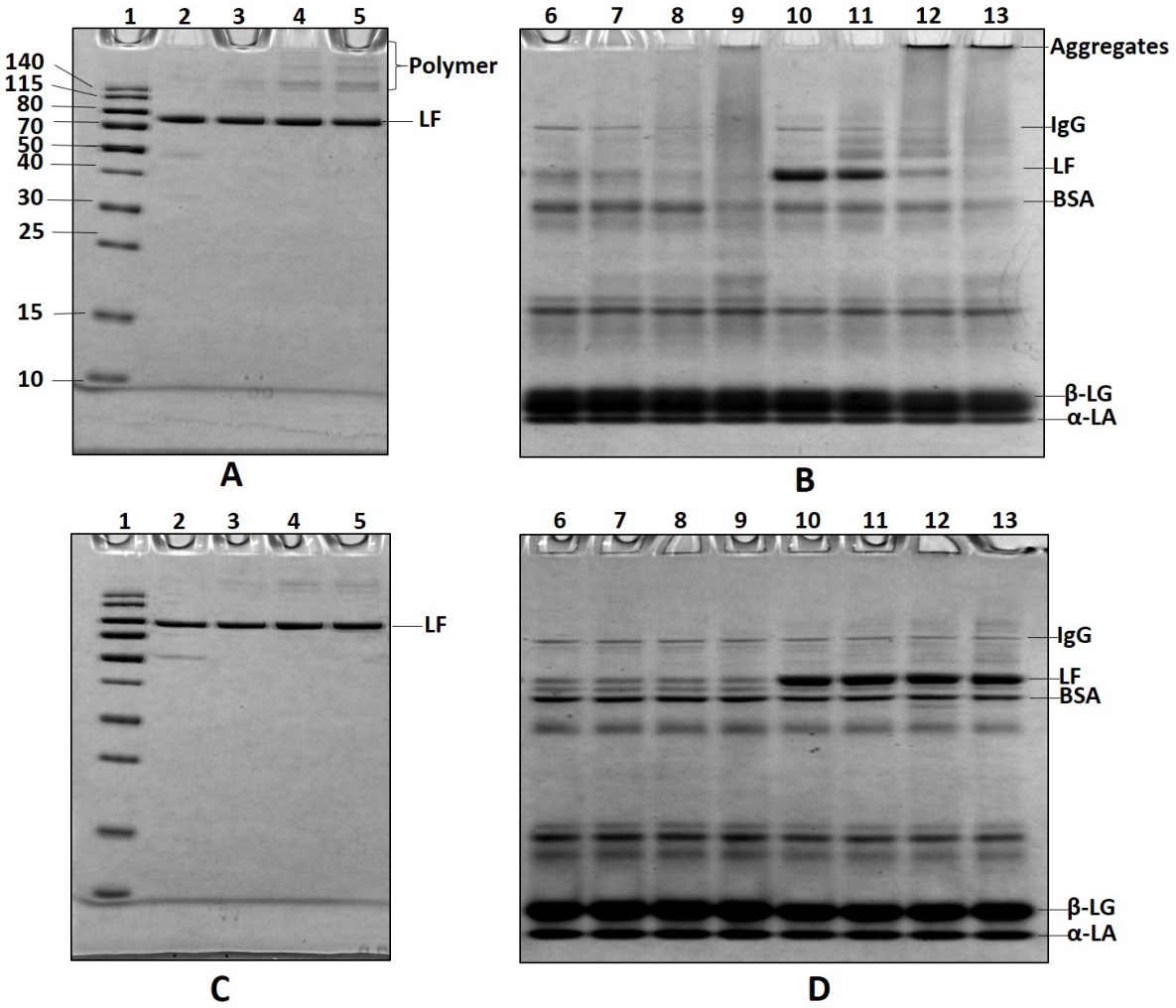
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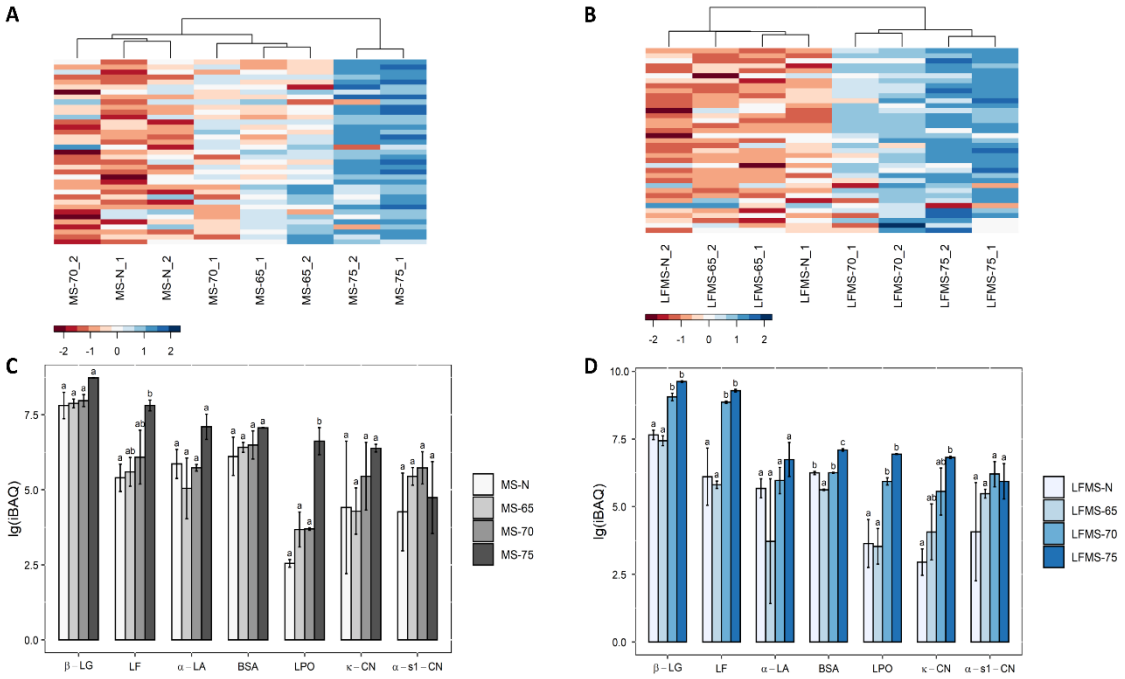
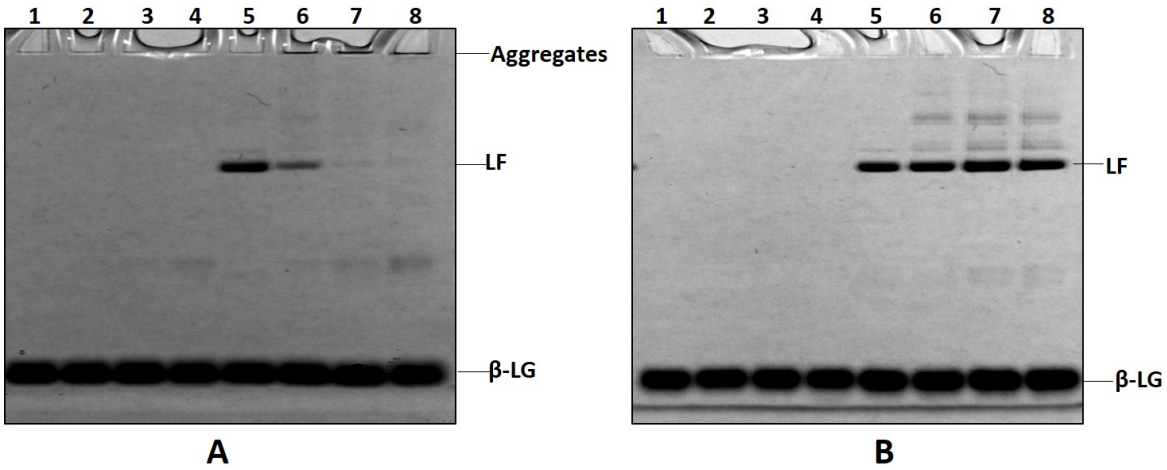

A
MS-70_2
MS-N_1
MS-N_2
MS-70_1
MS-65_1
MS-65_2
MS-75_2
MS-75_1
-2 -1 0 1 2
B
LFMS-N_2
LFMS-65_2
LFMS-65_1
LFMS-N_1
LFMS-70_1
LFMS-70_2
LFMS-75_2
LFMS-75_1
-2 -1 0 1 2
C
lg(iBAQ)
0.0
2.5
5.0
7.5
β－LG
LF
α－LA
BSA
LPO
κ－CN
α－s1－CN
MS-N
MS-65
MS-70
MS-75
D
lg(iBAQ)
0.0
2.5
5.0
7.5
10.0
β－LG
LF
α－LA
BSA
LPO
κ－CN
α－s1－CN
LFMS-N
LFMS-65
LFMS-70
LFMS-75

687

688



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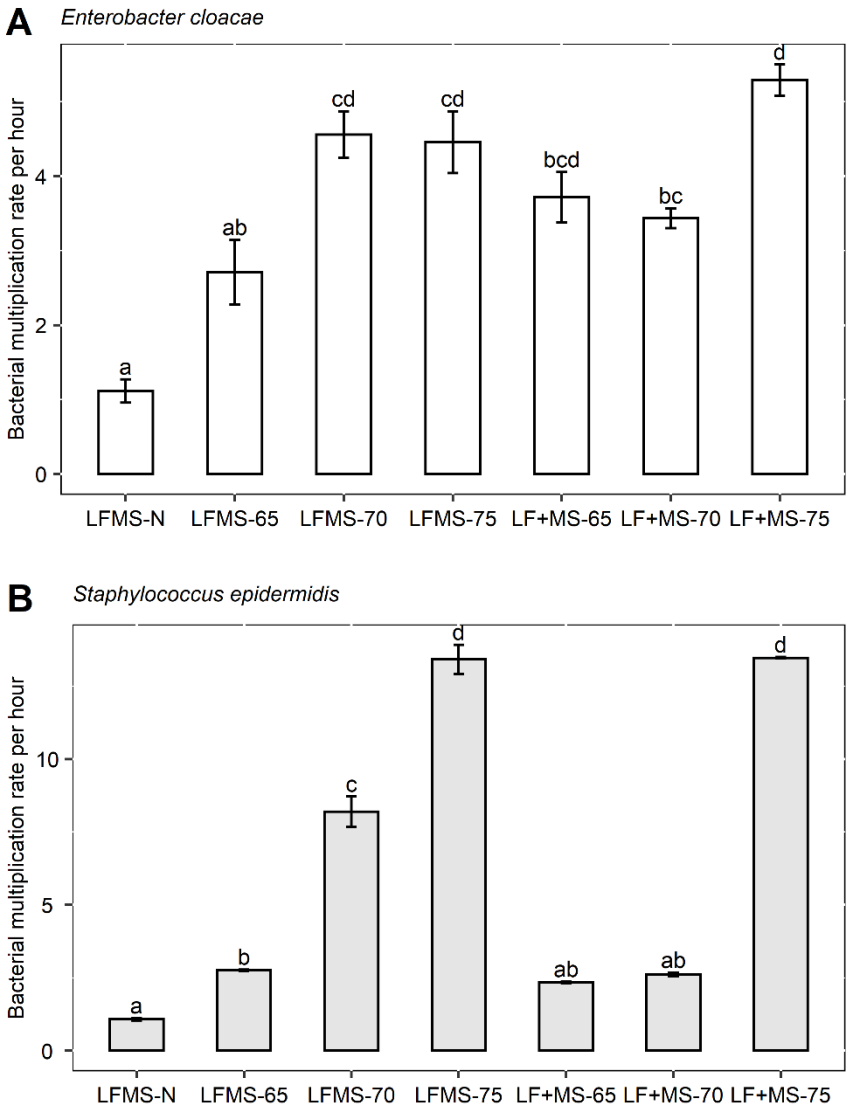
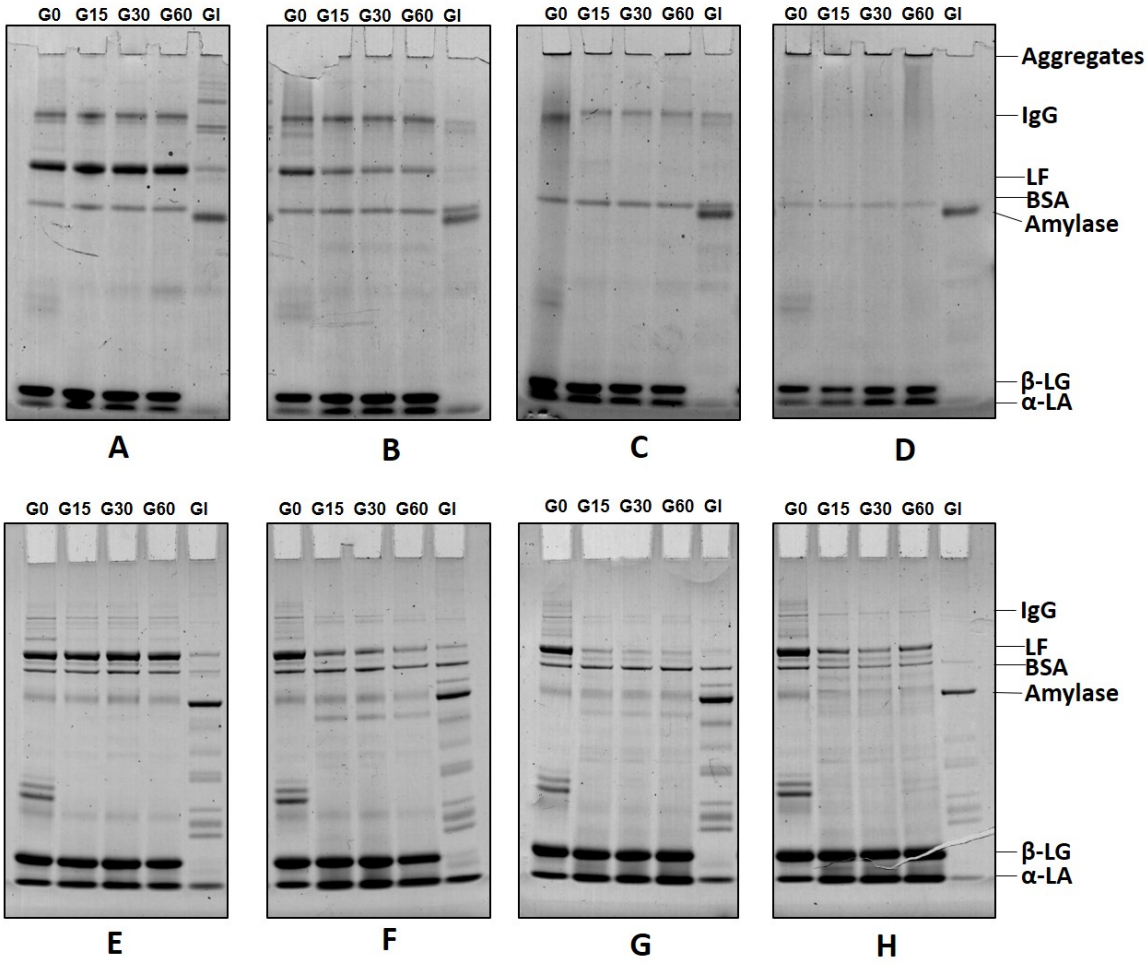

A
Enterobacter cloacae
Bacterial multiplication rate per hour
0
2
4
a
ab
cd
cd
bcd
bc
d
LFMS-N
LFMS-65
LFMS-70
LFMS-75
LF+MS-65
LF+MS-70
LF+MS-75
B
Staphylococcus epidermidis
Bacterial multiplication rate per hour
0
5
10
a
b
c
d
ab
ab
d
LFMS-N
LFMS-65
LFMS-70
LFMS-75
LF+MS-65
LF+MS-70
LF+MS-75

691

692


G0 G15 G30 G60 GI
Aggregates
IgG
LF
BSA
Amylase
β-LG
α-LA
A
B
C
D
E
F
G
H

693

694

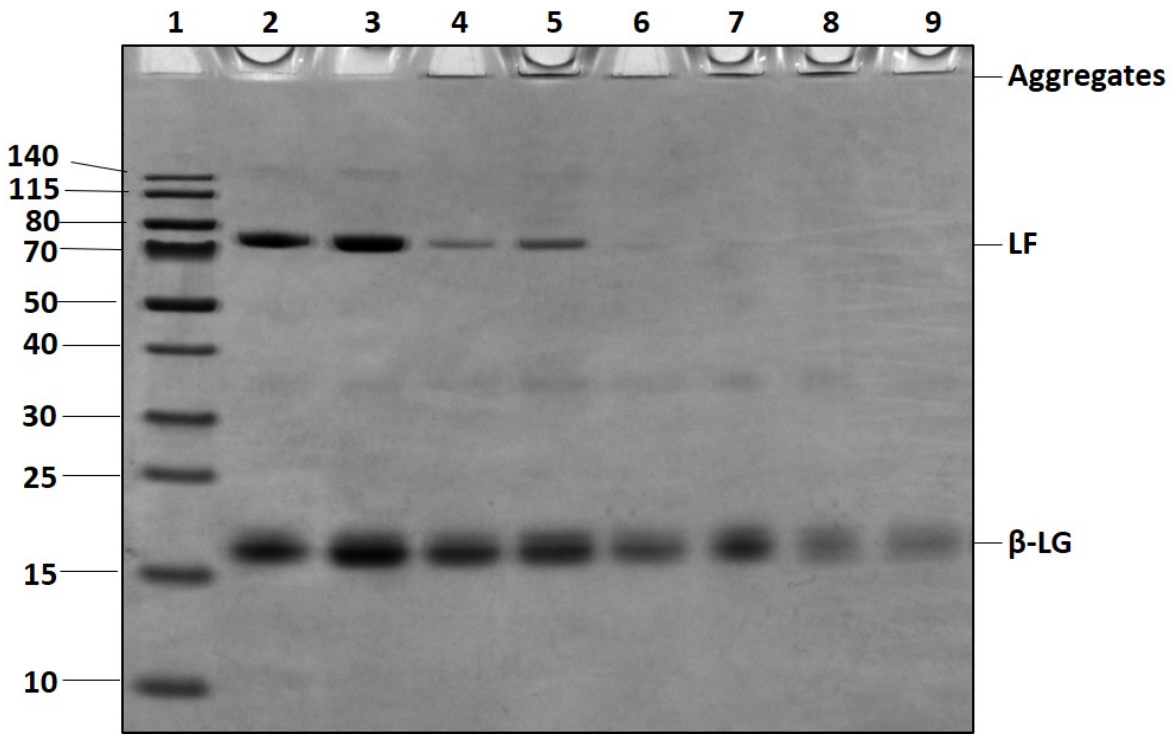
Supplementary figure 1 The effect of heating of lactoferrin in the absence and presence of β-lactoglobulin on its disulphide linked aggregation. Lane 1, protein marker; Lane 2, 3, mixture of lactoferrin and β-lactoglobulin without heat treatment; Lane 4, 5 mixture of lactoferrin and β-lactoglobulin being heated at 65 °C for 30 min; Lane 6, 7, mixture of lactoferrin and β-lactoglobulin being heated at 75 °C for 30 min;. Lane 8, 9, mixture of lactoferrin and β-lactoglobulin being heated at 85 °C for 30 min; Abbreviations: LF, lactoferrin; β-LG, β-lactoglobulin.

Supplementary figure 2 In vitro digestion of unheated LFMS samples after gastric digestion and gastrointestinal digestion. M, protein marker; G60 means digested samples collected after 60 min of gastric digestion; GI means samples collected after complete gastrointestinal digestion. Abbreviations: IgG, immunoglobulin G; LF, lactoferrin; BSA, bovine serum albumin; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

Supplementary figure 3 Cleavage pattern of lactoferrin in the absence and presence of milk serum protein on after infant gastric digestion. LFMS, milk serum with addition of 1 mg/ml lactoferrin; LF+MS, lactoferrin and milk serum samples were heated at same temperature separately, and then mixed before digestion. N, sample without heat treatment; 65/70/75 refer to samples being heated at 65/70/75 °C for 30 min, respectively.

Supplementary figure 4 Peptide profiles from lactoferrin in differently heated milk serum samples after infant digestion. LFMS, milk serum with addition of 1 mg/ml lactoferrin; LF+MS, lactoferrin and milk serum samples were heated at same temperature separately, and then mixed before digestion. N, sample without heat treatment; 65/70/75 refer to samples being heated at 65/70/75 °C for 30 min, respectively.

Supplementary figure 5 Change of turbidity of β-lactoglobulin after heating. N, sample without heat treatment; 65/70/75 refer to samples being heated at 65/70/75 °C for 30min, respectively. Abbreviations: β-LG, β-lactoglobulin. Statistical significance was analyzed using ANOVA. Means followed by the same letter were not significantly different ($p > 0.05$). Different letters indicate statistically significant difference among different heat treatments ($p < 0.05$) based on Tukey's HSD test.



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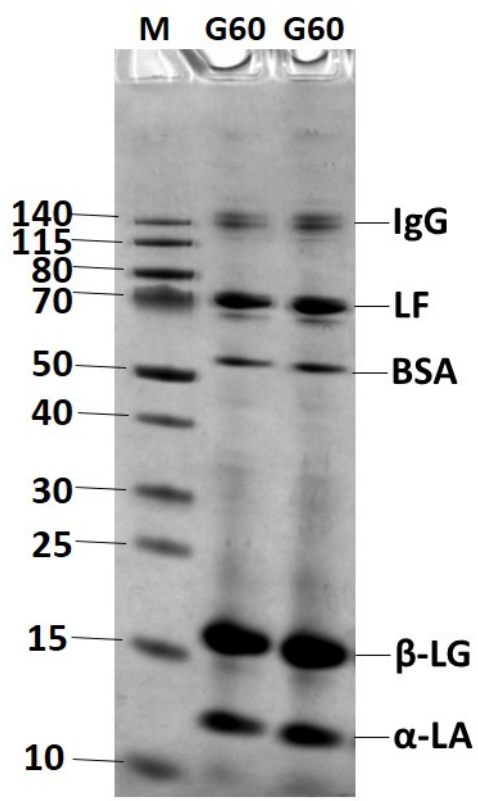
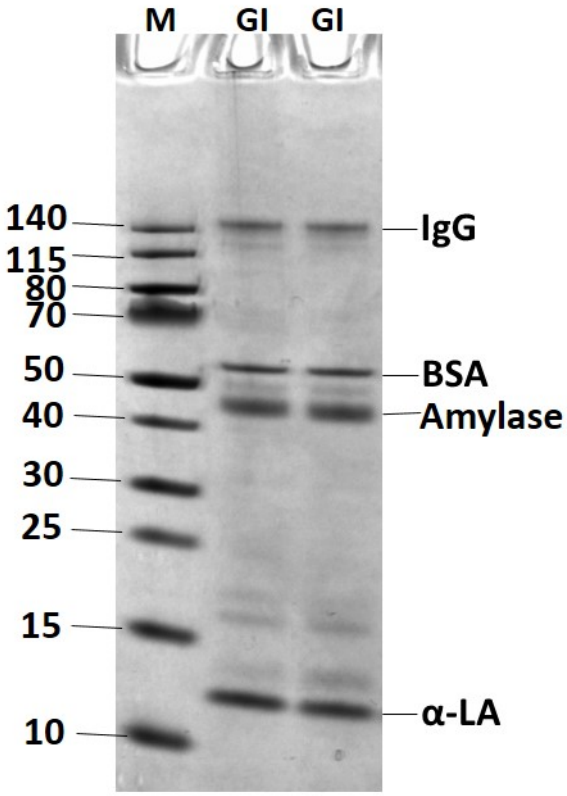
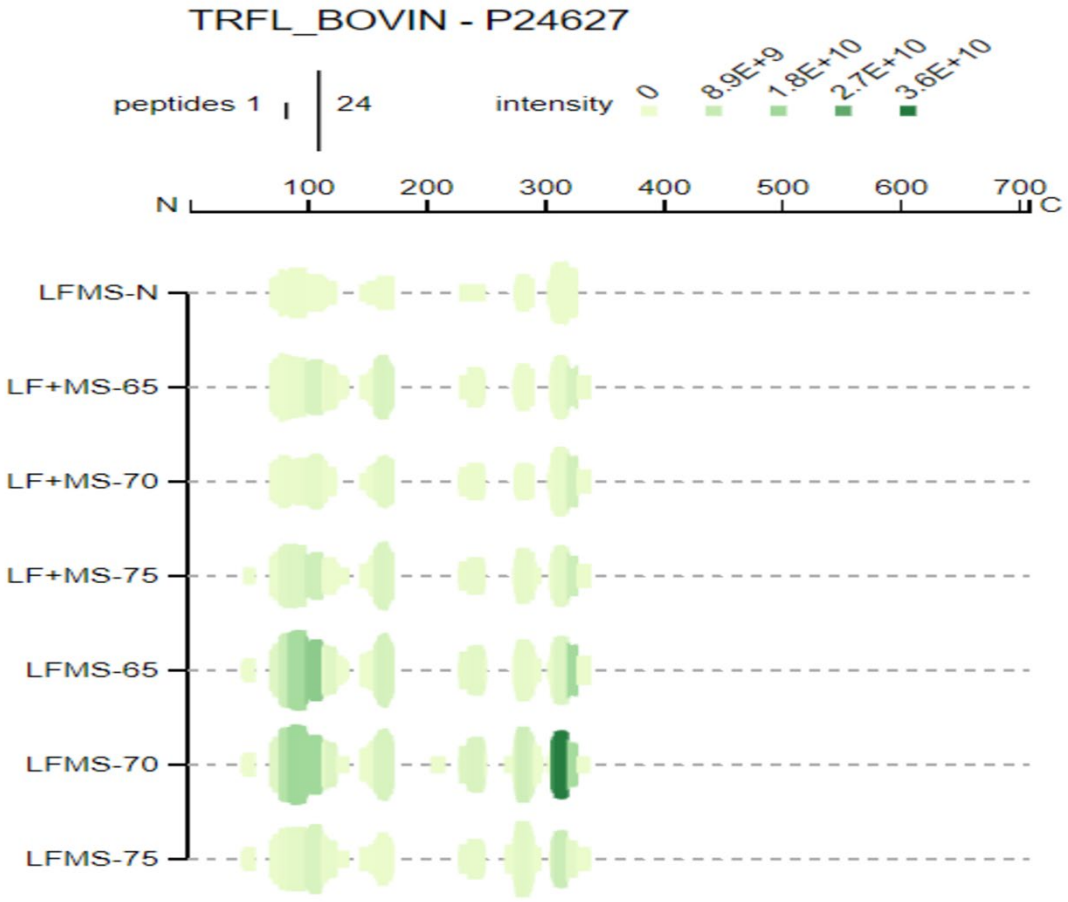

M
G60
G60
140
115
80
70
50
40
30
25
15
10
IgG
LF
BSA
β-LG
α-LA


M
GI
GI
140
115
80
70
50
40
30
25
15
10
IgG
BSA
Amylase
α-LA

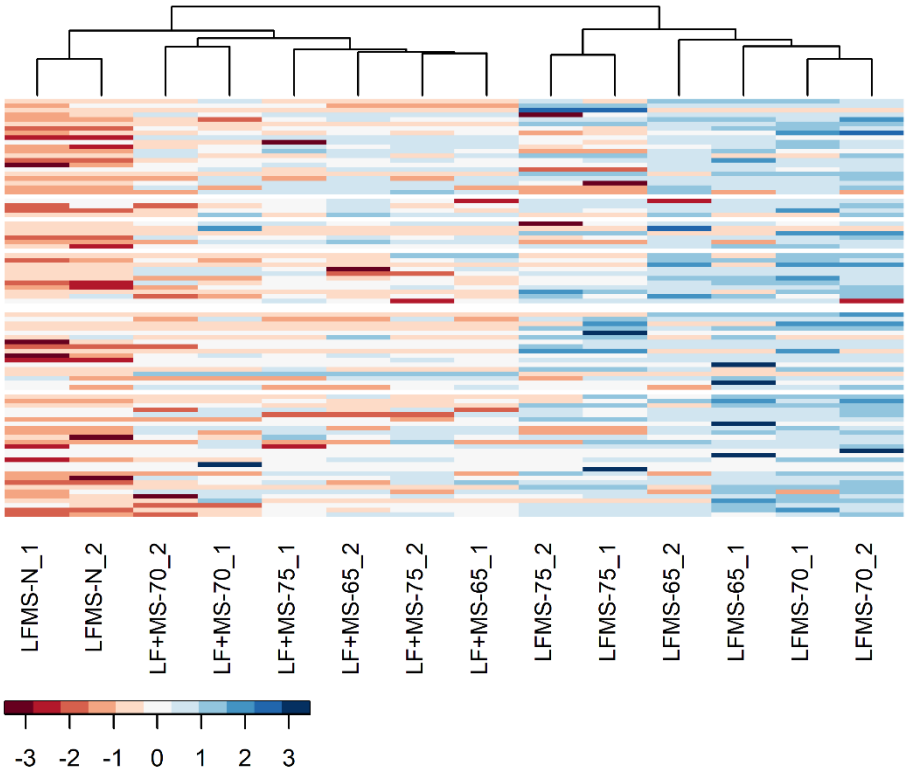
721

722


TRFL_BOVIN - P24627
peptides 1
24
intensity
0
8.9E+9
1.8E+10
2.7E+10
3.6E+10
N
100
200
300
400
500
600
700
C
LFMS-N
LF+MS-65
LF+MS-70
LF+MS-75
LFMS-65
LFMS-70
LFMS-75

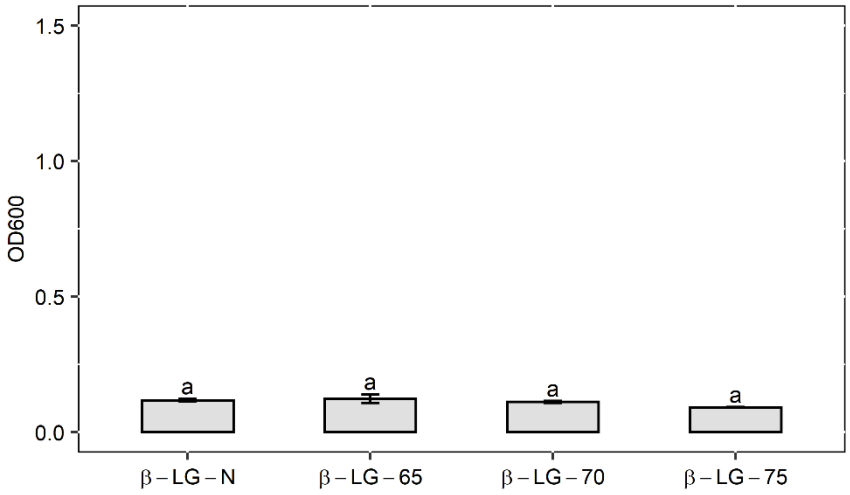
723

724


LFMS-N_1
LFMS-N_2
LF+MS-70_2
LF+MS-70_1
LF+MS-75_1
LF+MS-65_2
LF+MS-75_2
LF+MS-65_1
LFMS-75_2
LFMS-75_1
LFMS-65_2
LFMS-65_1
LFMS-70_1
LFMS-70_2
-3
-2
-1
0
1
2
3

725

726



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