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Short communication

Comparison of drying techniques for bovine lactoferrin: Iron binding and antimicrobial properties of dried lactoferrin

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ABSTRACT

The effects of pilot scale freeze- and spray-drying on some functional properties of lactoferrin were studied. Denaturation and loss of function relative to the fresh concentrate as received from a dairy factory was estimated in three ways: (a) from the enthalpy of the denaturation peak; (b) by iron binding capacity as measured by atomic absorption spectroscopy before and after incubation with an iron-rich solution; and (c) from anti-microbial properties by incubation of multiple dilutions against three microorganisms for 48 h at 37 °C. Spray-dried lactoferrin showed significantly larger extent of denaturation, and lower iron binding capacity, when compared with fresh or freeze-dried lactoferrin. For all treatments, lactoferrin exhibited bacteriostatic activity even at low lactoferrin concentrations of 0.01 mg mL^{-1}. No bactericidal activity was observed for any concentration or treatment of lactoferrin.

1. Introduction

Lactoferrin is a globular glycoprotein of approximately 80 kDa found in biological fluids including saliva, amniotic fluid, tears, and milk serum (Lönnerdal, 2003). It has iron binding and antimicrobial properties. Lactoferrin is more compact when saturated with iron, and consequently more heat stable (Franco, Pérez, Conesa, Calvo, & Sánchez, 2018).

Drying is required to convert lactoferrin concentrate to a shelf-stable form for transport and sale. Two drying methods used industrially are freeze-drying or spray drying. Previous authors reported little difference in the functionality of lactoferrin dried by either spray drying or freeze-drying (Wang, Timilsena, Blanch, & Adhikari, 2017a). However, the laboratory equipment used by these authors may not represent the equipment, particle thermal histories, or freeze-drying programmes used industrially. Air residence times can be ten times longer in pilot-scale spray dryers than in benchtop spray driers (Schmitz-Schug, Foerst, & Kulozik, 2013). Additionally, counter-current configuration, as used in this study, extends particle residence time over typical co-current benchtop units. Final freeze-dryer shelf temperatures typical of industry, where cycle time is critical, often exceed those presented in scientific literature. Studies that closely mimic the trajectories of industrial drying would be of interest to those selecting lactoferrin drying methods.

This current work investigated the effects of drying method on denaturation as determined calorimetrically, on iron binding capacity, and on antimicrobial activity of bovine lactoferrin. Drying was conducted using a pilot-scale production spray dryer, and a freeze-dryer that replicates industrial heating geometries, tray materials and dimensions, and heating programmes. The extent of denaturation was determined relative to "as-received" lactoferrin from a dairy factory. The impact on iron binding capacity was investigated by measuring the iron uptake of as-received and of dried lactoferrin upon incubation in an iron-rich solution. The antimicrobial activity was investigated by incubating three bacteria against serial dilutions of as-received or dried lactoferrin.

2. Materials and methods

2.1. Lactoferrin reception

Lactoferrin solution (16–18% TS, ≥95% lactoferrin) was provided by Tatua Dairy Company (Morrinsville, New Zealand) having been commercially extracted from fresh pasteurised skim milk using a strong cation exchanger with gradient elution, and finished by

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ultrafiltration then final filtration at 0.2 μm to reduce microbial loads prior to drying.

2.2. Spray drying

A Mobile Minor spray dryer (GEA Niro, Düsseldorf, Germany; 1500 mm high with 800 mm diameter) was used in fountain (counter-flow) mode. The lactoferrin solution was atomised in a two-fluid atomiser by air at a gauge pressure of 120 kPa. The dryer inlet temperature was held at 190 °C for all runs, and outlet temperature was maintained at either 75 °C or 95 °C by adjusting product feed-rate between 1.0 and 1.5 L h^{-1}. These conditions are coded 190/75 and 190/95. Each condition was run twice over two days with the running order reversed on the second day. Powder was collected in vessels at the base of the main chamber cone and cyclone. Samples for each condition were pooled across both days.

2.3. Freeze drying

The lactoferrin solution was freeze-dried using an FD-18 freeze-dryer (Cuddon, Blenheim, New Zealand) and with the product coded FD. This freeze-dryer is designed to scale directly to large industrial dryers, and has the same tray geometry and heating behaviour. Solution of depth 12–15 mm was frozen in place on freeze-dryer shelves at −40 °C. Product temperature during the freezing and drying was monitored at three different locations. The temperature/time programme (slightly over 48 h) mimicked that used on industrial scale. Three runs each dried a single tray of approximately 3.1 L lactoferrin solution. The dried lactoferrin cake was broken up manually and then pooled, sub-sampled and sealed in foil bags and stored at 0–2 °C.

2.4. Powder analysis

Powder moisture content was determined by drying in a vacuum oven at 100 °C under a vacuum of at least 85 kPa for 18 h. Aerobic and anaerobic plate counts were carried out by incubation of serial dilutions of powder samples on standard plate count agar for 48 h at 30 °C under aerobic and anaerobic conditions.

2.5. Drying effects on lactoferrin functional properties

2.5.1. Extent of denaturation

Dried lactoferrin was dissolved in MilliQ water to 15% (w/w). Samples of the solution (10–15 mg) were sealed in TZero aluminium hermetic pans and placed in a Q2000 differential scanning calorimeter (DSC) (TA instruments, New Castle, DE, USA) with an empty reference pan. Samples were heated from 20 °C to 100 °C at 5 °C min^{-1}. The extent of denaturation was calculated from the relative enthalpies of the first denaturation peak of the sample and the as-received lactoferrin (Wang, Timilsena, Blanch, & Adhikari, 2017b):

$$\%_{\text{Denatured}} = (1 - \Delta H_{\text{sample}}/\Delta H_{\text{as-receive}}) * 100 \quad (1)$$

2.5.2. Iron binding

Dried lactoferrin was dissolved at 40–50 mg mL−1 in 50 mm Tris buffer with 20 mm NaHCO3, which was then adjusted to pH 7.8. An 11 mm ferric solution was prepared from Fe(NO3)3.9H2O in 50 mm Tris buffer, pH 7.8. A 2.5× molar excess of nitrilotriacetic acid (NTA) in the form of Na2NTA was added to the Fe-rich solution. Previous authors found a LF:Fe:NTA ratio of 1:2:2 is sufficient to saturate lactoferrin (Wang et al., 2017b); others reported a LF:Fe:NTA ratio of 1:2:5 gives saturation comparable with that at a ratio of 1:4:4 (Majka et al., 2013). A ratio of 1:2:5 was selected for this study. Fe-rich solution was added to 5 mL aliquots of lactoferrin solution for a final LF:Fe:NTA ratio of 1:2:5 then samples incubated at room temperature for 1 h. Excess Fe was removed by gel filtration though Sephadex G25-150 (Mata, Sánchez, Headon, & Calvo, 1998).

Protein concentrations were measured in duplicate by Bradford protein assay, with the calibration curve generated using lyophilised lactoferrin (Sigma–Aldrich NZ, Auckland, NZ). Samples for Fe analysis were microwave digested by a microwave GO Plus digestion system (Anton Paar, Graz, Austria) according to AOAC official method 2015.06 (Pacquette & Thompson, 2018). Digested samples were diluted to an expected concentration of 20–100 ppb Fe with 2% HNO3.

A PinAAcle 900Z graphite-furnace atomic absorption spectrometer (PerkinElmer, Waltham, MA, USA) was used to determine Fe content according to Bass and Bosnak (2011). For each replicate, three consecutive measurements were recorded and averaged. A calibration curve ($R^2 = 0.999$) was prepared from stock solution of 100 ppb Fe in 2% HNO3 at dilutions giving 0–100 ppb Fe.

2.5.3. Antimicrobial activity

Lactoferrin samples were dissolved at 0.01, 0.03, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, or 3.0 mg mL^{-1} in sterile Milli-Q water and sterilised by passage through a 0.22 μm membrane filter at room temperature (Minisart Sartorius, Göttingen, Germany). Bacterial inocula of *Escherichia coli* NCTC 8196, *Cronobacter sakazakii* ASQ 5, and *Salmonella typhimurium* at concentrations of 10^4–10^5 cfu mL^{-1} were prepared by serial dilution in sterile 0.1% peptone water. Inocula and lactoferrin samples (100 μL of each) were pipetted into 96 well plates and incubated under aerobic conditions at 37 °C. Bacterial activity was measured by absorbance at 620 nm at 0, 8, 24, and 48 h using a SPECTROstar Nano plate reader (BMG Labtech, Ortenberg, Germany). After 48 h, 100 μL from each well was spread on to media (EMB agar for *E. coli*, XLD agar for *S. typhimurium*, and *C. sakazakii* agar for *C. sakazakii*), and incubated for 24 and 48 h to test for microbial survival or growth.

2.6. Statistical methods

All analyses were conducted in triplicate or duplicate where indicated. One-way ANOVA was performed using Minitab 18 statistical software (Minitab, State College, PA,USA).

3. Results and discussion

Lactoferrin as-received had a solids concentration of 17.4 ± 0.4%. This solution, and all subsequent spray dried and freeze-dried samples, had aerobic and anaerobic plate counts of <1 cfu g^{-1}. Table 2 shows the measured moisture content of the powders after drying. The moisture contents reported in this study are lower than those of Wang et al. (2017a) who reported levels of 5.2% and 8.6% for lactoferrin spray dried with exit temperatures of 95 °C and 70 °C, respectively. The current study is closer to the CFDA GB 1903.17–2016 standard for lactoferrin, which requires a moisture loss of less than 4.5% on drying (China, 2016).

3.1. Drying effects on lactoferrin functional properties

3.1.1. Extent of denaturation

DSC curves are shown in Fig. 1. In all samples, peaks were observed for denaturation of both the apo- (52–62 °C) and holo- (>79 °C) forms of bovine lactoferrin at native pH (Iafisco, Foltran, Di Foggia, Bonora, & Roveri, 2011). The presence of both these peaks indicates that the lactoferrin as-received and dried was partially iron-saturated (Iafisco et al., 2011). Table 1 summarises peak

Table 1
Measured differential scanning calorimetry (DSC) data and calculated extent of denaturation of dried lactoferrin.[a]

Sample	DSC peaks and denaturation						
	Peak 1 onset (°C)	Peak 1 max (°C)	Peak 2 onset (°C)	Peak 2 max (°C)	ΔH1 (J g^{-1})	ΔH2 (J g^{-1})	Denatured (%)
As-received	57.0 ± 0.1^{a}	62.1 ± 0.4^{a}**	85.4 ± 0.3^{a}	89.4 ± 0.1^{a}	19.6 ± 0.3^{a}**	1.9 ± 0.7^{a}	Reference^{a}**
FD	57.2 ± 0.2^{a}	62.7 ± 0.0^{b}**	85.1 ± 0.2^{a}	89.2 ± 0.2^{a}	18.3 ± 0.3^{b}**	2.3 ± 0.6^{a}	6.8 ± 0.1^{b}**
190/75	57.1 ± 0.4^{a}	62.6 ± 0.1^{b}**	85.4 ± 0.4^{a}	89.2 ± 0.2^{a}	16.9 ± 0.7^{c}**	2.0 ± 0.6^{a}	14.0 ± 0.7^{c}**
190/95	57.9 ± 1.2^{a}	62.7 ± 0.2^{b}**	85.5 ± 0.5^{a}	89.5 ± 0.1^{a}	16.5 ± 1.7^{c}**	2.0 ± 0.4^{a}	16.2 ± 1.3^{c}**

[a] Treatments that do not share a common superscript letter are significantly different, error bars are confidence intervals for the mean: ***P < 0.01; **P < 0.05; *P < 0.1.

Table 2
Measured moisture content, and Iron binding properties of lactoferrin samples.[a]

Sample	Moisture content (%, w/w)	Molar ratio Fe:LF	
		Pre-incubation	Post-incubation
As-received	82.6 ± 0.4^{a}***	0.38 ± 0.35^{a}	1.43 ± 0.09^{a,b}*
FD	0.55 ± 0.19^{b}***	0.25 ± 0.24^{a}	1.54 ± 0.23^{a}*
190/75	6.39 ± 0.07^{d}***	0.23 ± 0.02^{a}	1.18 ± 0.16^{c}*
190/95	3.92 ± 0.10^{c}***	0.44 ± 0.18^{a}	1.26 ± 0.07^{b,c}*

[a] Iron binding properties are expressed as molar ratios of iron to lactoferrin; treatments that do not share a common superscript letter are significantly different, error bars are confidence intervals for the mean: ***P < 0.01; **P < 0.05; *P < 0.1.

locations, enthalpies and the implicit extent of denaturation. There were no significant differences between any pair of treatments in the onset temperature of either denaturation peak, or in the location of the peak maximum, or enthalpy for the second denaturation peak.

There was a significant ($P < 0.05$) difference between treatments for the peak enthalpy of the first denaturation peak (ΔH_1), and implicitly the extent of denaturation of apo-lactoferrin. The as-received lactoferrin had the greatest ΔH_1, followed by the FD treatment and then the two spray dried treatments, as shown in Table 1. There was no significant difference ($P = 0.62$) between the two spray-dried treatments. The extent of denaturation for the two spray-dried treatments, 190/75 and 190/95, was significantly ($P < 0.05$) greater than for the FD treatment.

For the FD treatment, stresses due to freeze concentration, ice-induced denaturation (Bhatnagar, Bogner, & Pikal, 2007), protein dehydration (Carpenter, Prestrelski, & Arakawa, 1993), and cold denaturation (Ascolese & Graziano, 2008), may be responsible for the denaturation observed in FD samples.

The spray-dried samples showed greater denaturation than reported by Wang et al. (2017a), who observed 0.9⁻2.0% denaturation for lactoferrin spray-dried in a bench-top dryer at 180 °C inlet temperature and similar outlet temperatures to the current study. This indicates that samples produced in the current study experienced a harsher thermal history, as a result of the increased residence time in the pilot scale drier used in this study. Residence times in commercial spray dryers would typically be longer still, so a greater extent of denaturation may be seen in commercial spray spraying.

3.1.2. Iron binding

Table 2 lists molar ratios of iron to lactoferrin measured before and after incubation with ferric ions. There was no significant difference ($P = 0.11$) between the molar Fe:LF ratio of samples prior to incubation. After incubation the freeze-dried samples bound significantly more iron than either spray-dried sample.

The iron binding ability of lactoferrin results from specific iron binding sites in the N and C lobes of the protein that can reversibly bind to ionic iron (Levay & Viljoen, 1995). The molecule becomes more compact and resistant to thermal denaturation after binding iron (Wang et al., 2017b). Denaturation of lactoferrin alters the molecule's secondary, tertiary and quaternary structure, which can reduce the iron binding capacity of the molecule. This is observed in

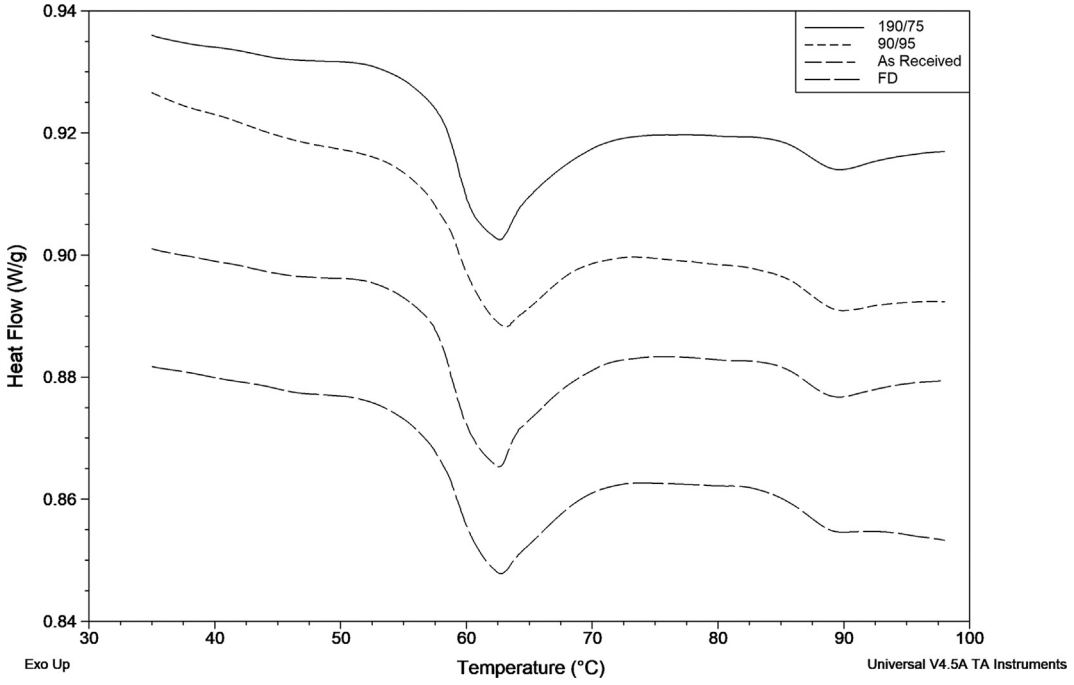

Fig. 1. Typical DSC curves for lactoferrin samples before and after drying treatments. From top to bottom: 190/75, 190/95, FD, as-received.

the current study, as spray-dried lactoferrin, which was denatured to a greater extent than freeze-dried lactoferrin displays significantly lower molar Fe:LF ratios and binding capacity than the freeze-dried lactoferrin.

3.1.3. Anti-microbial activity

The absorbance at 620 nm of all wells inoculated with lactoferrin solutions, at all dilutions and for all lactoferrin drying treatments, was reduced when compared to the control wells after 8 and 24 h incubation. This is shown in Fig. 2. This indicates that even at extremely low lactoferrin concentrations, spray dried and freeze-dried lactoferrin both acted as microbial inhibitors.

Lactoferrin as-received, and all dried treatments showed bacteriostatic activity against *E. coli* NCTC 8196, *C. sakazakii* ASQ 5, and *S. typhimurium* at concentrations from 0.01 to 3 mg mL^{-1}. The 190/95 samples appear to show less inhibitory activity than the as-received lactoferrin. Growth was observed on all plates spread with 48 h cultures from each combination of treatment, dilution and organism, therefore, no sample showed bactericidal activity.

Lactoferrin has antimicrobial properties independent of its ability to sequester iron, linked to interactions with the surface of

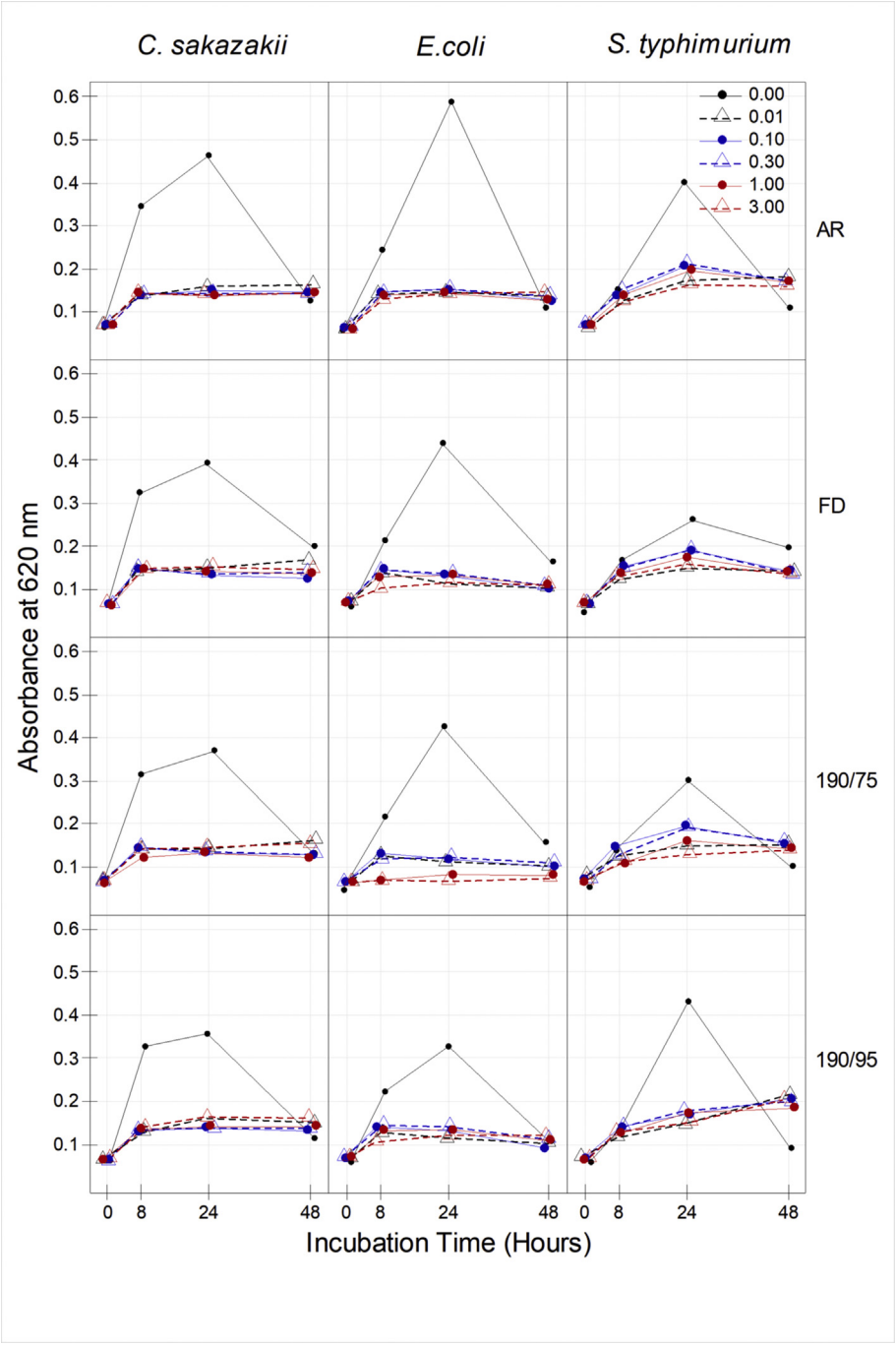

Fig. 2. Optical density at 620 nm of *C. sakazakii* ASQ 5, *E. coli* NCTC 8196 and *S. typhimurium* cultures over 48 h in the presence of lactoferrin concentrations of 0, 0.01, 0.1, 0.3, 1, and 3 mg mL^{-1}. Each plot represents one combination of lactoferrin drying treatment and culture. AR is the lactoferrin as-received, FD is the freeze-dried lactoferrin, 190/75 and 190/75 are spray dried treatments.

microbial cells (Farnaud & Evans, 2003). This may explain why differences in iron binding capacity seen in this study did not translate directly to differences in antimicrobial activity.

4. Conclusion

This study showed that the method of drying, when undertaken at time/temperature trajectories mimicking industrial scale, can have a significant effect on the extent of denaturation and iron binding properties of lactoferrin. Spray-dried lactoferrin was denatured to a greater extent than freeze-dried or fresh and showed lower iron-binding capacity. Drying method had no effect on the antimicrobial ability of dried lactoferrin. These results imply that at larger scales of equipment, freeze drying results in a closer to native dried lactoferrin than does spray drying. Furthermore, laboratory scale investigation does not always adequately mimic industrial scale drying.

Declaration of competing interest

Tatua Co-operative Dairy Company Limited is a dairy company that manufactures and sells lactoferrin powder. Cuddon Freeze Dry Ltd is a manufacturer of industrial freeze drying equipment. Both were interested in careful measurement of the relative impacts of industrially-relevant freeze-and spray-drying on lactoferrin properties.

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References

Ascolese, E., & Graziano, G. (2008). On the cold denaturation of globular proteins. *Chemical Physics Letters, 467*, 150–153.

Bass, D., & Bosnak, C. P. (2011). The determination of toxic, trace, and essential elements in food matrices using THGA coupled with longitudinal Zeeman background correction *(Application Report)*. Waltham MA, USA: PerkinElmer, Inc. Retrieved from https://www.perkinelmer.com/lab-solutions/resources/docs/APP_PinAAcleMetalsinFood.pdf.

Bhatnagar, B. S., Bogner, R. H., & Pikal, M. J. (2007). Protein stability during freezing: Separation of stresses and mechanisms of protein stabilization. *Pharmaceutical Development and Technology, 12*, 505–523.

Carpenter, J. F., Prestrelski, S. J., & Arakawa, T. (1993). Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. I. Enzyme activity and calorimetric studies. *Archives of Biochemistry and Biophysics, 303*, 456–464.

China. (2016). Food nutrition enhancer lactoferrin. In *National Food Safety Standards (Vol. GB 1903.17-2016)*. Beijing, China: National Health and Family Planning Commission of the People's Republic of China, & China Food and Drug Administration.

Farnaud, S., & Evans, R. W. (2003). Lactoferrin—a multifunctional protein with antimicrobial properties. *Molecular Immunology, 40*, 395–405.

Franco, I., Pérez, M. D., Conesa, C., Calvo, M., & Sánchez, L. (2018). Effect of technological treatments on bovine lactoferrin: An overview. *Food Research International, 106*, 173–182.

Iafisco, M., Foltran, I., Di Foggia, M., Bonora, S., & Roveri, N. (2011). Calorimetric and Raman investigation of cow's milk lactoferrin. *Journal of Thermal Analysis and Calorimetry, 103*, 41–47.

Levay, P. F., & Viljoen, M. (1995). Lactoferrin: A general review. *Haematologica, 80*, 252–267.

Lönnerdal, B. (2003). Lactoferrin. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry* (Vol. 1, pp. 449–466). Boston, MA, USA: Springer US. Proteins.

Majka, G., Śpiewak, K., Kurpiewska, K., Heczko, P., Stochel, G., Strus, M., et al. (2013). A high-throughput method for the quantification of iron saturation in lactoferrin preparations. *Analytical and Bioanalytical Chemistry, 405*, 5191–5200.

Mata, L., Sánchez, L., Headon, D. R., & Calvo, M. (1998). Thermal denaturation of human lactoferrin and its effect on the ability to bind iron. *Journal of Agricultural and Food Chemistry, 46*, 3964–3970.

Pacquette, L. H., & Thompson, J. J. (2018). Minerals and trace elements in milk, milk products, infant formula, and adult/pediatric nutritional formula, ICP-MS method collaborative study, AOAC Final Action 2015.06, ISO/DIS 21424, IDF 243. *Journal of AOAC International, 101*, 536–561.

Schmitz-Schug, I., Foerst, P., & Kulozik, U. (2013). Impact of the spray drying conditions and residence time distribution on lysine loss in spray dried infant formula. *Dairy Science & Technology, 93*, 443–462.

Wang, B., Timilsena, Y. P., Blanch, E., & Adhikari, B. (2017a). Characteristics of bovine lactoferrin powders produced through spray and freeze drying processes. *International Journal of Biological Macromolecules, 95*, 985–994.

Wang, B., Timilsena, Y. P., Blanch, E., & Adhikari, B. (2017b). Drying and denaturation characteristics of three forms of bovine lactoferrin. *Drying Technology, 35*, 606–615.