Effect of drying methods on the physicochemical and bioactivities of bovine colostrum and lactoferrin

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Master of Food Technology

Massey University, Auckland, New Zealand

Sinong Wu

2024

Abstract

The aim of this study was to use low temperature electrostatic spray drying to produce bovine colostrum and lactoferrin powders with improved functional properties. The objectives were to examine the effects of spray, freeze and electrostatic spray drying on the physicochemical properties, bioactivity, antimicrobial and antioxidant activity of the bovine colostrum and lactoferrin.

Liquid bovine colostrum and lactoferrin was spray dried at two different inlet temperatures: 165 and 180°C and was electrostatic spray dried at two lower inlet temperatures: 75 and 150°C. Powders collected were analysed for the following characteristics: moisture content, water activity, crude protein content, solubility, particle size, absorbance spectrum from Fourier-transform infrared spectrophotometry (FTIR), bioactive immunoglobulin and lactoferrin content (enzyme-linked immunosorbent assay (ELISA)) bioactivity, iron-binding ability (two methods), antioxidant ability using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and antimicrobial ability.

All analysed colostrum and lactoferrin powders had a moisture content below 4.5% (w/w) and water activity below 2.6. There was generally a decrease in the moisture content and water activity with increasing drying temperature. Drying temperatures also had an effect on the powder solubility and particle size. With higher drying temperatures, the solubility and volumetric median particle size (D_v50) of the powders decreased. The secondary structure of the protein investigated using FTIR showed that high temperature spray dried colostrum had greater protein degradation than low temperature electrostatic spray dried. ELISA analysis indicated that electrostatic spray dried colostrum retained more bioactive lactoferrin protein than freeze dried or high temperature spray dried. From both iron-binding methods tested, found that high temperature spray dried and low temperature electrostatic spray dried colostrum and lactoferrin had similar iron-bind ratios, ranging between 3 to 4 moles Fe/moles protein for colostrum, and 1.3 to 1.5 moles Fe/moles protein for lactoferrin. Low temperature electrostatic spray dried colostrum and lactoferrin showed better DPPH radical scavenging ability, followed by freeze dried and lastly high temperature spray dried. Colostrum electrostatic spray dried at 75°C and freeze dried lactoferrin showed better antimicrobial ability to *Escherichia coli* and *Staphylococcus aureus* than others.

In conclusion, low temperature electrostatic spray drying was able to produce bovine colostrum and lactoferrin powders with slightly better bioactive and functional properties.

Acknowledgements

I would like to firstly acknowledge and thank Fluid Air and Spraying Systems for funding this project and providing me with the scholarship. I would especially like to thank Dr Bogdan Zisu for his support and help in this project.

I would like to thank my supervisors Professor Marie Wong and Dr Don Otter, for their guidance, help, patience and enormous support throughout this project. Also, thanks to Dr Zhi Yang and Dr Yacine Hemar, though they left this project part way through, I would like to thank them for getting this project started.

Thanks to Magnolia Dairy Ltd., Auckland, New Zealand, for proving the bovine colostrum, and thanks to Westland Milk Products, Hokitika, New Zealand, for providing the bovine lactoferrin.

Thanks so much to Arthur Xu, Rachel Liu, Dr Kay Rutherfurd and Alex Burton for their help with my experiments. Thanks so much to Dr Loc Pham for helping me with the electrostatic spray drying in Spraying Systems, Melbourne, Australia. Special thanks to Roy Wang for helping me with my data analysis.

Thank you to my lovely and caring friends, Yusi, Vina, Lan, Alisa and Anja: thank you for your love, encouragement, and all the positive energy that kept me uplifted.

I would like to really thank my mum and dad, for trusting that I can handle the decisions I have made by my very self, for motivating and supporting me immensely since day one I came to this world. I am beyond grateful for how I have always been surrounded by your love.

To my silly angelic partner Jacky: thank you for your support, companionship and all the joy you have brought into my life.

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

Bovine colostrum and lactoferrin are well known for their health benefits and a wide range of applications in various industries. Colostrum is the first milk cows produce after parturition that repletes with immunoglobulins (Igs), growth factors and nutrients, making it highly valued in nutritional, sports, medical and therapeutical uses (Ceniti *et al.*, 2023; Chandwe & Kelly, 2021; Rathe *et al.*, 2014). Lactoferrin is an iron-binding whey protein that commercially recovered from whey or skim milk, it is prized for its iron-binding and antimicrobial properties (Baker & Baker, 2004; Jenssen & Hancock, 2009). Lactoferrin has been added in infant formula and incorporated into functional foods and beverages for its health benefits (Aly *et al.*, 2013; Li *et al.*, 2024a). The global colostrum and markets were valued over $US3000 and $US600 million in 2023 and is expected to grow larger (Anon, 2024a; 2024b).

The growing interest in human consumption of bovine colostrum and lactoferrin focusing on their nutritional and functional aspects has enlarged the production of the ingredient (Krolitzki *et al.*, 2022). It was reported that freeze drying colostrum showed a negative impact on the colostral fat as a large proportion of the fat globules were destroyed, however, an optimal drying temperature below 55°C was recommended to maintain its biological functionality (Puppel *et al.*, 2019). MAINER *et al.* (1999) found that the biological activity of colostrum was impacted when it was treated over 65°C. Sánchez *et al.*(1992b) found that heat-induced degradation of bovine lactoferrin protein was observed over a temperature range of 72-85°C.

Spray drying is the most frequently applied drying technique to dehydrate dairy products but this method involves high temperatures (Schuck *et al.*, 2016). Freeze drying is valued for its low-temperature drying conditions that minimizes the degradative reactions such as protein denaturation, producing the highest quality food product (Liapis & Bruttini, 2020). However, compared to spray drying, freeze drying requires 88% more capital cost and 80% more operational cost (Ratti, 2012).

Electrostatic spray drying works by applying the electrostatic effect on the droplets and prolongs the constant rate drying period, hence able to dry the products at lower temperatures than spray drying (Mutukuri *et al.*, 2021). Electrostatic spray drying has been applied to dry various materials include agricultural, food, pharmaceutical, and biomedical material. It has been shown that electrostatic spray dried colostrum and lactoferrin preserved more functional and bioactive properties of the bioactive proteins (Masum *et al.*, 2022).

The overall aim of this project was to apply electrostatic spray drying to obtain colostrum and lactoferrin powders with better biological activities than conventional spry drying.

In order to meet this aim, the objectives for this project were:

1. To produce freeze, spray and electrostatic spray dried powders using fresh colostrum and lactoferrin.
2. To measure the physicochemical properties and to characterise colostrum and lactoferrin powders produced by freeze, spray and electrostatic spray drying.
3. To determine the bioactivity, antimicrobial and antioxidant activity of colostrum and lactoferrin powders produced by freeze, spray and electrostatic spray drying.

Chapter 2. Literature Review

This literature review chapter covers the general properties of bovine colostrum and lactoferrin, followed by the introduction of three different drying techniques. The research methodologies employed in this research are also outlined and discussed.

2.1 Bovine milk

Bovine milk contains the nutrients needed for the growth and development of calves, providing lipids, proteins, amino acids, vitamins and minerals (Haug *et al.*, 2007). At the macronutrient level, bovine milk comprises 85-87% water, 3.8-5.5% fat, 2.9-3.5% protein and 5% carbohydrates. Additionally, at the micronutrient level, bovine milk contains various of bioactive components, including immunoglobulins, vitamins, minerals, biogenic amines, organic acids, nucleotides and oligosaccharides (Foroutan *et al.*, 2019). The content of these components in milk varies based on factors such as the breed of cows, lactation stage, diet, parity, and season (Auldist *et al.*, 1998; DePeters & Cant, 1992).

In general, bovine milk proteins can be classified into four different groups: caseins (α_{s1}, α_{s2}, β and k caseins), serum proteins [α-lactalbumin (α-La), β-lactoglobulin (β-Lg)], bovine serum albumin (BSA), immunoglobulins (Igs) and a range of other minor whey proteins (Le *et al.*, 2017). Caseins are characterised as proteins that coagulate and precipitate from skim milk when the pH is adjusted to pH 4.6 at 20°C. Whey protein, on the other hand, remains soluble at pH 4.6 after the precipitation of caseins (Ali *et al.*, 2019). Casein typically constitutes approximately 76 to 86% of the total protein in milk, with the rest being whey proteins (DePeters & Cant, 1992).

Commercially available bovine milk is the "mature milk" from cows. In dairy cattle, the initial milk secreted immediately following parturition is called colostrum. Colostrum contains high levels of antibodies that benefit the immune and digestive health of calves. Over time post-calving, colostrum is gradually replaced by transition milk. Transition milk is significantly lower in immunoglobulin G (IgG) compared to colostrum and calves cannot absorb this antibody from the small intestine through to the blood, but transition milk also contains many immune properties at a greater level than mature milk (McGrath *et al.*, 2016).

2.2 Bovine colostrum

Bovine colostrum is the pre-milk fluid produced by cows during the first 96 h postpartum (Copestake *et al.*, 2006). It is a rich natural source of bioactive components, i.e., immunoglobulins (Igs), lactoferrin, lysozyme, lactoperoxidase and peptides. In ruminants, it is the primary source of initial acquired immunity for offspring (Stelwagen *et al.*, 2009). It is designed to provide the calf with the necessary nutrients and passive immunity against pathogens. It is also considered significantly invaluable in revitalizing weak or sickly calves attributed to its high level of bioactive components (Nagy, 2009; Singh *et al.*, 2011).

2.3 Bioactive compounds in bovine colostrum

Colostrum is a complex fluid rich in antibodies and growth factors. These antibodies confer passive immunity to neonates, whereas the growth factors play a crucial role in stimulating their gut development. The other bioactive components of colostrum, including lactoferrin, lysozyme and lactoperoxidase contribute greatly to survival of calves and shaping the development of the gastrointestinal system in the early life of calves (Elfstrand *et al.*, 2002; Pakkanen & Aalto, 1997; Playford *et al.*, 2001).

Nutritionally, bovine colostrum contains essential nutrients such as proteins, fats, vitamins, and minerals **(Table 2.1)**, each with well-characterized roles in the development and growth of calves (Kehoe *et al.*, 2007; Playford & Weiser, 2021). From the literatures, there are four main groups of antibodies reported, including immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA) and immunoglobulin E (IgE). Among these, IgG constitutes approximately 80% of the total immunoglobulins present in bovine colostrum (Król *et al.*, 2012; Wąsowska & Puppel, 2018).

Table 2.1 Summary of Immunoglobulin G (IgG) and Lactoferrin content present in bovine colostrum.

IgG (mg/mL)	Lactoferrin (mg/mL)	Reference
98	0.388	(Bar *et al.*, 2010)
30 ± 3	1	(Abd El-Fattah *et al.*, 2014)
115	-	(Borad *et al.*, 2019)
35	0.8	(Kehoe *et al.*, 2007)
35.6 - 93	1.5 - 5	(Playford & Weiser, 2021)

In general, colostrum contains more fat, protein and growth factors compared to transitional or mature milk **(Table 2.2)**. However, the levels of bioactive compounds in and nutritive values of bovine colostrum diminish rapidly over time following calving. Contrary to this the content of lactose changes in an inverse manner to these components by increasing with successive milkings until it reaches a concentration of about 4.4% (McGrath *et al.*, 2016).

Table 2.2 Comparison of mature bovine milk and colostrum (Playford & Weiser, 2021).

Component	Bovine colostrum	Bovine mature milk
Total Solids (%)	24-28	12.9
Fat (%)	6-7	3.6-4.0
Total Protein (%)	14-16	3.1-3.2
Casein (%)	4.8	2.5-2.6
Albumin (%)	6.0	0.4-0.5
Immunoglobulin (mg/mL)	42-90	0.4-0.9
IgG (g/L)	32	1
Lactose (%)	2-3	4.7-5.0
Lactoferrin (g/L)	1.5-5	0.02-0.75

The amount of colostrum produced and its composition is highly variable due to a number of factors, such as breed, age, parity, productivity, feeding intensity, season of the year, length of the dry period of cows and time postpartum, as older calves produce more colostrum and the concentration of antibodies are higher (Puppel *et al.*, 2019; Zarcula *et al.*, 2010).

2.2.2 Functional properties of bovine colostrum

The colostral immunoglobulins (Igs) play a pivotal role in providing passive immunity to newborn calves (Copestake *et al.*, 2006). This initial milk is instrumental in endowing newborns with immunity, shielding them from diseases, particularly through the transmission of immune cells and antibodies, a process known as passive immunity (Kaplan *et al.*, 2022). Given that the initial immune system of calves is not fully developed at birth, it is imperative that colostrum is administered promptly thereafter (Bar *et al.*, 2010). At birth, calves possess the ability to absorb immunoglobulins from maternal colostrum through their small intestine. However, the small intestine of calves remains permeable for only a short duration. Calves need to receive an adequate supply of colostrum shortly after birth, as their lifelong immunity may be compromised (Lopez & Heinrichs, 2022).

Previous studies found out that as a calf ages over 12 h after birth, their intestinal permeability to the immunoglobulins starts closing, further by the 24 h mark, an intestinal barrier appears and the calf's intestine becomes impermeable to large proteins therefore the absorption of immunoglobulins from the colostrum ceases entirely (Bush & Staley, 1980; Stott *et al.*, 1979).

2.2.3 Applications of bovine colostrum

In addition to its vital role in supporting the health of calves, bovine colostrum has been used for human consumption due to its high nutritive values. Bovine colostrum is often processed into powder form via freeze- or spray drying and transformed into health supplements available in both powder and pill form. These supplements are reported to offer a myriad of benefits for human health (Kaplan *et al.*, 2022). Igs are the prominent immune components of colostrum, freeze dried powders can impart the health advantages into functional beverages, infant formulas and nutraceuticals (Umar *et al.*, 2023).

Colostrum is held in high regard as a multifaceted food ingredient owing to its rich composition of essential nutrients, high concentrations of bioactive proteins, vitamins, minerals, growth factors, as well as free and conjugated oligosaccharides. This versatile substance finds application in an array of culinary creations such as cheeses, jellies, yogurts, ice creams, nutritional bars, and beverages (Playford & Weiser, 2021).

Furthermore, colostrum in powdered form is frequently incorporated into various products, including infant formulas and cosmetics, to harness its beneficial properties and enhance their nutritional profiles (Costa *et al.*, 2023). Guberti *et al.* (2021) confirmed that the consumption of bovine colostrum brings some general benefits on intestinal and respiratory recovery without causing adverse effects. Their research indicates that bovine colostrum can potentially enhance and modulate the immune system, fostering both local and systemic responses in diverse clinical and non-clinical conditions.

2.2.4 Processing of bovine colostrum

Bovine colostrum is typically collected from individual farms and shipped to central processing facilities, where it undergoes pasteurization, optional defatting and lactose removal. Then the colostrum is spray- or freeze dried into powder for foods or dietary use (Playford & Weiser, 2021). Processes involved in the production of bovine colostrum for human consumption play a pivotal role in preserving and maintaining the activity of the bioactive components. Found that the bioactive components in colostrum were not damaged after freeze-drying (Umar *et al.*, 2023). And the IgG and lactoferrin protein in colostrum degraded when treated above 60°C (Bar *et al.*, 2010). Many of the active compounds are heat sensitive, therefore different production techniques, especially high temperature processes impose effects on their bioactivities to different degrees (Bar *et al.*, 2010).

2.3 Lactoferrin

Bovine lactoferrin is a glycoprotein with a molecular weight of about 80 kDa (Groves, 1960). This protein was first isolated as an unknown "red protein" from bovine milk in 1939 (Sorensen & Sorensen, 1939). Lactoferrin protein is a member of the transferrin family, along with serum transferrin, ovotransferrin, melanotransferrin and the inhibitor of carbonic anhydrase, which has a specific ability to bind iron (González-Chávez *et al.*, 2009). Investigations of bovine lactoferrin have been reported to show anti-microbial, anti-carcinogenic and anti-inflammatory effects (Legrand *et al.*, 2005).

Commercially, lactoferrin is isolated and purified on an industrial scale from cheese whey and skim milk (Tomita *et al.*, 2002). At present, global lactoferrin production was expected to be higher than 300 metric tonnes per year in 2021, ten-times expand than a decade ago (Krolitzki *et al.*, 2022).

2.3.1 Composition and structural features of bovine lactoferrin

Lactoferrin is an iron-binding glycoprotein present in milk as well as other exocrine secretions and neutrophil granules in mammals, also with substantially higher levels being found in colostrum and human breast milk (Baker & Baker, 2005; González-Chávez *et al.*, 2009).
Bovine lactoferrin contains a single polypeptide chain of 696 amino acid residues. The polypeptide chain is folded into two symmetrical lobes (the N-lobe and C-lobe) joined by a short α- helix. N and C lobe is further divided into two domains: N1 and N2 or C1 and C2, respectively, with the iron-binding site located between two domains **(Figure 2.1)** (Baker & Baker, 2012).

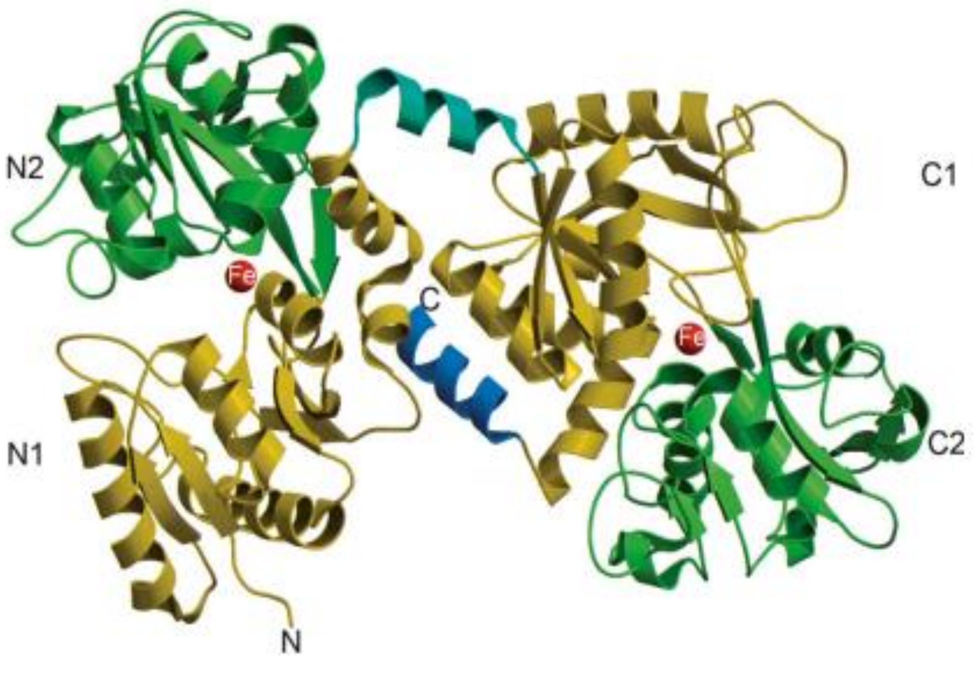

Figure 2.1 The tertiary structures of lactoferrin determined by X-ray crystallography. The iron-bound form of bovine lactoferrin. The N-lobe is on the left, with its 2 domains N1 (gold) and N2 (green) closed over the bound iron atom (red sphere), and the C-lobe is on the right with its 2 domains C1 (gold) and C2 (green) also closed over the iron atom. The a-helix that joins the 2 lobes is shown in turquoise and the C-terminal helix in blue (Baker & Baker, 2012).

In lactoferrin, domain N1 is composed of amino acid residues 1-90 & 251-333; N2 is made up of amino acid residues 91-250. Similarly, domain C1 is comprised of 345-431 & 593-676 amino acid residues and C2 is comprised of 432-592 amino acid residues. The amino acid residues 334-344 form a small 3-turn helix that functions as a flexible hinge during opening and closing of the domains (Baker & Baker, 2005; Steijns & Van Hooijdonk, 2000).

2.3.2 Lactoferrins affinity with iron

Lactoferrin has a great iron-binding affinity in which two ferric ions (Fe^{3+}) can bind to the one single lactoferrin molecule. Each symmetrical lobe (N or C lobe) can bind one Fe^{3+} in the presence of carbonate ion (CO_3^{2-}) (González-Chávez *et al.*, 2009). The iron-binding site is situated inside the interdomain cleft in each lobe and consists of four residues 2 tyrosines (Tyr), 1 aspartate (Asp), and 1 histidine (His) residues (Sharma *et al.*, 2013) (**Figure 2.2**).

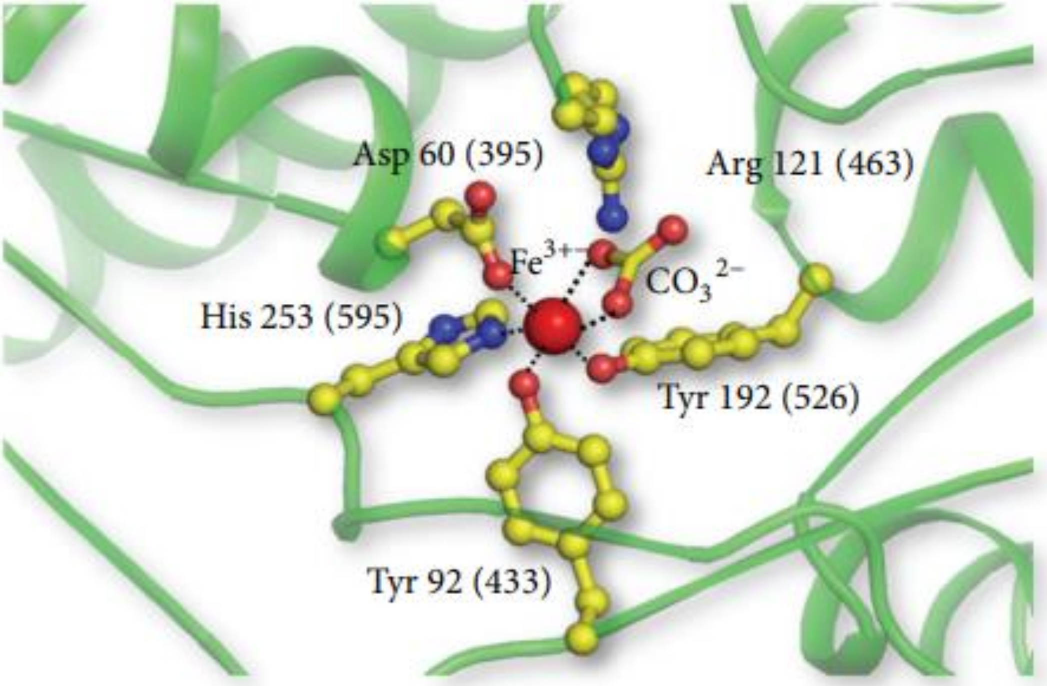

Figure 2.2 Schematic figure of the iron-binding site of the C-lobe of lactoferrin. The iron atom is shown as a red sphere, while the interacting amino acid residues (2 Tyr, Asp and His) of lactoferrin are in yellow. And the corresponding amino acid residue numbers of C-lobe are in brackets (Sharma *et al.*, 2013).

Each lactoferrin molecule can bind up to two Fe^{3+}, based on the Fe^{3+}saturation levels, lactoferrin has been found to exist in three different forms: fully iron saturated form (holo). Partially iron saturated form (mono), and fully iron depleted form (apo) (Wang *et al.*, 2019).

Commercially available lactoferrin is the native form where a mixture of these three different forms. The iron saturation level of commercially available (native), apo and holo forms of lactoferrin were found to be 12.6 ± 1.1%, 1.4 ± 0.5% and 100.0 ± 0.3%, respectively (Wang *et al.*, 2017b).

It has been reported that in the N-lobe, as iron is bound within the deep cleft formed by two domains (N1 and N2), domain N2 rotates by 53° and close (Schanbacher *et al.*, 1993). In the iron-saturated (holo) lactoferrin, two domains of each lobe enclose the bound iron and form this highly sable and relatively rigid structure that effectively sequestered away from the external environment. This holo lactoferrin conformation is difficult to remove the bound metal without first destabilizing the protein structure, for example, at very low pH or perhaps by receptor action (Baker & Baker, 2005).

Whereas the iron-free (apo) lactoferrin adopts an open form in which the two domains of each lobe are wide open that makes it less compact and stable than the holo lactoferrin (Baker & Baker, 2012).

It is estimated that only 6-8% of milk lactoferrin is iron saturated (Sánchez *et al.*, 1992a), which correlates to the finding of Makino & Nishimura (1992) that 95% of milk lactoferrin is in the monoferric and/or apolactoferrin form (Levay & Viljoen, 1995). Iron binding and release are associated with domain movements, in which the two domains of each lobe close over the bound iron ion or open to release it. This is influenced by cooperative interactions between the lobes (Baker & Baker, 2005).

2.3.3 Denaturation of lactoferrin

Lactoferrin is heat-sensitive as the heat-induced degradation of lactoferrin was observed above 72°C (Sánchez *et al.*, 1992b). When in aqueous form, lactoferrin is stabilized by hydrogen bonding, hydrophobic/hydrophilic interactions, disulphide bonds and ligand binding (Brisson *et al.*, 2007). These structure stabilizing forces can be affected by pH and temperature conditions.

The pH affects salt bridges and hydrogen bonding in lactoferrin whereas temperature alters the kinetic energy of hydrogen bonds, non-polar hydrophobic and intermolecular thiol/disulphide interactions (Brisson *et al.*, 2007; Hendsch & Tidor, 1994). The effects of pH and thermal changes on lactoferrin are presented below in **Table 2.3**.

Table 2.3 Effect of pH on the optical characteristics of apo-lactoferrin solutions[1] (Abe *et al.*, 1991).

	pH									
°C	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
80	-	-	-	-	-	-	±	+	±	-[2]
90	-	-	-	-	-	±	+	+	+	-[2]
100	-	-	-	-	±	±	+	+	+	-[2]
120	-	-	-	-	±	+	+	+	+	-[2]

[1]Clear solution (-), turbid solution (±), or gel (+).

[2]Gelled when the pH was adjusted to near neutral.

Table 2.3 showed that the apo lactoferrin treated with different temperature remained soluble from pH values 2 to 5. However, higher temperature and more alkaline conditions tend to denature the apo lactoferrin as the solutions become turbid or gel formed.

2.3.4 Functional properties and applications of lactoferrin

Lactoferrin has been reported to exhibit wide spectrum of functions expect for iron-bind ability, including antimicrobial, antioxidant, anti-inflammatory, immunoregulatory activities and regulation of cellular growth and differentiation that protect against cancer development (Jahani *et al.*, 2015; Niaz *et al.*, 2019; Vogel, 2012; Wakabayashi *et al.*, 2006; Ward *et al.*, 2005). Bovine lactoferrin has a net positive charge that allows the full hydrogen bonding potential of the carbonate ion, which therefore the carbonate ion can bind the Fe^{3+} (Moore *et al.*, 1997). It is suggested to exhibit physiological properties such as iron transport, antioxidant, anticarcinogenic and anti-inflammatory properties; and protection against microbial infection, which is the most widely studied function to date. The antimicrobial activity of lactoferrin is driven primarily by two mechanisms. The first is iron sequestration in sites of infection, which deprives the microorganism of this nutrient, thus creating a bacteriostatic effect. The other mechanism is the direct interaction of the lactoferrin molecule with the infectious agent. The positive amino acids in lactoferrin can interact with anionic molecules on some bacterial, viral, fungal and parasite surfaces, causing cell lysis (González-Chávez *et al.*, 2009).

2.3.5 Antibacterial activity of lactoferrin

Lactoferrin is considered to be the most polyvalent protein present in a host's defence against tissue injuries and infections in vertebrates. The structural characteristics of lactoferrin provide functionality in addition to the Fe^{3+} homeostasis function, there are also microbiostatic activities against a broad spectrum of bacteria, fungi, yeasts, viruses (Rodríguez-Franco *et al.*, 2005). Owing to the propensity of its basic N-terminal domain to interact with various microbial and host targets, lactoferrin not only has antimicrobial properties but also modulates the innate and adaptive immune responses (Legrand, 2016). Lactoferrin involved in a large spectrum of biological properties including antibacterial activity against Gram-negative and Gram-positive bacteria (Ellison & Giehl, 1991).

Initially iron chelation was considered as a major mechanism for its antimicrobial action. Now it is well established that iron-independent mechanisms such as direct interaction with bacteria leading to membrane destabilization, modulation of bacteria motility, aggregation or endocytosis into host cells, inhibition of adherence and biofilm formation are also responsible for its antimicrobial properties (Farnaud & Evans, 2003).

The bactericidal effect of lactoferrin is mediated by damaging of the outer bacterial membrane, with subsequent alteration of its permeability. Yamauchi *et al.* (2006) had demonstrated that bovine lactoferrin can disrupt the outer cell membrane of the Gram-negative bacterial. Lactoferrin binds to the lipid component of lipopolysaccharide (LPS) of the bacteria hence making the membrane more susceptible to factors that alter the cation-LPS relationship (Jahani *et al.*, 2015).

The ability of lactoferrin to inhibit bacterial growth *in vitro* was one of the earliest functions described for this protein. In 1972, Bullen *et al.* (1972) demonstrated that the antibacterial activity of lactoferrin depends on its capacity of sequestering iron necessary for bacterial survival and growth. Almost all bacteria need iron for growth, a lack of iron inhibits the growth of iron-dependent bacteria such as *Escherichia coli* (Yen *et al.*, 2011). However, lactoferrin can also serve as an iron donor, and in this manner to support the growth of some bacteria with lower iron demands such as *Lactobacillus sp. or Bifidobacterium sp.,* generally considered as beneficial (Petschow *et al.*, 1999; Sherman *et al.*, 2004).

Lactoferrin is capable of binding certain DNA and RNA viruses. The lactoferrin antiviral effect is not related to lactoferrin withholding iron from the environment. It is generally accepted that the inhibiting activity takes place during the early phases of viral infection by the lactoferrin binding with glycosaminoglycans. In this manner lactoferrin prevents viruses from entering cells and infection is stopped at an early stage (Ward *et al.*, 2005). Such a mechanism has been demonstrated as being effective against the herpes simplex virus and cytomegalovirus (Andersen *et al.*, 2001; Fujihara & Hayashi, 1995; Marchetti *et al.*, 1996). The isolated N-terminal basic peptides-lactoferricin, which is a lactoferrin-derived peptide released by pepsin digestion of lactoferrin, is a more potent antibacterial compound than the native protein (Roseanu *et al.*, 2010). **Table 2.4** presents the bacteria that can be inhibited by lactoferrin and corresponding mechanisms.

Table 2.4 The bacteria against which lactoferrin has shown an inhibitory effect and the mechanism used by lactoferrin to exert its effect.

Bacteria	Mode of Action	Reference
Gram-positive		
Bacillus stearothermophilus	Iron sequestering	(O'Leary & Busta, 1974)
Staphylococcus aureus	Iron sequestering	(Bhimani *et al.*, 1999)
Streptococcus mutans	Iron-independent interaction with cell surface	(Arnold *et al.*, 1981)
Staphylococcus epidemidis	Interaction with lipoteichoic acid on bacterial surface	(Roseanu *et al.*, 2010)
Gram-negative		
Chlamydophila psittaci	Interferes with cell adhesion	(Beeckman *et al.*, 2007)
Helicobacter pylori	Iron-independent mechanism of inhibition	(Wang *et al.*, 2001)
Legionella pneumophila	Prevent intracellular proliferation	(Goldoni *et al.*, 2000)

2.3.6 Antiviral activity of lactoferrin

Lactoferrin displays antiviral activity against both DNA- and RNA-viruses, including rotavirus, respiratory syncytial virus, herpes viruses, human immunodeficiency virus (HIV) (Van der Strate *et al.*, 2001) and poliovirus (Marchetti *et al.*, 1999), predominantly in *in vitro* systems.

Anti-herpesvirus activity is mediated mainly by the N-lobe, but the C-lobe and even other members of the transferrin family also demonstrate some activity, suggesting that antiviral activity may be a property that developed early in the evolution of the transferrin family of proteins (Giansanti *et al.*, 2002; Siciliano *et al.*, 1999). Andersen *et al.* (2001) reported that lactoferrins and its N-terminal peptide lactoferricin (particularly the cyclic form) inhibit expression of early and late antigens, as well as production of infectious viral progeny during human cytomegalovirus (HCMV) infection *in vitro*.

The antiviral activity of lactoferrin lies in the early phase of infection. It has been reported that lactoferrin binds to and blocks glycosaminoglycan viral receptors, especially heparan sulphate. The binding of lactoferrin and heparan sulphate can prevent the first contact between virus and host cell and therefore prevents infection (Van der Strate *et al.*, 2001).

2.3.7 Applications of bovine lactoferrin

Lactoferrin was first added to infant formula in 1986 (Wakabayashi *et al.*, 2006). Five hundred milligrams per kilogram lactoferrin is recommended as the level of supplementation in powdered infant formula by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) (Hao *et al.*, 2019). Also, lactoferrin has been added to a broad variety of products, such as immune system-enhancing nutraceuticals, cosmetics, pet care supplements, drinks, fermented milks, chewing gums, and toothpaste (García-Montoya *et al.*, 2012; González-Chávez *et al.*, 2009; Hao *et al.*, 2019; Mulder *et al.*, 2008; Tomita *et al.*, 2002).

Pizarro *et al.* (1991) found that iron deficiency and low iron levels were most common among infants fed a non-iron-fortified cow milk formula but were uncommon among infants fed an iron-fortified cow milk formula. Infants consistently fed iron-fortified formula had less than a 1-in-25 chance of having anaemia, and less than 1% had iron-deficiency anaemia. In addition, studies in models of neonatal piglets founded that lactoferrin-fortified diet significantly reduced the incidence of diarrhea and improved the humoral immunity (Li *et al.*, 2014).

Duran *et al.* (2002) reported that the dietary supplements containing bovine lactoferrin had antibacterial, antiviral and antioxidant properties and enhanced the immune system response. Mulder *et al.* (2008) studied the potential immune modulating properties and antioxidant activities of an oral supplementation of bovine lactoferrin in humans, their results showed that an oral supplementation of bovine lactoferrin, in particular 100 mg for seven days followed by 200 mg for a further seven days, enhanced total T-cell activation and hydrophilic antioxidant status.

Mohamed & Schaalan (2018) showed that the antidiabetic efficacy of lactoferrin was mechanistically evidenced by amelioration of the glycolytic homeostasis, dyslipidemic profile, and anti-inflammatory clinical outcomes.

2.4 Freeze drying

2.4.1 Introduction of freeze drying

Freeze drying, also known as lyophilisation, is a drying process that removes water from the frozen state of the material. Freeze drying was introduced as a commercial practice during World War II due to the huge demand for dried human blood plasma in treatments in the field. By the end of the war, freeze drying became an accepted technique for drying medicine and biological materials (Harper & Tappel, 1957). Freeze drying was first introduced to process food in the 1940s (Devahastin & Jinorose, 2020). A complete freeze drying process includes three major stages: freezing, primary drying and secondary drying (Kasper & Friess, 2011). **Figure 2.2** shows an overview of the freeze drying process.

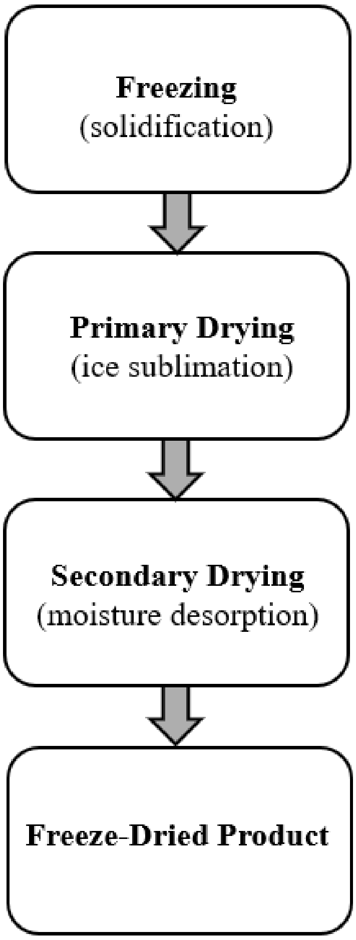

Figure 2.3 Freeze drying process.

2.4.2 Three stages of freeze drying

Freezing stage

The overall performance of the freeze-drying process depends significantly on the freezing stage (Kasper & Friess, 2011). At this stage, the liquid is cooled until below the solidification temperature of the material system and ice nucleation starts, followed by ice growth (Nail *et al.*, 2002).

Primary drying stage

During the primary drying stage, the crystalline ice formed during freezing is removed by sublimation. This requires the chamber pressure to below the vapor pressure of the crystalized solvent. The sublimation rate of the ice is a function of the area over which sublimation takes place and the thickness (Franks, 1998). The thicker the layer of the material on the tray, the thinner the available superficial area for contact which leads to a decrease in the drying rate (Boss *et al.*, 2004). At the completion of primary drying stage, 15-20% of the water is left undried (sorbed or bound water), which is then removed in the secondary drying stage.

Secondary drying stage

During the secondary drying stage, the solvent is removed from the chamber and a small amount of water can be removed by desorption. The bound water is removed by heating the product under vacuum (Boss *et al.*, 2004). Pores on the dried layers are formed when bound (unfrozen) water is removed from the solute (Sadikoglu & Liapis, 1997).

2.4.4 Merits and limitations of freeze drying

Often freeze drying is compared to conventional spray drying for its following major advantages:

a. Low temperature drying can minimise the degradative reactions that normally occur in the spray drying process, such as the Maillard reaction (Zhou & Langrish, 2021), protein denaturation and enzymatic reactions (Liapis & Bruttini, 2020).
b. High recovery of the product, low moisture content and water activity, enhanced stability of the products (Ciurzynska & Lenart, 2011).
c. Freeze dried products are porous and reconstitute easily in water (Harper & Tappel, 1957).
d. Better to conserve the sensorial properties of produced foods in terms of flavour, aroma, colour and texture (Valentina *et al.*, 2016).

Disadvantages:

a. Time-consuming, slow drying rate and the specific requirement of using very low pressures and high vacuum (Liapis & Bruttini, 2020).
b. Very expensive due to its high energy consumption and high costs of both operation and maintenance (Ciurzynska & Lenart, 2011).

2.5 Spray drying of dairy products

The removal of water from milk prevents the growth of micro-organisms and facilitates preservation and storage of milk constituents (Schuck, 2002). Various drying techniques have historically been employed to prolong the shelf life of dairy products. However, the quality of dried dairy products is affected particularly by the drying technique itself (Caric & Kalab, 1987; Franks, 1998). Since the 1980s, the dairy industry has successfully recovered proteins such as caseins, caseinates, whey proteins, and various derivatives such as micellar casein concentrates, whey concentrates and native phosphocaseinate suspension, from milk. These milk proteins were dehydrated primarily by evaporation followed by spray drying (Schuck, 2002).

Among the dehydration techniques to process fresh milk, spray drying is one of the most convenient techniques for the dehydration of dairy products or derivates. Spray drying is a continuous drying operation of converting a liquid feed into a powder with a hot gas being the drying medium (King Dean, 1987). Applications of spray drying within the dairy industry date from the early 1800s, yet it was not until the 1850s that it became technologically feasible to implement this unit operation industrially, and it was first patented in 1872 (O'Sullivan *et al.*, 2019).

Spray drying is currently the mostly used method in industrial milk production due to its drying efficiency and high process control accuracy (Li *et al.*, 2024b). World production of dry dairy products has increased constantly in recent years, due to the main advantages of the powders which:

a. retain high quality, without special storage conditions.
b. reduce mass and volume compared to fluid products.
c. provide balance between milk supply and consumption.
d. provide an irreplaceable food component in hot climate regions.
e. are a valuable food reserve for emergencies.
f. are suitable for various tailor-made food products (Schuck *et al.*, 2016).

To describe the spray drying process, there are three major phases which comprise the process. First of all, the concentrated liquid feed (generally by evaporation) is pumped into the dryer and is converted to discrete solid particles, through the dispersion of the liquid stream by a process known as atomization, into individual droplets that having a high surface to mass ratio. Because the rate of evaporation is directly proportional to the droplet surface area (Launder, 1978; Zang *et al.*, 2019). Then these individual droplets come into close contact with a hot gaseous drying medium, where the solvent (usually water) within the dispersion droplets is vaporized, resulting in rapid and almost complete moisture evaporation, and thus drying. The last phase being product separation and recovery, where the dried particles become separated from the drying medium (usually air, inert gas is used in some conditions to prevent chemical reactions. Depending on the air flow as well as the chamber design, separation can be carried out altogether in the collection unit or firstly at the chamber bottom and secondly in the collection part (Cal & Sollohub, 2010; O'Sullivan *et al.*, 2019). The drying process is shown in **Figure 2.4.**

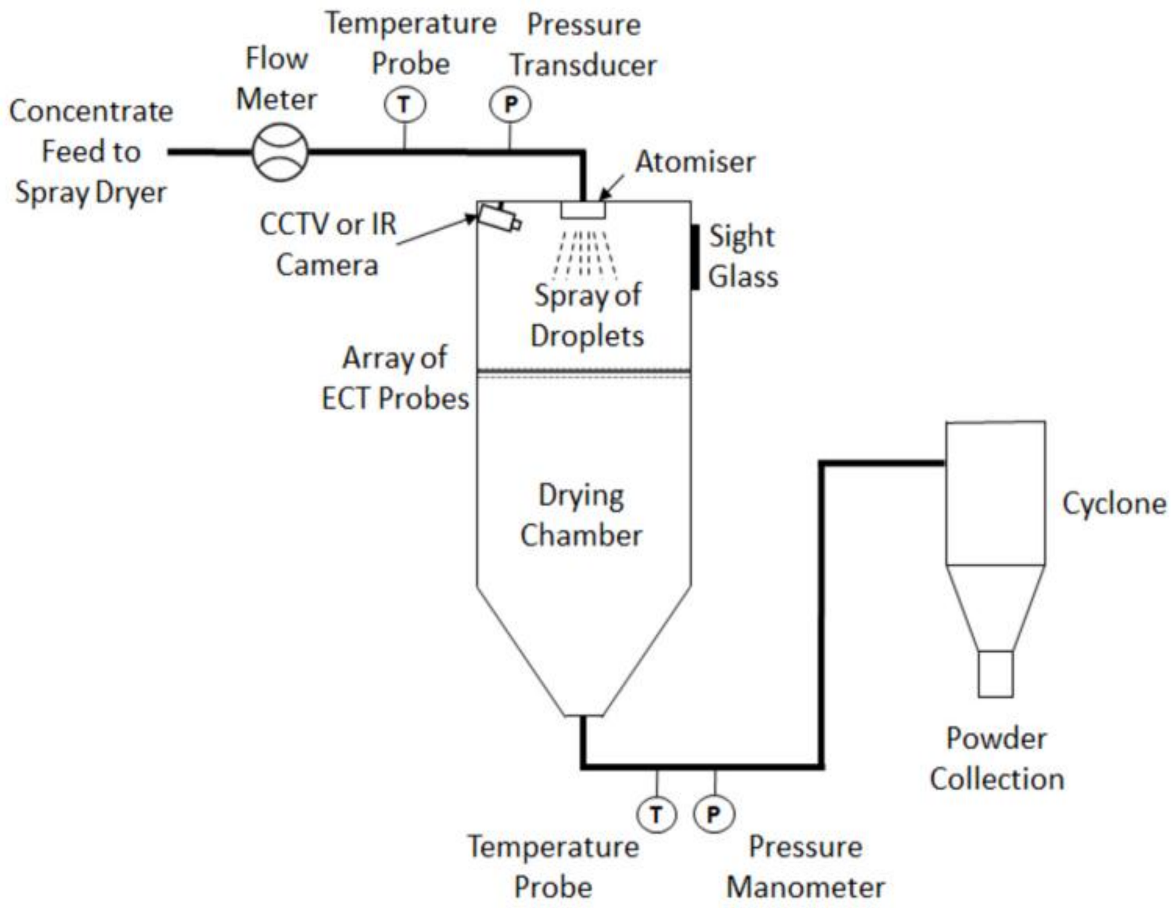

Figure 2.4 Overview of the spray drying unit operation and its associated process control systems for monitoring the performance of the atomization process (O'Sullivan *et al.*, 2019).

2.5.3 Spray drying theory

Thermal drying is a simultaneous heat and mass transfer process. The energy necessary for water evaporation is supplied to the material, meanwhile, the moisture is transported to the ambient medium (often hot air). The drying medium picks up the water vapour from the surface of the drying material and carries it away. The main mechanisms of drying are surface diffusion or liquid diffusion on the pore surfaces, liquid or vapor diffusion due to moisture concentration differences, and capillary action in granular and porous foods due to surface forces (Kowalski, 2012).

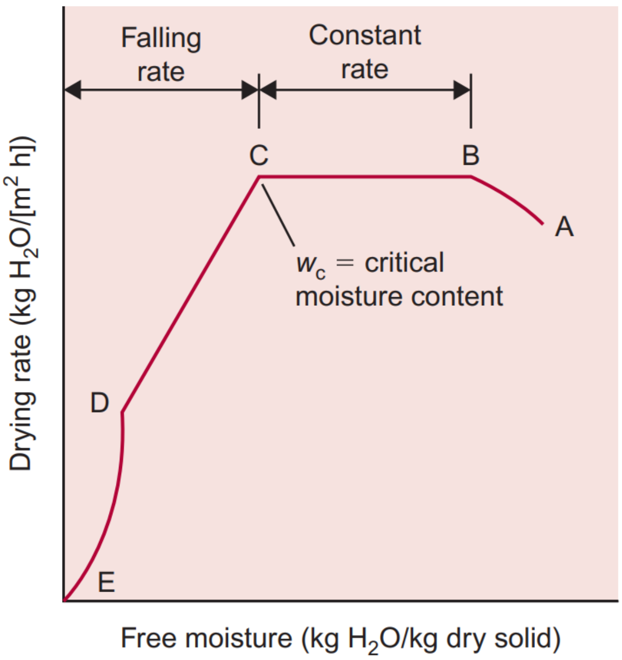

Figure 2.5 Illustration of constant rate and falling rate drying periods (Singh *et al.*, 2014).

Three drying periods are distinguished during the drying process (**Figure2.5**). During an initial adjustment period of the drying (AB), water is transported in liquid form to the droplet surface where it evaporates and escapes as vapour to the ambient air. The surface moisture of the droplets quickly drops to the wet-bulb temperature as the temperature rises. Once the product arrives at the wet bulb temperature of the air, the constant rate drying period (BC) proceeds consequently, where removal of the moisture takes place from the interior porous structure as well as remaining wet spots on the surface. Significant decrease of moisture content occurs at a constant rate. The moisture removal rate decreases with time at moisture levels below the critical moisture content. The falling rate drying period (CE) then proceeds, during which the temperature is raised to fully evaporate the remaining solvent (Basmadjian, 2007; Singh *et al.*, 2025). However, with most of the moisture removed from the previous periods, the solid content of the outer layer of the droplet develops a dry shell and reduces the shrinkage of the particle (Perré & May, 2007). This can cause problems for temperature-sensitive activities from microorganisms, probiotics, or proteins.

2.5.5 Critical parameters during the spray drying process

- Inlet temperature of air: The inlet temperature, ranging from 90-160°C in practice (Bhandari *et al.*, 1993), is the temperature of the drying medium when first in contact with the feed. It is related to the product's powder moisture, solubility, density (bulk and packed), and hygroscopicity (Goula *et al.*, 2004). The powder is subjected to higher temperature, which may affect the chemical/physical properties of heat sensitive product.

- Outlet temperature of air: It is a consequence of the heat exchange in a drying air–feed system. In practice, outlet temperature is the highest temperature to which the product may be heated. It governs the sizing of powder recovery equipment; the higher is the outlet air temperature the larger will be the size of powder recovery equipment and the conveying ducts and plenums (Maury *et al.*, 2005).

- Viscosity of feed material: A higher viscosity of the feed ingredient can lead to an increased spray drop size and hence to an increased drying time. As the viscosity is lowered, less energy or pressure is required to form a particular spray pattern. Viscosity can range from 0.001 to 0.308 Pa·s. Pneumatic nozzles can atomize highly viscous feeds easier than rotary or pressure nozzles, and the new effervescent nozzle has better performance dealing with food liquids with high viscosity (Stähle *et al.*, 2015).

- Solid content of feed material: A higher solid content of the feed is correlated with lower powder solubility, better powder wettability and more intact spherical shape of the particle (Wu *et al.*, 2014).

- Feed temperature: with the temperature of the feed increases, the solution is may easily dry as it brings more energy to the system (Patel *et al.*, 2009).

- Wall deposition: Partially dried product falls on the wall during spray-air movement in the drying chamber. Wall deposition is a key processing problem in the spray dryer particles that indirectly affects the quality and quantity of the product. The degree of wall deposition is affected by several factors including operating parameters, type and size of spray dryer and the spray dryer wall properties (Keshani *et al.*, 2015).

2.5.6 Merits and limitations of spray drying

Advantages:

a. Spray drying is a rapid, continuous, cost-effective, and scalable dehydration process (Sosnik & Seremeta, 2015).

b. Achievable in producing powders with narrow size distribution (Bhandari *et al.*, 2008).

c. High encapsulation efficiency and encapsulated product stability (García-Carrasco *et al.*, 2023).

Disadvantages:

Before spray drying, the milk undergoes several processes (such as heat treatment, cream separation, membrane processes, vacuum evaporation and homogenization). The chemical, physical, technological, nutritional, functional and microbiological properties of final products are influenced by a number of factors such as operating conditions, properties of the dairy products and storage conditions.

a. Protein denaturation due to high-temperature processing.

b. Reduced product solubility (Malumba *et al.*, 2008).

c. Difficult to handle and clean (Samantha *et al.*, 2015).

2.6 Other spray drying methods in the development

2.6.1 Foam spray drying

Foaming of liquid and semi-liquid materials has long been recognized as one of the methods to shorten drying time (Kudra & Ratti, 2006). Foam spray drying is based on the drying of purposely foamed droplets, these being formed before, during, or after atomization and before or during the drying itself (Ratti & Kudra, 2006). In foam spray drying, the product to be dried is first converted into a stable foam by whipping, bubbling, or shaking in the presence of a foaming agent and foam stabilizer. The foam has been significantly increased the surface area of the product and thereby decreasing the time, temperature, and cost needed for drying (Çalışkan Koç *et al.*, 2022).

Foam spray drying method produces a quickly dispersible product during drying without a subsequent agglomerating step; it permits a greatly increased dryer output. Gas injection improves the efficiency of water removal and makes possible the drying of the product (in the case of skim milk concentrates up to 60% solids); it hence reduces costs of instant powder manufacture by requiring only minor equipment changes which do not affect the efficiency of the dryer to produce a standard powder (Patel *et al.*, 2009).

In the application of foam drying, it has been applied to obtain high-quality powders from yoghurt, tomato paste and a variety of fruit pulps and juices (Hardy & Jideani, 2017; Kudra & Ratti, 2006).

2.6.2 Spray freeze drying

Instead of using hot atmosphere to drive moisture from atomized fluid particles, freezing air is employed and brought into contact with the spray, whereupon the individual droplets are frozen for subsequent moisture removal through sublimation under vacuum. This process must be performed under the triple point of water. As the water is transferred directly from the solid to vapor in the sublimation process, the drying rate is mainly dependent on the vapor pressure (Wang *et al.*, 2006).

Spray freeze drying process **(Figure 2.6)** involves three major units-droplet formation, freezing and sublimation (Ishwarya *et al.*, 2015).

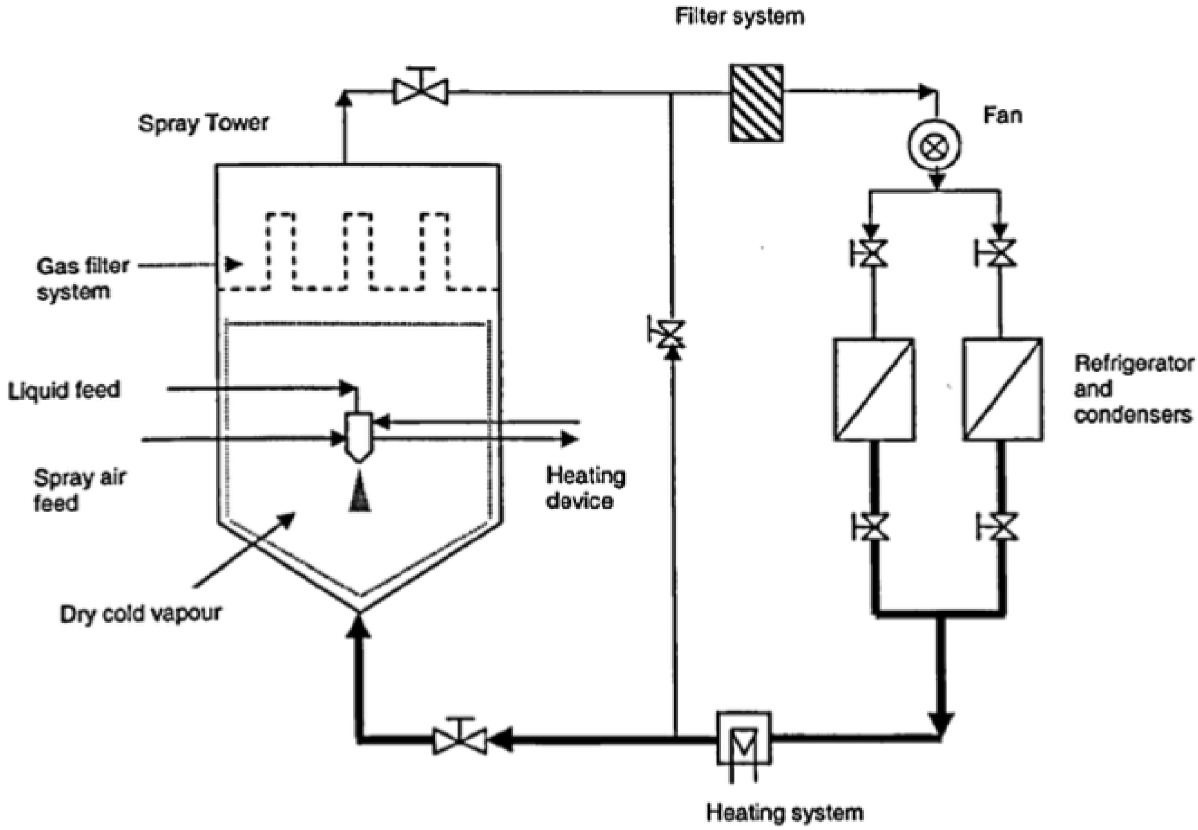

Figure 2.6 A schematic diagram of spray freeze drying apparatus (Ishwarya *et al.*, 2015).

During the freezing stage, atomized fine droplets are quickly frozen upon contact with a cryogen to form icy particles. This can further be demonstrated by several consecutive steps:

1) initial cooling to a super cooled temperature below the normal freezing temperature,

2) nucleation followed by growth of ice crystals and the subsequent rapid evolution of latent heat associated with fast crystal growth, termed recalescence,

3) further, slower crystal growth which is limited by heat transfer from the cryogenic fluid, during which some freezing point depression may occur and once freezing is complete, cooling of the frozen particle down to the cryogen temperature.

However, substances other than pure water will not solidify completely owing to the freeze concentration of solution in the interstices between ice crystals.

2.7 Electrostatic spray drying

Electrostatic spray drying delivers an electrostatic charge during the atomization process of liquid droplets and water is removed at lower temperatures. This drying technique applies nitrogen instead of oxygen as a drying medium which expands applicability to oxygen sensitive materials (Masum *et al.*, 2022). The electrostatic spray drying system consists of four major components: a pumping system (often a syringe pump), a metal nozzle, high voltage power supply, and inert drying gas. During the atomization, the conductive liquid feed is injected by the pump through the nozzle to which the electrical potential is applied. With sufficient voltage supplied, the free charges at the surface of the liquid exiting the nozzle cause an electrical stress which breaks up the stream into primary droplets. Next these primary droplets experience the Coulomb fission which is a process where a highly charged "primary drop" undergoes successive breakup to result in a multitude of smaller "secondary drops" (Gu *et al.*, 2007). Followed by the solvent evaporation that causes the primary droplets to shrink, leading to the increase in charge concentration so the primary droplets finally will break up into smaller offspring droplets (**Figure 2.7**).

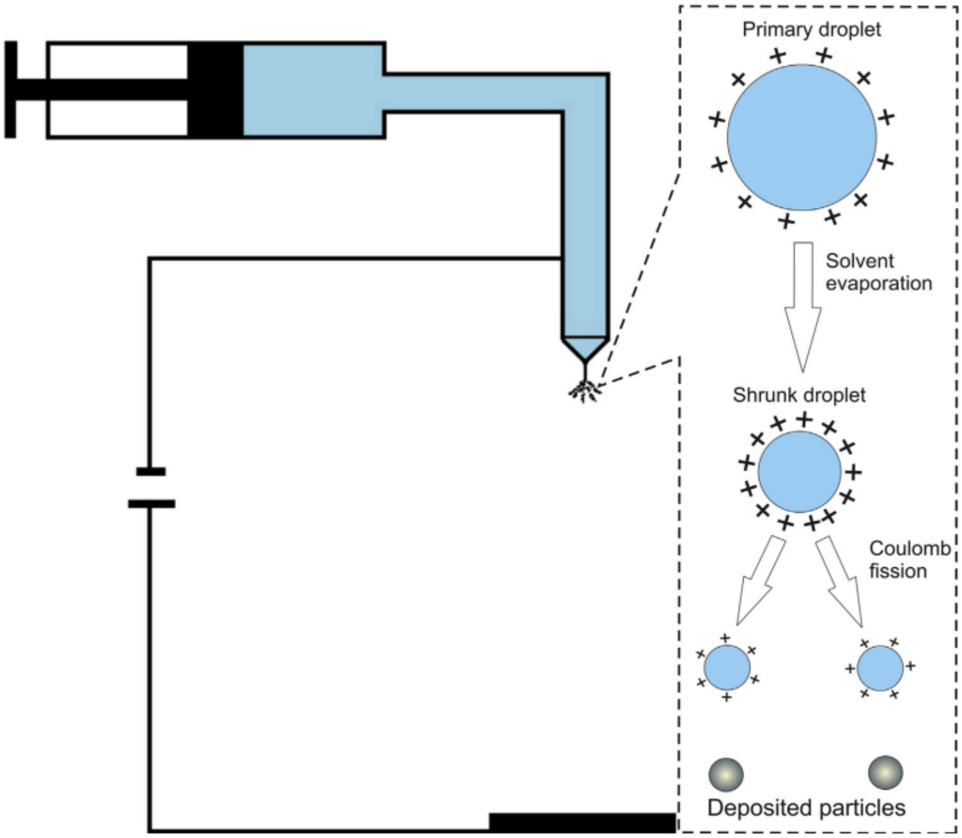

Figure 2.7 Schematic illustration of a standard electrostatic spray drying setup (Nguyen *et al.*, 2016).

In the electrostatic spray drying process, the electrostatic effect stratifies the components of atomized droplets based on the polarity of the materials. The application of an electronic charge results in the polar solvent picking up more electrons than the solute. With feedstock in a polar solvent, solid materials are driven to the inside of the droplet and the solvent is driven to the outside. This prevents early shell formation of the particles and eliminates the need for the falling-rate drying period, allowing fast, efficient drying at a lower temperature (**Figure 2.8**) (Anon, 2024c).

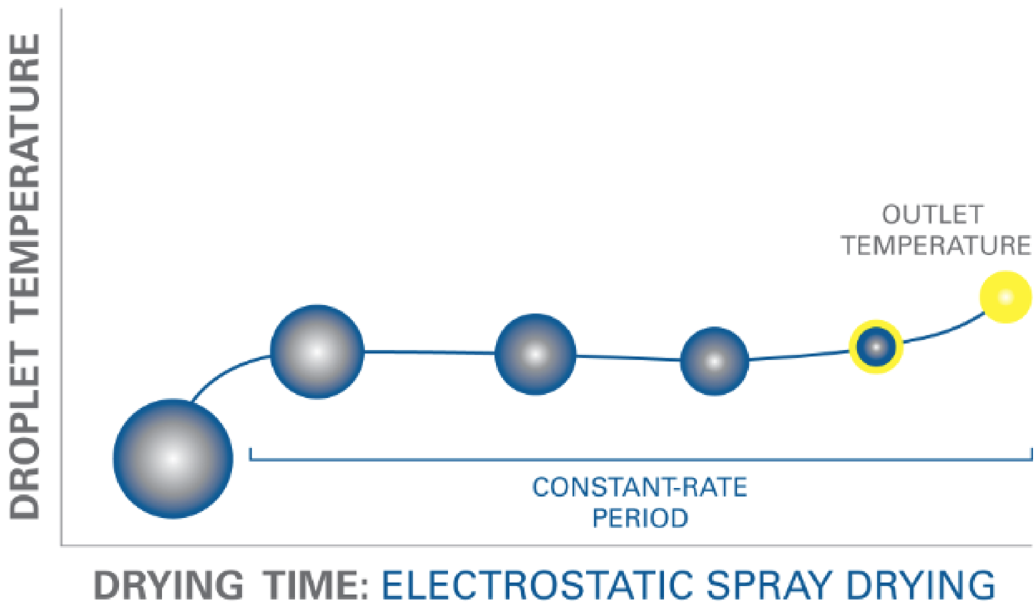

Figure 2.8 Illustration of constant rate and falling rate drying periods of electrostatic spray drying (Anon, 2024c).

From the previous studies, Thakkar & Misra (2020) confirmed an improved therapeutic potential of electrostatic spray dried nanocrystals. (Mutukuri *et al.*, 2021) used electrostatic spray drying to produce solid monoclonal antibody formulation with improved physical stability than traditional spray drying.

2.8 Lactoferrin recovery and preparation on laboratory and industrial scale

Lactoferrin in aqueous form is very sensitive to chemical or microbial degradation and starts to lose its functional properties when it is stored at ambient temperature for more than four weeks (Wakabayashi *et al.*, 2006). Therefore, it is generally converted into powder form to extend its shelf-life and to preserve its functional properties.

The extraction and drying process to produce lactoferrin powder and processing parameters of lactoferrin-containing products have to be optimized to minimize any undesired denaturation (Wang *et al.*, 2017b). Skim milk and sweet cheese whey (pH 5.2-6.7) that have not undergone excessive heating can be sources of lactoferrin.

At present, lactoferrin is isolated and purified on an industrial scale from cheese whey and skim milk. The concentration of lactoferrin in cheese whey is roughly 100 mgL^{-1} (Tomita *et al.*, 2002). The feedstock containing lactoferrin is usually concentrated via ultrafiltration and diafiltration prior to introduction into the spray dryer. Since lactoferrin exists as a cationic protein in whey, it is readily adsorbed to a cation-exchange resin and then eluted using salt solutions. The eluted crude lactoferrin is desalted and concentrated using ultrafiltration and after which it is subjected to pasteurization. Purified lactoferrin powder with a purity of 95% or higher is finally obtained by freeze drying. In an alternative process, microfiltration and spray drying are performed instead of pasteurization and freeze drying, respectively (Tomita *et al.*, 2002).

On the laboratory scale, spray dried lactoferrin powders are usually produced using a laboratory scale spray dryer. According to Wang *et al.* (2017b), for the isolation of lactoferrin, industrially obtained fresh lactoferrin solution (16–18%, w/v) is used as starting material. The inlet temperature was maintained at 180°C. In order to investigate the extent of denaturation of the spray dried powders, two outlet temperatures (70°C and 95°C) were chosen. The selected air inlet and outlet temperatures were within the recommended inlet (180–220°C) and outlet (90–110°C) temperature ranges used for spray drying of heat sensitive products at normal atmospheric pressure. These two inlet and outlet temperature ranges are also commonly used in the industrial production of protein powders through spray drying. The feed flow rate during the spray drying process was adjusted to between 1000 and 1200 mLh^{-1} to maintain the set outlet temperatures. Powders collected from the cyclone were used for further tests and the powders remaining in the chambers were discarded.

In contrast to spray drying, according to Ratti (2012), freeze drying requires 88% more capital cost and 80% more operational cost than spray drying. The challenge of spray drying is that lactoferrin encounters thermal, interfacial and rapid evaporation-related stresses. The process parameters involved in extraction and drying of proteins induce certain degree of changes in the protein structure and ultimately affect their functional attributes and nutritional value (Wang *et al.*, 2017b).

2.9 Effects of spray drying on colostrum and lactoferrin

Uniform and repeatable product characteristics are critical in the performance and acceptance of consumer products, and the spray drying process can have a major influence on achieving these characteristics (Huntington, 2004). To better understand the influence of different drying treatments on colostrum and lactoferrin, literature investigations were carried out in terms of physicochemical, structural and biological attributes.

From the literatures, physicochemical properties including the water activity, moisture content, particle size, solubility, antioxidant capacity and iron-binding ability were studied and summarized. Findings about biological properties including active protein and antimicrobial ability of colostrum and lactoferrin were presented. The extent of denaturation and changes in the secondary structural features of spray dried colostrum and lactoferrin powders were also studied (Wang *et al.*, 2017b).

According to Wang *et al.* (2017a), spray dried lactoferrin powders with an outlet temperature of 95°C had a moisture content and water activity double that of freeze dried lactoferrin powder. Spray dried powders with an outlet temperature of 70°C had moisture content and water activity four times greater than freeze dried. This indicated that the spray drying conditions retained more moisture than freeze drying. The protein and residual moisture contents, water activity, solubility, particle size and colour parameters of both spray dried lactoferrin (SDLf) and freeze dried lactoferrin (FDLf) are presented in **Table 2.5.**

Table 2.5 Physicochemical properties of obtained lactoferrin powders. SD_{95} = Spray dried lactoferrin at 95°C outlet temperature, FD = Freeze dried lactoferrin, SD_{70} = Spray dried lactoferrin at 70°C outlet temperature (Wang *et al.*, 2017a).

Sample	Protein content (%)	Moisture content (%)	Water activity (aw)	Solubility (%)	Particle size (µm)
FD	90 ± 2.3^{a}	2.7 ± 0.5^{a}	0.1 ± 0.0^{a}	94.3 ± 0.8^{a}	48.0 ± 0.7^{a}
SD_{70}	92.2 ± 2.2^{a}	8.6 ± 0.3^{b}	0.4 ± 0.0^{b}	99.3 ± 0.8^{b}	12.9 ± 0.2^{b}
SD_{95}	94.0 ± 2.6^{a}	5.2 ± 0.8^{c}	0.2 ± 0.0^{c}	98.4 ± 2.1^{b}	12.8 ± 0.1^{b}

All the data are expressed as mean ± SD and are the mean of triplicates.

Mean values with different lower-case letters in superscript at the same column are significantly different ($p<0.05$)

Kawakami *et al.* (1992) found a 15−30% loss of iron-binding capacity of bovine native lactoferrin after heating at 90°C for 4 min. Similarly, Mata *et al.* (1998) observed a 50% loss of iron-binding ability of human native lactoferrin in buffer after heating at 85°C for 20 min. Based on the previous research carried out, high temperature treatment can actually reduce the iron-binding capacity of lactoferrin.

Immunoglobulins present in colostrum are thermolabile, and denaturation initiates at operating temperature above 65°C (Costa *et al.*, 2023). Godden *et al.* (2003) reported an IgG concentration reduction by 23.6-58.5% due to the pasteurization process. Heat can negatively affect the immunoglobulin content in colostrum.

2.10 Fourier-transform infrared spectroscopy (FTIR) to determine the secondary structural changes of lactoferrin and colostrum

The first structures of protein at an atomic resolution were determined in the late 1950s (Kendrew *et al.*, 1958). Proteins are linear biological polymers for which the monomeric units are amino acids (Gallagher, 2009). A protein exhibits four levels of structure: primary, secondary, tertiary, and quaternary. Primary structure refers to the sequence of amino acids. The secondary structure of a protein refers to the periodical spatial arrangement of amino acid residues at certain segments of the polypeptide chain. Among these, they are stabilized by hydrogen bonds between peptide amide and carbonyl groups (Schulz *et al.*, 1979).

In general, two forms of periodic secondary structures are found in proteins: helical and extended sheet-like structures. Specifically, the polypeptides can form *a*-helix, *b*-sheets, *b*-turns or random coils. Among these, *a*-helix and *b*-sheet structures are very stable, especially proteins that contain large fractions of *b*-sheet structure usually exhibit high denaturation temperatures (Damodaran *et al.*, 2007).

The three-dimensional folding of the globular protein is called the tertiary structure. The structures of aggregates of globular proteins are the quaternary structures There are a few different techniques to determine the structure of proteins, such as X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared (FTIR) spectroscopy. X-ray crystallography and NMR spectroscopy can provide the greatest level of detail about a protein's structure and a number of proteins' structure were deciphered primarily using X-ray crystallography (Gallagher, 2009).

FTIR has long been established as a powerful analytical technique to investigate protein secondary structure and local conformational changes. FTIR is an absorption spectroscopy that analyses the frequencies of amide bands to determine the distribution of the secondary structure of proteins (Pelton & McLean, 2000). The FTIR spectrum presents the absorption of energy by vibrating the stretching and bending of chemical bonds.

FTIR spectroscopy works by shining infrared radiation on a sample and seeing which wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. The polypeptide and protein repeating units give rise to nine characteristic IR absorption bands, namely amide A, B and I-VII. Among these, absorption associated with the amide I and II leads prominent to the vibrations of the bonds (Kong & Yu, 2007).

According to Stanciuc *et al.* (2013) the elements of the secondary structure of lactoferrin mainly altered by the thermal treatment, changes in the secondary structure features of lactoferrin are presented in **Table 2.6**.

Table 2.6 Structural indicators of lactoferrin molecule thermally treated at 25 and 80°C (Stanciuc *et al.*, 2013).

Descriptors	Temperature	
	25℃	80℃
Secondary Structure Descriptors		
Strand, %	17.3 ± 0.02	16.1 ± 0.06
Alfa helix, %	23.5 ± 0.15	21.2 ± 0.20
Helix 3-10, %	3.8 ± 0.06	7.7 ± 0.06

The average structural properties of lactoferrin highly depend on the temperature. Analysis of the two structures equilibrated at 25 and 80°C indicated that a temperature increases results in the decrease of the elements of the regular secondary structure, such as strand and α-helix content. Regardless of temperature, the secondary structure is dominated by the α-helix motif (23.5% at 25°C and 21.2% at 80°C). Although the total number of residues involved in helix formation decreased with temperature, the number of helices increased from 28% at 25°C to 37% at 80°C (Stanciuc *et al.*, 2013).

Chapter 3. Materials and Methods

Materials, three different drying techniques, chemical reagents and the analytical methodologies used in this research are described in this chapter. The physicochemical properties of liquid lactoferrin, colostrum and their powders produced from three different drying treatments were determined, including moisture content, water activity, secondary structure profile, iron-binding and antioxidant abilities. The biological activities of these samples were also investigated regarding the proteins' bioactivity and anti-microbial activity.

3.1 Materials

3.1.1 Raw bovine colostrum

Raw bovine colostrum was donated by Magnolia Dairy Ltd., Auckland, New Zealand. It was produced in July 2022 from Jersey breed cows within 12 h after calving. After collection of the colostrum, it was immediately transported to the food technology laboratories at Massey University, Auckland, and gently mixed using an overhead stirrer (RW 20 digital, IKA, USA) at 200 rpm for ~ 20 minutes. Then the colostrum was divided into 2.5 L plastic containers and stored at -20°C before use.

3.1.2 Bovine lactoferrin stream

Bovine lactoferrin stream was provided by Westland Milk Products, Hokitika, New Zealand. The lactoferrin was recovered from skim milk using ultrafiltration with diafiltration. The lactoferrin stream was received at Massey University, Auckland in October 2022, and stored at -20°C in 2.5 L plastic containers before use.

3.1.3 Commercial lactoferrin powder samples

For comparison purposes, two batches of freeze dried lactoferrin powders supplied by Westland Milk Products, Hokitika, New Zealand in 2022 were analysed in this project. Received powder samples were stored in foil barrier bags at -20°C before use.

3.2 Proximate analysis of liquid materials

Proximate analysis of the liquid colostrum and lactoferrin was completed in November 2022, by the Nutrition Laboratory, Massey University, Palmerston North, New Zealand.

Total solids was determined by the Dairy method, AOAC 990.19, 990.20. Ash was determined by the furnace method at 550°C AOAC 942.05 (Feed, meat). Crude protein was determined by method AOAC 968.06 (Dumas method), the conversion factor for nitrogen-to-protein in colostrum was 6.38 and that for lactoferrin was 6.14. Lactose was determined by an enzymatic method. Fat in colostrum was determined by the Mojonnier method (Flour, Baked, extruded products) AOAC 922.06; fat in lactoferrin was determined by the Mojonnier method, (Dairy) AOAC 989.05. Moisture was calculated from total solids. Carbohydrate was calculated by difference.

3.3 Experimental design

Lactoferrin and colostrum were dried using three different drying methods; freeze drying (F), spray drying (S) and electrostatic spray drying (ES). Freeze-drying was completed in one batch. For spray drying, lactoferrin and colostrum were spray dried at two different temperatures in duplicates, inlet 180°C/outlet 90°C and inlet 165°C/outlet 90°C. As for electrostatic spray drying, two different temperatures were used: inlet 75°C/outlet 35°C, and inlet 150°C/outlet 60°C. Two liquid materials and all the dried sample powders and are listed in **Table 3.1**.

Table 3.1 Liquid and powder colostrum and lactoferrin samples evaluated.

No.	Abbr.	Sample
1	LQCL	Liquid colostrum
2	LQLF	Liquid lactoferrin
3	FCL	Freeze dried colostrum
4	FLF	Freeze dried lactoferrin
5	SCL165B1	165°C Spray dried colostrum Batch 1
6	SCL165B2	165°C Spray dried colostrum Batch 2
7	SCL180B1	180°C Spray dried colostrum Batch 1
8	SCL180B2	180°C Spray dried colostrum Batch 2
9	SLF165B1	165°C Spray dried lactoferrin Batch 1
10	SLF165B2	165°C Spray dried lactoferrin Batch 2
11	SLF180B1	180°C Spray dried lactoferrin Batch 1
12	SLF180B2	180°C Spray dried lactoferrin Batch 2
13	ESCL75B1	75°C Electrostatic spray dried colostrum Batch 1
14	ESCL75B2	75°C Electrostatic spray dried colostrum Batch 2
15	ESCL150B1	150°C Electrostatic spray dried colostrum Batch 1
16	ESCL150B2	150°C Electrostatic spray dried colostrum Batch 2
17	ESLF75B1	75°C Electrostatic spray dried lactoferrin Batch 1
18	ESLF75B2	75°C Electrostatic spray dried lactoferrin Batch 2
19	ESLF150B1	150°C Electrostatic spray dried lactoferrin Batch 1
20	ESLF150B2	150°C Electrostatic spray dried lactoferrin Batch 2
21	HG28 (Batch No.)	Westland freeze dried lactoferrin
22	CG17 (Batch No.)	Westland freeze dried lactoferrin

3.4 Three different drying treatments

3.4.1 Freeze drying of bovine colostrum and lactoferrin

Freezing drying was completed using a Labconco freeze dryer shown in **Figure 3.1**.

Figure 3.1 Labconco freeze dryer (Model 7753034, Labconco Corporation, USA).

Preparation of materials for freeze drying

One litre of lactoferrin or colostrum was divided into three aluminum foil trays with each one containing approximately 330 mL of material, the top of the tray was covered by a layer of foil to prevent contamination during storage. All trays of lactoferrin and colostrum were frozen at -20 ± 2°C for 72 h before freeze drying. This is necessary as during the freezing stage, most of the water is converted into solid by forming a network of ice crystals so that it can undergo sublimation (Kasper & Friess, 2011). Before loading to the freeze dryer, the top foil cover of each tray was punctured with small holes to allow water vapour to escape the tray during freeze-drying.

Freeze drying conditions

Vacuum pressure varied from 0.28 to 2 mbar; tray temperatures varied from -12°C to 19°C depending on the freeze dryer's vacuum cycle. The condenser temperature was between -54°C to -47°C during the freeze-drying process. All samples were freeze dried for 300 h.

Powder collection and storage

Freeze dried colostrum and lactoferrin formed a "cake" appearance and contained uneven pores after freeze-drying due to ice crystal formation at the freezing stage (Badal Tejedor *et al.*, 2020). The "cake" was ground into powder. To do so, the samples were carefully transferred to a Ziploc bag and crushed manually to powders using a wooden rolling pin instead of an electric grinder due to potential extra heating on the freeze dried samples. After crushing and grinding the freeze dried samples were vacuum packed in individual foil bags and stored at -20°C before analysis. Freeze dried colostrum and lactoferrin showed light yellow and pink colours as shown in **Figure 3.2**.

Figure 3.2 Freeze dried colostrum (left) and lactoferrin (right) powders.

3.4.2 Spray drying of bovine colostrum and lactoferrin

Spray drying was carried out using a Saurin pilot scale spray dryer (**Figure 3.3**) (SL-10, Saurin, Australia).

Figure 3.3 SL-10 Saurin spray dryer (SL-10, Saurin, Australia).

Preparation of materials for spray drying

Frozen colostrum or lactoferrin was thawed at room temperature (20 ± 2°C) overnight before spray drying. The thawed colostrum or lactoferrin was continuously stirred at 300 rpm using an overhead stirrer (IKA, RW 20 digital, USA) while pumping into the spray dryer to make sure the feed was homogenous. A peristaltic pump (Masterflex, Easy-load, Model 07516-00, USA) was used to pump the feed into the spray dryer at an 8.2 mL/min flow rate.

Spray drying conditions

Two inlet air temperatures were selected to carry out spray drying: 180°C and 165°C, outlet temperature was set at 90°C. Conditions for all spray drying runs are recorded in **Table 3.2**.

Table 3.2 Spray drying of bovine colostrum and lactoferrin conditions.

Material	Feed Volume (L)	Feed Temperature (°C)	Inlet Temperature (°C)	Outlet Temperature (°C)	Flow Rate (mL/min)	Air Pressure (bar)
Colostrum	1.25	22 ± 1	180 ± 2	90 ± 2	8.2	4
	1.25	22 ± 1	165 ± 2	90 ± 2	8.2	4
Lactoferrin	1.25	22 ± 1	180 ± 2	90 ± 2	8.2	4
	1.25	22 ± 1	165 ± 2	90 ± 2	8.2	4

Powder collection and storage

At the end of the drying process, when the last feed material was pumped into the dryer, the heating program was stopped with compressed air still in the dryer until the outlet temperature decreased to less than 35°C. The lid was unlatched, and the powder was carefully recovered from the collector and the cyclone, then powders recovered from the collector and cyclone were pooled and vacuum packed in foil bags (VMS 103, AUDIONVAC, Weesp, Netherlands). All powders were stored at -20 ± 2°C before further analysis.

Spray dryer CIP (cleaning in place) cleaning

At the end of each spray drying run, the collection chamber was dismantled to recover all the powders. The spray nozzle was then dismantled and checked for any potential blockage. The spray dryer was reassembled and switched on. About 200 mL of hot water (~ 80°C) was pumped in to clean the feeding tube and also the nozzle. Then 1% caustic cleaning solution was pumped through the dryer to clean any parts which came into contact with the dairy ingredients. Finally, a hot water hose was used to flush thoroughly the dryer to remove any remaining caustic solution. After cleaning, all parts were air dried completely before reassembly and the next run.

3.4.3 Electrostatic spray drying of bovine colostrum and lactoferrin

Electrostatic spray drying was completed using a PolarDry® Model 001 dryer (**Figure 3.4**) (Fluid Air, IL, United States). Located at Fluid Air, Melbourne, Australia.

Figure 3.4 PolarDry® Model 001 Electrostatic spray dryer (Fluid Air, Melbourne, Australia).

<u>Preparation of materials for electrostatic spray drying</u>

Frozen colostrum and lactoferrin materials were packed with ice in a chilly bin and transported to Australia as check-in luggage. On arrival at Fluid Air, the colostrum and lactoferrin remained frozen and they were immediately transferred to a -20°C freezer on site before electrostatic spray drying. Colostrum or lactoferrin was thawed at room temperature (20 ± 2°C) overnight before electrostatic spray drying. The liquid colostrum or lactoferrin was continuously stirred using a magnetic stirring plate (Cimarec+™, Thermo Scientific, USA) at about 300 rpm while pumping to the electrostatic spray dryer.

Electrostatic spray drying conditions

Two inlet temperatures were applied: 150°C and inlet 75°C. Conditions used for electrostatic spray dryings are listed in **Table 3.3**.

Table 3.3 Electrostatic spray drying of bovine colostrum and lactoferrin conditions.

Material	Feed Temperature (°C)	Inlet Temperature (°C)	Outlet Temperature (°C)	Flow Rate (mL/min)	Air Pressure (bar)	Voltage (kV)
Colostrum	18	75	35	3	8	5
	18	150	60	3	8	5
Lactoferrin	18	75	35	3	8	5
	18	150	60	3	8	5

Powder collection and transport

All powders from the collection bottle and cyclone were recovered and sealed in foil barrier bags, stored at -20°C immediately after each drying. All powders were transported back to Massey University, Auckland, New Zealand for analysis.

Electrostatic spray dryer cleaning

At the end of the production run and cooling, the collection chamber was dismantled to recover all the powder. The spray nozzle was carefully removed to check for any blockages. The dryer parts were washed and air dried.

3.5 Drying yield

From each drying, the drying yield was calculated as the percentage of recovered powders in total solids of the original feed (**Equation 3.1**). For spray and electrostatic spray drying, the yield of powders was calculated from the collected powders from the collector and cyclone.

$$\%\ \text{Drying Yield} = \frac{Mass\ (g) of\ recovered\ powder}{Mass\ (g)\ of\ the\ total\ solids\ in\ the\ original\ feed} \times 100\ \% \qquad (3.1)$$

3.6 Powders protein content by Kjeldahl

The crude protein content of colostrum and lactoferrin powders were determined by a Kjeldahl method (Kjeldahl method, AOAC, 930.29, 2023) with a modification. After digestion, the samples were diluted to 100g with Milli-Q® H_2O (Milli-Q® Reference Water Purification System, Merck, Germany) and 40 g of this diluted sample was then distilled and titrated.

3.6.1 Solution preparation for Kjeldahl

0.1% (w/v) methyl red in ethanol

In a volumetric flask, 0.1000 g of methyl red (Pure indicator grade, Thermo Fisher Scientific, USA) was accurately weighed and dissolved in ethanol (>99.8%, Fisher Chemical™, USA) to 100 mL.

0.1% (w/v) bromocresol green

In a volumetric flask, 0.1000 g of bromocresol green (Pure indicator grade, Thermo Fisher Scientific, USA) was accurately weighed and dissolved in ethanol (>99.8%, Fisher Chemical™, USA) to 100 mL.

4% (w/v) boric acid preparation

In a 2-L beaker, 80 g of boric acid (LabServ, Thermo Fisher Scientific, Australia) was dissolved completely in about 800 mL of hot distilled H_2O (80 ± 5°C) and about 500 mL of cold distilled H_2O was added to drop the solution's temperature to about 30°C. Twenty millilitres of 0.1% (w/v) bromocresol green and 14 mL of 0.1% (w/v) methyl red were added to the solution and transferred to a volumetric flask, more cold distilled H_2O was added to a final volume of 2 L.

3.6.2 Sample digestion for Kjeldahl

Whole milk powder (WMP) was used as a control. About 1.0 g of WMP was weighed into a digestion flask. About 0.2 g of lactoferrin or 0.5 g of colostrum powder was weighed into designated digestion flasks. The exact weight (4 decimal places) of the powders were recorded. Two Kjeltabs catalysts (each containing 3.5 g K_2SO_4 and 0.0035 g Se) (FOSS Analytical A/S, Hillerød, Denmark) and 25 mL of concentrated H_2SO_4 (96%, Ajax Finechem™, Thermo Fisher Scientific, USA) were added to each flask. Then the samples were digested using a digestor (Tecator™, Digestor Auto 20, FOSS Analytical A/S, Hillerød, Denmark) at 420℃ for 3 h. After digestion, the flasks were carefully removed from the heating unit and cooled down in the fume hood before handling. Each digested sample was diluted with Milli-Q® H_2O to 100 g and 40 g of which was transferred back to a clean digestion flask for distillation.

3.6.3 Distillation

Fifty millilitres of 4% (w/v) boric acid solution was added to a 250 mL conical flask and placed on the receiving platform of the distillation unit (Kjeltec 8100 Distillation Unit, FOSS Analytical A/S, Hillerød, Denmark). In each digestion flask, with 40 g digest, 70 mL of 40% (w/v) NaOH was added and the flask was distilled for 5 min.

3.6.4 Titration and calculation

After distillation, the distilled samples were titrated with 0.1M HCl (NIST Standard Concentrate, Fisher Scientific, LE11 5RG, UK) to a grey-mauve endpoint. **Equation 3.2** was used to calculate the % nitrogen and protein content in samples.

$$\%\,Nitrogen = \frac{(A*B)*14*100}{1000*C} \qquad (3.2)$$

Where:

A = mLs HCl used

B = exact molarity HCl

C = weight (g) of original sample used.

Protein (%) = % nitrogen × conversion factor (The conversion factor for nitrogen to protein in milk & dairy products is 6.38).

3.7 Physicochemical properties of dried powders

3.7.1 Moisture content

The moisture content of the powders was measured using the Niro No. A-1b method powder moisture routine method (GEA, 2024). Aluminium pans were dried in the oven at 103 ± 2℃ for 1 h to remove any moisture and cooled in a desiccator for 20 min. The exact weight of the dried aluminum pan was recorded as 'Weight of pan'. Approximately 3 g of powder was weighed on the pan and the exact weight was recorded as 'Weight of pan + powder'. The powders were dried in an oven (Heratherm OMS100, Thermo Scientific, USA) at 103 ± 2℃ for 16 h until a constant weight. The pans were cooled in a desiccator for 20 min and weighed again, recorded as "Weight of pan + dried powders".

The moisture content of the powder was calculated using **Equation 3.3**:

$$\%\ \text{Moisture Content} = \frac{(\text{Weight of pan + powder}) - (\text{Weight of pan+dried powder})}{(\text{Weight of pan + powder}) - (\text{Weight of pan})} \times 100 \qquad (3.3)$$

The moisture content for each powder was determined in duplicate.

3.7.2 Water activity

The water activity of powders was measured using a water activity meter (AquaLab 4TEV, Addium, WA, USA). Before measurement, the instrument was calibrated with 0.250 aw, 0.500 aw and 0.760 aw verification standards supplied by the manufacturer. A small plastic spatula was used to sample the sample cups. To start the measurement, a sample cup was filled with sample powder to half-full (approximately 0.8 g) to ensure the bottom of the cup was completely covered. The water activity of each powder was measured in duplicate. Cleaning in between measurements was conducted to ensure the measurement chamber was not contaminated by powders.

3.7.3 Solubility

The solubility test was implemented according to method given by Anema *et al. (*2006*)* on measuring milk protein concentrate. Aqueous solutions of 5% (w/w) powder in Milli-Q® water were made by mixing the powder in Milli-Q® water with a propeller-blade attached to an overhead stirrer (Heidolph RZR 2050 Overhead Stirrer, Heidolph Elektro GmbH and Co. KG, Kelheim, Germany). During mixing, the solution was placed in a water bath at 30°C. This solution was stirred for 30 min to allow for complete dispersion before analysis.

Ten grams of the solution was transferred to a 15-mL Eppendorf tube and centrifuged using an MSE Mistral 1000 Centrifuge (Sanyo-Gallenkamp, Global Science and Technology Ltd, Auckland, New Zealand), at 700×*g* for 10 min. About 3 g of the supernatant was pipetted onto a pre-weighed moisture dish and total weight was recorded. The moisture dish was dried for about 18 h in an oven at 105 ± 2°C (Heratherm OMS100, Thermo Scientific, USA). After drying, the dishes were removed to a desiccator (to avoid condensation affecting the results) to sit for 20 min and then reweighed. The amount of soluble material, σ, in the powders was calculated based on **Equation 3.4**:

$$\sigma=\frac{weight\ of\ dry\ material}{weight\ of\ solution}\times 100 \qquad (3.4)$$

3.7.4 Particle size analysis

Particle size distribution and volumetric median particle size (D_v50) were determined using a Mastersizer (Mastersizer 3000, Malvern Instruments Ltd., UK). Both dry and wet methods were applied. For the dry method, powders were dispersed into the testing chamber by vacuum. As for the wet method, the powders were dispersed in pure isopropanol and the particle size distribution was determined (Nugroho *et al.*, 2021). The reason why a wet method was introduced to investigate the particle size was because the electrostatic spray dried colostrum powders (produced in Fluid Air, Australia) were tracked by the NZ Ministry of Primary Industries when couriered from Australia to New Zealand and hence unavailable to be tested in their powder form due to the requirement for biological and physical containment of hazardous samples.

The colostrum and lactoferrin powders freeze dried were not considered to be measured for particle profiles as these powders were initially manually ground so the particles were not evenly distributed, hence the results would not be regarded as representative.

3.7.4.1 Dry method

Sample powders were transferred out of a -20℃ freezer to warm to room temperature for about 2 h before testing. About 3g of powders were sieved (commercial stainless steel mesh, 400 μm) and loaded into the Aero S dry cell. A particle refractive index of 1.45 was used for the measurement. Air pressure was set at 2 bar and the feed rate was adjusted to 50%. After each measurement, the sample chamber was dismantled to clean, any residual powders were brushed out of the chamber. The chamber was then carefully reassembled to test the next sample. Volumetric median particle size (D_v50) values and particle distribution were measured (Wang *et al.*, 2017a).

3.7.4.2 Wet method

Isopropanol (≥ 95%, Sigma-Aldrich, Missouri, USA) was used as a dispersing medium based on the method reported by (Nugroho *et al.*, 2021). A 2% w/w suspension of powder in isopropanol was prepared by adding 0.1 g sample powder to isopropanol to a total mass of 5 g. The stirrer speed was set to 200 rpm. A particle refractive index of 1.39 was used to measure lactoferrin powder samples. For colostrum powders, a particle refractive index of 1.53 was used. A dispersant refractive index of 1.39, and an absorption coefficient of 0.01 was used for both powders (Nugroho *et al.*, 2021). The isopropanol suspension was mixed well to ensuring the powders were evenly suspended in the isopropanol. A few drops of powder suspension were immediately pipetted to the Hydro SM sample cell until the laser obscuration was within the ideal range. The results were the average of three measurements. After measurements, the system was cleaned with 10% w/w Decon (Decon 90, Decon Laboratories, USA) and flushed five times with distilled water. Each sample was measured in duplicate, results were shown as particle size distribution and volumetric median particle size D_v50.

3.7.5 Fourier transfer infrared spectrophotometry (FTIR)

Proteins are linear biological polymers that are made up of amino acids. When a protein folds to form a well-defined 3-dimensional structure it exhibits three levels of structure: primary, secondary and tertiary (Gallagher, 2009). FTIR has long been used to investigate the secondary structure of the proteins. A Bruker FTIR (Bruker Alpha, Bruker, Ettlingen, Germany) was used to analyse the samples. Before measurement, a Kimwipe wet with MeOH was used to wipe and clean the crystal plate. Sample powder was placed directly on the diamond crystal plate to coat the crystal with approximately 1 mm thickness of material. The pressure arm was positioned over the sample and pressure was applied to the sample. All spectra were average of 64 scans from 4000cm^{-1} to 650cm^{-1} at a resolution of 4cm^{-1} (Wang *et al.*, 2017a). All powders were analysed in duplicates. Freeze dried sample was used as a control to compare the shifts of determined peaks. Three characteristic IR absorption peaks were analysed, including at 1645 cm^{-1} (amide I, C=O stretching and C-N stretching). 1542 cm^{-1} (amide II, C-N stretching and N-H bending), and 1250 cm^{-1} (amide III, C-N stretching and N-H bending). A typical FTIR spectra is shown in **Figure 3.5**.

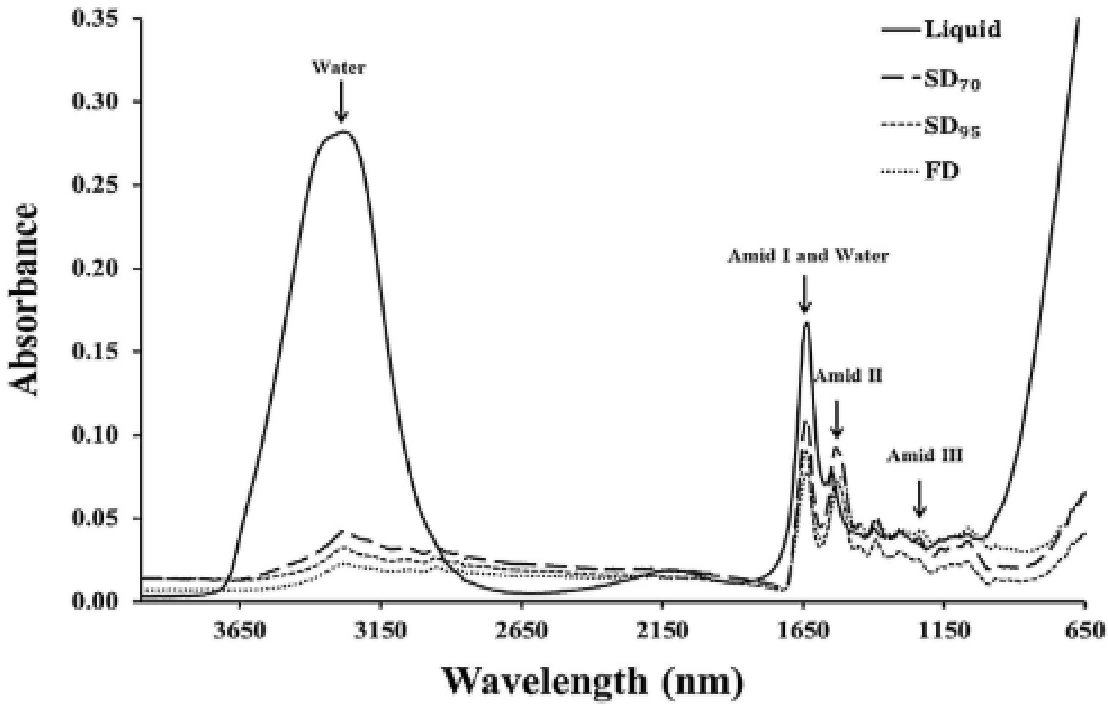

Figure 3.5 A typical FTIR spectra of liquid, freeze dried (FD), and spray dried (SD) lactoferrin. SD_{70}=spray dried lactoferrin at outlet temperature of 70°C, SD_{95}=spray dried lactoferrin at outlet temperature of 95°C (Wang *et al.*, 2017a).

3.8 Enzyme-linked immunosorbent assay (ELISA) bioactivity of lactoferrin and IgG protein

Bethyl enzyme-linked immunosorbent assay kits (ELISA) (Cat. No. E11-118 and E11-126, Bethyl Laboratories, USA) were used to quantify the lactoferrin and IgG activity in both liquid and powdered samples. Components supplied in each kit:

- Pre-coated 96-well strip plate
- Protein standard
- Detection antibody
- 20× Dilution buffer
- HRP Solution C
- TMB Substrate
- Stop Solution
- 20× Wash Buffer
- Sealing Tape

A 500 ng protein standard was provided in each individual test kit and the standard curve of lactoferrin, or IgG was obtained by serially diluting the protein standard to a series of concentrations needed. All ELISA test reagents from the kits were removed from the fridge about 1h prior to the experiment, because all reagents must be at room temperature (20-25°C) before use.

3.8.1 Buffer preparation

<u>Dilution buffer</u>

To prepare the dilution buffer, 25 mL of the concentrated buffer (20×) was diluted to 500mL with Milli-Q® H_2O.

Wash buffer

To prepare the wash buffer, 50 mL of the concentrated wash buffer (20×) was diluted to 1 L with Milli-Q® H_2O.

3.8.2 Sample preparation

Sample preparation for bovine lactoferrin or bovine IgG ELISA assay followed the same procedure and sample dilutions.

For powders

To prepare 5 mg/g stock solutions, 0.05 g was weighed and dissolved in Milli-Q® H_2O to a total mass of 10 ± 0.1 g, the exact weight of each powder was recorded. The solutions were mixed on a magnetic stirring plate (IKA® RT 10 P, USA) for two hours to hydrate well before use.

Stock solutions were first diluted 1/1000 times: 10 μL stock solution was diluted to 10 mL with dilution buffer in a 15 mL falcon tube and mixed well by vortexing (ZX3 Vortex mixer, VELP Scientifica, Italy). Afterwards, 100 μL of the 1000 × diluted sample was mixed with 900 μL dilution buffer in a 1.5 mL centrifuge tube to further dilute the sample 10 times. Finally, the sample stock solutions were diluted 1/10,000 times.

Liquid lactoferrin or colostrum

Liquid lactoferrin and colostrum were diluted 1/10,000 times in total. Firstly, 10 μL liquid lactoferrin or colostrum and 10 mL sample dilution buffer were added to a 15 mL falcon tube providing a 1/1000 dilution. Then 100 μL of this dilution was added to 900 μL dilution buffer, therefore the liquid lactoferrin or colostrum was further diluted 1/10 times. In total, liquid lactoferrin or colostrum was diluted 1/10,000 times.

3.8.3 Standard preparation

To prepare the lactoferrin or IgG protein standards at different concentrations, serial dilutions were carried out following this procedure. The top standard was prepared by reconstituting the 500-ng lactoferrin or IgG standard in vial with 1.0 mL of 1 × dilution buffer to achieve a final concentration of 500 ng/mL. Seven 1.5 mL centrifuge tubes were prepared, 300 μL of dilution buffer was added into each of the seven tubes. Repeatedly, 150 μL of the previous standard was added into 300 μL of dilution buffer in the next tube until the sixth tube as described in **Table 3.4.**

Table 3.4 Dilution of lactoferrin or IgG protein standards.

Concentration Required (ng/mL)	Previous Standard Volume (μL)	Diluent Volume (μL)
167	150	300
55.6	150	300
18.5	150	300
6.17	150	300
2.06	150	300
0.69	150	300

The seventh tube containing only 300 μL of 1× dilution buffer served as the blank.

3.8.4 Measurement procedure

Each standard or sample was analysed in triplicate. A multichannel pipette was used to add 100 μL standards or samples to designated wells. The plate was then covered by a sheet of sealing tape and incubated at room temperature (20-25°C) for one hour. After incubation, the plate was washed four times by a plate washer (ELX405, Bio-Tek, USA) and any remaining water in the wells removed from the plate by tapping it upside-down on paper towel. 100 μL anti-lactoferrin antibody (100 μL) was added to each well, the plate was covered again and incubated at room temperature for one hour. After incubation, the plate was washed four times and water was removed from the plate by tapping it upside-down on a paper towel. Then HRP solution C (100 μL) was added to each well and the plate was again covered and incubated at room temperature for 30 min. Then the plate was washed four times and water was removed from the plate by tapping it upside-down on a paper towel. TMB substrate solution (100 μL) was added to each well and the plate was incubated in the dark at 25°C for 30 min. At this point the colour in the wells started developing a blue colour. After 30-min incubation, the reaction was stopped by adding 100 μL of stop solution to each well, once the stop solution was added, the colour changed immediately to yellow.

The absorbance of the solution in each well was read at an absorbance of 450 nm using a plate reader (Synergy 2, Bio-Tek, USA). Concentrations of targeted protein were calculated based on the standard curve and results were reported as mg of active protein/g powder. The ELISA standard curves are presented in Appendix 1 and 2.

3.9 Iron-binding ability-A relative colorimetric method

Two methods were used to evaluate the iron binding status of the powder samples. The first method was carried out as described by (Kawakami *et al.*, 1992) which is a colorimetric method that generates relative results of iron bound to protein compared to freeze dried samples as a control.

<u>Hydrochloric acid (HCl) solutions</u>

HCl (0.1M) was prepared by adding 8.3 mL 36% (w/w) HCl to a volumetric flask, and it was made up to 1L with Milli-Q® H_2O. This 0.1 M HCl was further diluted to 0.05 M by transferring 50 mL of 0.1 M HCl to a 100 mL volumetric flask and adding Milli-Q® H_2O to 100 mL.

<u>Sodium hydroxide (NaOH) solutions</u>

To prepare 0.05 M NaOH, 0.2000 g NaOH was initially dissolved in a beaker with about 80 mL Milli-Q® H_2O, then transferred to a 100 mL volumetric flask. The inside of the beaker was rinsed three times, and the solution was transferred to the flask, more Milli-Q® H_2O was added to a final volume of 100 mL.

<u>Sample Buffer Preparation</u>

Sodium bicarbonate ($NaHCO_3$) (1% w/v) was prepared by dissolving 1g $NaHCO_3$ (ACS grade, Sigma-Aldrich, USA) in Milli-Q® H_2O to a final volume of 100 mL in a 100 mL volumetric flask.

3.9.1 Iron solution preparation

Iron (III) chloride ($FeCl_3$) (0.5% w/v) was prepared by dissolving 0.5 g $FeCl_3$ (Reagent grade, Sigma-Aldrich, USA) in Milli-Q® H_2O to a final volume of 100 mL in a 100 mL volumetric flask.

3.9.2 Preparation of sample stock solutions

For lactoferrin powder samples, to make 1% (w/v) sample stock solution, 0.1000 g of powder was dissolved in about 4 g of Milli-Q® H_2O. Then stirred for two h on a magnetic stirring plate (IKA® RT 10 P, USA) to allow lactoferrin to hydrate well. After mixing, the pH was adjusted to 7.0 with 0.05 M HCl or 0.05 M NaOH and transferred to a 10 mL volumetric flask to make up a final volume of 10 mL.

For liquid lactoferrin, 1 g of lactoferrin or colostrum stream was added to 8 g of Milli-Q® H_2O, adjusted the pH to 7.0 with 0.05 M HCl or 0.05 M NaOH and more Milli-Q® H_2O added to get a final mass of 10 g.

To each test tube, 2 mL sample solution was mixed with 40 μL $NaHCO_3$ and 40 μL Milli-Q® H_2O or $FeCl_3$ solution. A vortex mixer (ZX3 Vortex mixer, VELP Scientifica, Italy) was used to mix the solutions. Two test tubes were prepared for each sample.

3.9.3 Measurement of absorbance

To measure the absorbance of the sample with no added iron, the spectrophotometer was zeroed with two blanks containing 2 mL of Milli-Q® H_2O, 40 μL of $NaHCO_3$ and 40 μL of Milli-Q® H_2O.
To measure the absorbance of the sample with added iron, the spectrophotometer was zeroed with two blanks containing 2 mL of Milli-Q® H_2O, 40 μL of $NaHCO_3$ and 40 μL of $FeCl_3$.

The iron-binding ability was assessed by reading the absorbance of the solution at 465 nm
The iron-binding ability (%) was calculated using **Equation 3.5**:

$$Iron - binding\ \% = \frac{Abs(sample\ with\ iron) - Abs(sample\ without\ iron)}{Abs(Freeze-dried\ Lf\ or\ CL\ with\ iron) - Abs(Freeze-dried\ Lf\ or\ CL\ without\ iron)} \times 100\% \qquad (3.5)$$

3.10 Iron binding ability-A qualification method using microwave plasma atomic emission spectroscopy (MP-AES)

To quantify the concentration of Fe^{3+} bound to the protein present in the sample, the method used was based on the method described by (Morel *et al.*, 2022). The principle of this method was to saturate the lactoferrin protein with extra Fe^{3+}, then to isolate the iron-bound protein from excess free iron. Then the protein concentration and iron content attached to the protein was quantified obtain the ratio of iron bound to lactoferrin protein. Sephadex™ G-25 desalting columns (PD-10, Cytiva, New Zealand) were used to separate iron-bound lactoferrin protein from excess ferric solution. This method involved four steps:

- Saturation of the lactoferrin protein with excess iron
- Separation of iron-bound proteins from unbound excess iron
- Quantification of the protein concentration using the Bradford method (Bradford, 1976)
- Quantification of the iron concentration bound to protein using Microwave Plasma Atomic Emission Spectroscopy (MP-AES)

3.10.1 Solution preparation

Column Equilibration buffer-50 mM Tris Buffer with 20 mM $NaHCO_3$ (*E-buffer*)

In a 1 L beaker, 1.68 g $NaHCO_3$ (ACS grade, Sigma-Aldrich, Missouri, USA) was dissolved in approximately 900 mL Milli-Q® H_2O, one pouch of pre-made Tris powder (Sigma-Aldrich, Missouri, USA) was added and the pH value was adjusted to 7.8 using 6 M HCl. The solution was transferred to a 1 L volumetric flask and the Milli-Q® H_2O used to rinse the beaker was also transferred to the volumetric flask, more Milli-Q® H_2O was added until the final volume of 1 L.

Lactoferrin or colostrum sample stock solutions

To prepare 10 mL 50 mg/mL lactoferrin or colostrum sample stock solutions: 500 mg lactoferrin or colostrum was weighed into a 10 mL volumetric flask, about 5 mL of the *E-buffer* was added and the solution was mixed with vortex mixer (ZX3 Vortex mixer, VELP Scientifica, Italy) then the solution was left at 4°C for 1 h, finally more of the *E-buffer* was to the final volume of 10 mL and the solution was gently mixed by tipping the flask upside down a few times. For liquid lactoferrin or colostrum, 2 g of liquid lactoferrin or colostrum was diluted in a volumetric flask with the *E-buffer* to 10 mL.

Iron solution (*Solution A*)

An 11 mM ferric solution was prepared by dissolving 0.2222 g $Fe(NO_3)_3{\cdot}9H_2O$ (Sigma-Aldrich, Missouri, USA) in *E-buffer* to a final volume of 50 mL. A 27.5 mM nitrilotriacetic acid (NTA) solution was prepared by dissolving 0.293 g Na_2NTA (Sigma-Aldrich, Missouri, USA) in *E-buffer* to a final volume of 50 mL.

Column washing buffer-0.2 M NaOH (*Solution B*)

To prepare the 0.2 M NaOH column washing buffer, 8 g of NaOH was dissolved in Milli-Q® H_2O in a volumetric flask and made up to 1 L.

3.10.2 Saturation of sample with excess iron

In a 5 mL Eppendorf tube, to 2 mL lactoferrin or colostrum stock solution, 0.23 mL iron solution was added and mixed gently with a pipette tip. The solution was left to incubate at 25 ± 2°C for 1 hour. This solution after incubation is called *Solution C*.

3.10.3 Removal of excess iron and collection of eluates

Preparation of the column

The Sephadex™ G-25 column was fixed on a stand and test tubes were placed underneath the column as illustrated in **Figure 3.6**. The column was equilibrated according to the product user manual, briefly, 5 mL of the *E-buffer* was pipetted to fill up the column and allowed to enter the packed bed completely and drain. Waste flow-throghs were discarded. Approximately 25 mL of *E-buffer* was used for equilibration per column.

Sample loading

After the column was equilibrated, 2.23 mL of *Solution C* was added to the column. After the sample had entered the column bed completely, 0.27 mL of *E-buffer* was added to the column to adjust the total loading volume to 2.5 mL. The first 2.5 mL of eluate from the column was discarded. Three and a half millilitres of more *E-buffer* was loaded onto the column and the eluate was collected in a test tube as shown in **Figure 3.6**. For each sample, the column extraction was carried out in duplicate. Initial trials were carried out to determine the volume of eluate to collect to ensure all that all excess iron was flushed from the protein sample.

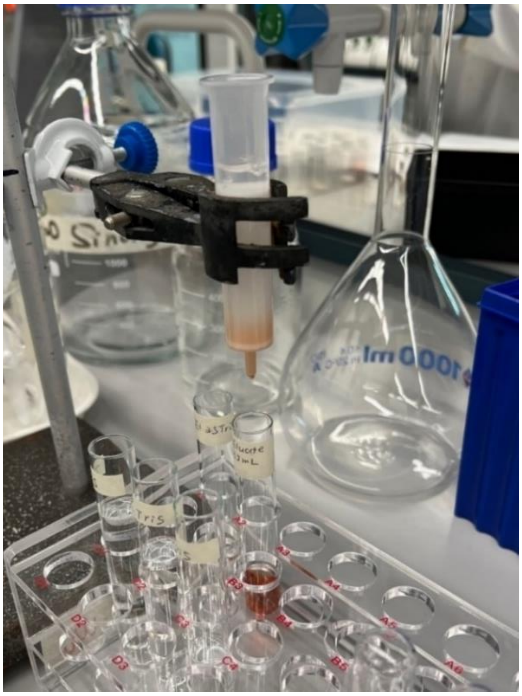

Figure 3.6 A Sephadex™ G-25 column set-up for protein extraction.

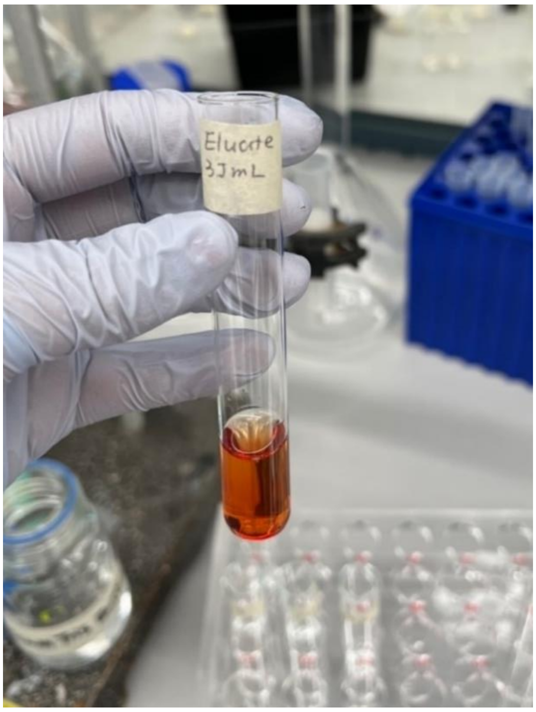

Figure 3.7 Eluate (3.5 mL) collected after each extraction and was red in colour.

Eluate collection

From each extraction, 3.5 mL eluate was collected into a 25 mL test tube as shown in **Figure 3.7**. Each eluate was divided into two parts for two further analyses: one part of eluate was subject to Bradford to determine the protein content, and the other to MP-AES to determine the Fe content.

By combining the results from Bradford and MP-AES, the ratio of the Fe bound to protein can be calculated for each sample.

To simplify the understanding of this column extraction method, a flow chart outlining the process is provided in **Figure 3.8**.

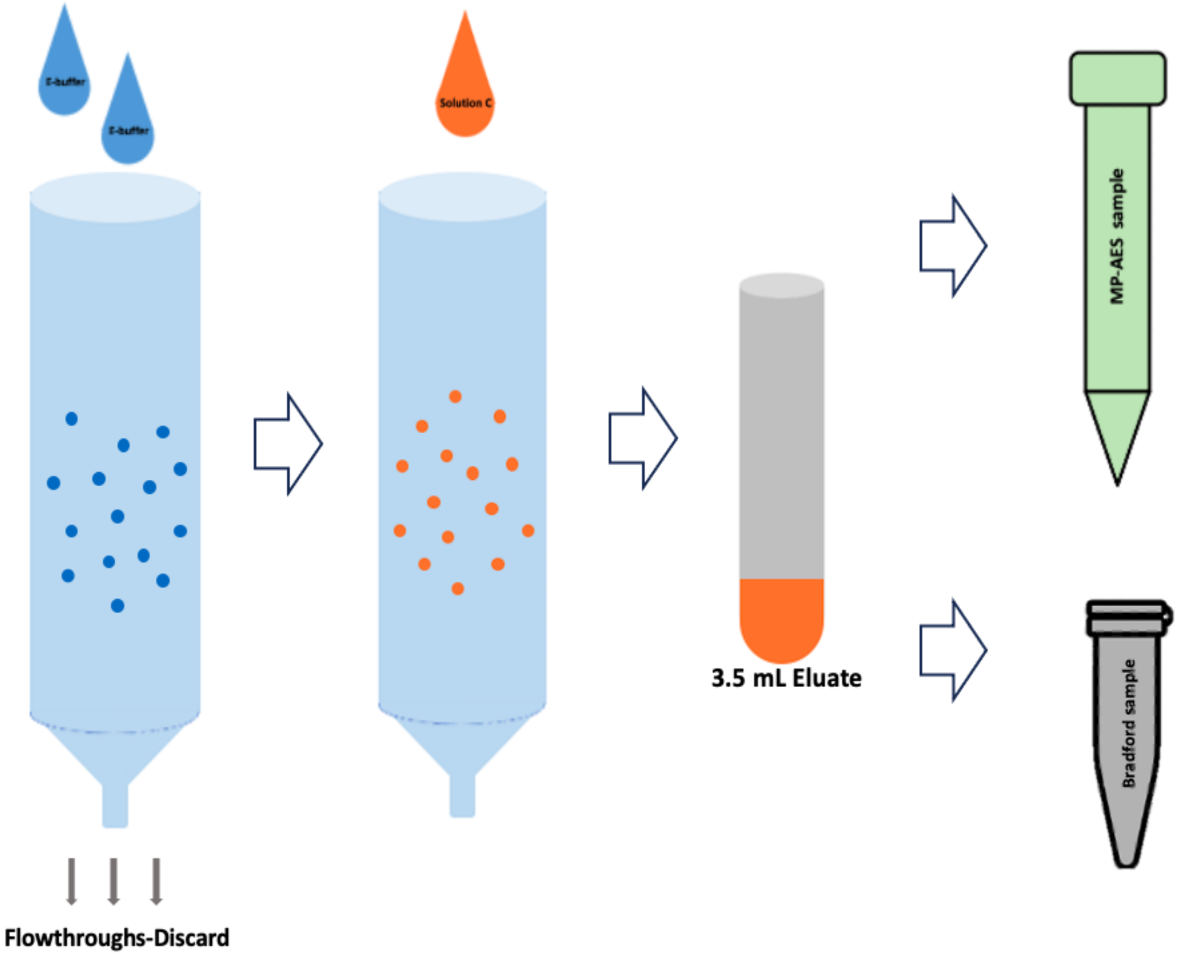

Figure 3.8 Illustration of a complete Sephadex™ G-25 column extraction process. E-*buffer* (blue) was loaded to equilibrate the column and *Solution C* (orange) was added to separate the protein. 3.5 mL eluate was finally collected in a test tube and was divided into two parts for further analysis.

3.10.4 Column washing and regenerating

At least two column volumes (about 20 mL) of *Solution B* was used to wash the column. Waste flowthroughs were discarded. However, to make sure the column was able to be reused, which means there should not be any remaining Fe trapped in the column after each purification.

To verify this, further analysis was carried. A commercial free iron detection test kit (IC-HI3834, Hanna Instruments, USA) was used, it works by using the phenanthroline method to measure total iron in the medium range of 0-5 mg/L. The phenanthroline complexes with the Fe^{2+} in presence of the solution and a form a coloured complex with λ_{max} = 510 nm (Yegorov *et al.*, 1993). Briefly, all potential ferric ions are reduced by sodium sulphite to ferrous ions, phenanthroline complexes with ferrous ion can form an orange-coloured solution. The colour intensity of the solution determines the iron concentration of the sample.

<u>Verification of the column washing flowthroughs</u>

The column was washed five times, therefore five flowthroughs (5 mL per wash) were collected in separate test tubes to be analysed. To the first 5 mL *Solution B* flowthrough, half packet of reagent HI3834 was added and after mixing the solution showed a red colour, this was repeated until to the fifth of the *Solution B* flowthrough, the solution was clear (**Figure 3.9**).

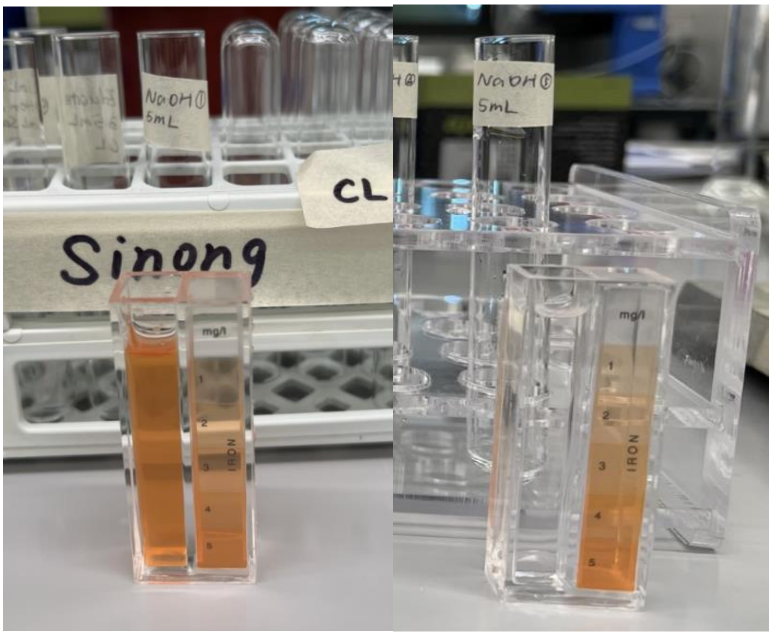

Figure 3.9 Colour change of the 1st *Solution B* washing (left) and 5th *Solution B* washing (right).

Spectroscopy verification

From comparison with the standard colours provided with the kit after the fifth time of washing, there was no more iron in the column. However, to verify this, the first *Solution B* flowthrough was scanned from 400-700 nm (UV-1700, Shimadzu, Japan) to identify its peak and matching wavelength. It was found to be 511 nm, which agree to the absorption of wavelength of ferrous phenanthroline complexes (510 nm).

Flowthroughs from the first to fifth times of washing were measured for absorbance against 511 nm (UV-1700, Shimadzu, Japan), results are shown in **Figure 3.10.**

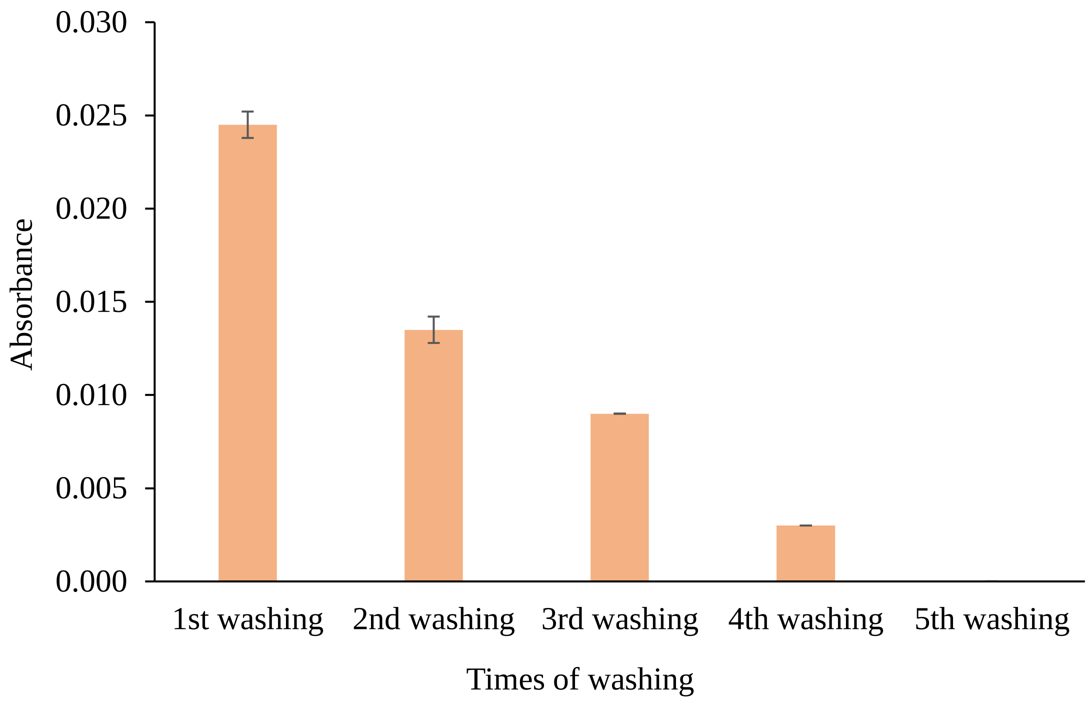

Figure 3.10 Change of iron content in the *Solution B* washings from the first to the fifth time.

Figure 3.10 shows that the iron content decreases as more washing was carried out, and after the fifth washing, there was no more iron detected. The columns were proved to be able to be regenerated and reused for more extractions.

Used columns were stored at 4 ± 3°C in 0.02% sodium azide (NaN_3) (ACS Reagent, Sigma-Aldrich, USA) and can be used again once equilibrated as described in Section 3.10.3.

3.10.5 Protein content determination by Bradford method

The Bradford method was used to quantify the protein concentration in the eluates (Bradford, 1976). Two hundred millilitres of commercial Bradford dye reagent (Sigma-Aldrich, USA) was transferred to a Schott bottle to avoid contamination when used, the bottle was covered carefully with foil to avoid light. Lyophilised bovine lactoferrin (≥ 85%, Sigma-Aldrich, USA) was used as the protein standard.

Bradford standard curve preparation

Seven concentrations of lyophilised bovine lactoferrin standards were prepared: 0.125, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mg/mL (w/v). The protein eluates collected from column extraction were thawed and diluted before analysis. Eluates from colostrum powder or liquid colostrum samples were diluted ten times by adding 100 µL eluate to 900 µL Milli-Q® H_2O. The eluates of from lactoferrin powder or liquid lactoferrin samples were diluted forty times (lactoferrin samples have more concentrated protein hence diluted more than colostrum samples) by adding 100 µL of eluate to 3.9 mL of Milli-Q® H_2O. Standard curve for Bradford is presented in Appendix 3.

Measurement procedure

Five millilitres of the Bradford dye reagent was added to a 15 mL test tube. Then 100 µL of diluted sample eluate was added and mixed immediately by vortexing (ZX3 Vortex mixer, VELP Scientifica, Italy), then incubated at 20 ± 2°C for 5 min. After incubation, approximately 1.5 mL solution was transferred to cuvettes (Semi-micro cuvette, Fisher Scientific, New Zealand) and the absorbance was measured at 595 nm (UV-1700, Shimadzu, Japan). Five millilitres of Bradford dye reagent and 100 µL Milli-Q® H_2O was used as a blank. The determined protein content of colostrum and lactoferrin eluates can be found in Appendix 4.

3.10.6 Sample iron content determination

The iron concentration in the sample eluates was analysed using Microwave Plasma Atomic Emission Spectroscopy (MP-AES) (4200 MP-AES, Agilent Technologies, USA).

Fe standard curve preparation

A Fe standard curve (Appendix 5) was prepared using a Fe standard of 1000 µg/L, in 5% HNO_3 (ICP-OES standards, single element, 7782-61-8, Agilent Technologies, USA). The 1000 µg/L Fe standard was diluted to five different concentrations from 0–0.8 mg/L (w/v). Each standard and sample was loaded once and measured by MP-AES in twenty replicates.

Sample digestion

Approximately 0.96 g of liquid sample eluates or 0.2 g of standard skim milk powder (ERM®-BD151, European Reference Materials, Belgium) was accurately weighed into each digestion tube and the weight was recorded.

To the solid powder sample, 3 mL Milli-Q® H_2O was added. To the liquid sample, 2 mL Milli-Q® H_2O was added. Then 4 mL 68% (w/w) nitric acid (Thermo Fisher Scientific, MA, USA) was added to each tube, the digestion vessel was then closed and transferred to the microwave reaction system (Multiwave 5000, Anton Paar, New Zealand). Samples were heated from ambient temperature to 190°C within 15 min, held at 190°C for 20 min and cooled to 65°C within 29 min. After the digestion process, the rotor was carefully removed from the microwave reaction system. Nitrogen dioxide gases were carefully released from each digestion tube, and then the vessel caps were removed. The digested samples were transferred to 30 mL beakers from the digestion tubes first. Then all the digests were transferred to a 25 mL volumetric flask and diluted with Milli-Q® H_2O to 25 mL.

Titration for Fe concentration determination

One mL of the digest was pipetted into a 250 mL conical flask, diluted with 50 mL Milli-Q® H_2O. Then a few drops of 0.5% w/v bromothymol blue (ACS Reagent, Dye Content ≥ 95%, Sigma-Aldrich, USA) was added to determine the endpoint. The solution was titrated with 0.1961M NaOH until the colour changed from yellow to blue. **Equation 3.6** was used to calculate the concentration of nitric acid:

$$Concentration\ of\ nitric\ acid\ \left(\frac{mol}{L}\right) = \frac{C_{NaOH} * V_{NaOH}}{V_{sample}} \qquad (3.6)$$

Where:

C_{NaOH}: Concentration of NaOH used in the titration (0.1961 M)

V_{NaOH}: Volume of NaOH used to titrate the sample (L)

V_{sample}: Volume of sample transferred into the conical flask (L)

Equation 3.7 was used to calculate total mass of Fe present in the eluates:

$$\text{Mass of Fe} = \left(\frac{mg\ Fe}{g\ Sample}\right) = \left[\frac{mg\frac{Fe}{L}\,(from\ std\ curve)}{1000}\right] * D * \text{V} \qquad (3.7)$$

Where:

D: Digest times after dilution of the digest

V: Further diluted volume (mL)

MP-AES measurement validation

The MP-AES method is validated by comparing the determined iron content of the standard skim milk powder (ERM®-BD151, European Reference Materials, Belgium) with the product certificate of analysis (European Commission, Joint Research Centre, Consumers and Reference Materials, Belgium) (Appendix 6).

3.11 DPPH antioxidant ability

The antioxidant ability of lactoferrin and colostrum samples was evaluated by the radical scavenging method in a citrate-phosphate buffer system. DPPH 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical appearing purple and has a maximum absorption at 515 nm. The DPPH assay has been widely used to determine the antioxidant ability of milk proteins (Chiang & Chang, 2005; Qureshi *et al.*, 2020; Stobiecka *et al.*, 2022). The method described by (Wang *et al.*, 2017a) was used with modifications for this experiment where 96-well microplates were used to carry out the analysis.

3.11.1 Citrate-phosphate buffer preparation

A citrate-phosphate buffer of pH 3 was prepared by mixing in the correct ratio of 0.1 M citric acid solution (Solution A) and 0.2 M di-sodium hydrogen phosphate dihydrate solution (Solution B) (Gomori, 1955). First, to prepare 100 mL solution A, 1.9212 g citric acid (Sigma-Aldrich, Missouri, USA) was dissolved in Milli-Q® H_2O in a 100 mL volumetric flask. Solution B was prepared by dissolving 3.56 g $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich, Missouri, USA) in Milli-Q® H_2O in a 100 mL volumetric flask to provide 0.2M di-sodium hydrogen phosphate dihydrate solution. To prepare pH 3 citrate-phosphate buffer, 39.8 mL of Solution A was mixed with 10.2 mL Solution B in a 100 mL volumetric flask and Milli-Q® H_2O was added to achieve a final volume of 100 mL. The pH of prepared citrate-phosphate buffer was verified to be 3.06 at 18.7℃.

3.11.2 Methanolic DPPH solution preparation

A 2 mM DPPH in methanol solution was freshly prepared by dissolving 39.432 mg DPPH (Sigma-Aldrich, Missouri, USA) in methanol (Thermo Fisher Scientific, MA, USA) in a 50 mL volumetric flask. The flask was covered in aluminium foil to prevent photochemical decomposition as in the presence of light, molecular oxygen reacts slightly with DPPH causing its reduction (Egerton & Mattinson, 2007; Pitts *et al.*, 1964).

3.11.3 Sample stock solution preparation for DPPH assay

For colostrum and lactoferrin powders, to make 20 mg/mL (w/v) sample stock solutions, approximately 500 mg of powder was accurately weighed and dissolved in Milli-Q® H_2O in a 25 mL volumetric flask and then transferred to 40 ml plastic containers. For each sample stock solution, a serial dilution was carried out by diluting the 20 mg/mL sample stock solution to produce seven different concentrations. Seven 15 mL falcon tubes were labelled, one for each concentration: 15, 10, 7.5, 5, 2.5, 1.25 and 0.625 mg/mL (w/v).

<u>Dilution procedure</u>

Three millilitres of the original 20 mg/mL (w/v) stock solution was added to 1 mL Milli-Q® H_2O in the first falcon tube to get 15 mg/mL (w/v) sample solution. Two millilitres of the original 20 mg/mL (w/v) stock solution was added to 2 mL Milli-Q® H_2O in the second falcon tube to get 10 mg/mL (w/v) sample solution. Two millilitres of the 15 mg/mL (w/v) sample solution was added to 2 mL Milli-Q® H_2O in the third falcon tube to get 7.5 mg/mL (w/v) sample solution. Two millilitres of the 10 mg/mL (w/v) sample solution was added to 2 mL Milli-Q® H_2O in the fourth falcon tube to get 5 mg/mL (w/v) sample solution. Two millilitres of the 5 mg/mL (w/v) sample solution was added to 2 mL Milli-Q® H_2O in the fifth falcon tube to get 2.5 mg/mL (w/v) sample solution. Two millilitres of the 2.5 mg/mL (w/v) sample solution was added to 2 mL Milli-Q® H_2O in the sixth falcon tube to get 1.25 mg/mL (w/v) sample solution. Two millilitres of the 1.25 mg/mL (w/v) sample solution was added to 2 mL Milli-Q® H_2O in the seventh falcon tube to get 0.625 mg/mL (w/v) sample solution.

For colostrum and lactoferrin raw liquid, samples were diluted with Milli-Q® H_2O to 10, 20 and 40 times in water. To prepared 10 times diluted colostrum or lactoferrin, 1 mL of the raw liquid was added to 9 mL Milli-Q® H_2O in a 15 mL-falcon tube and mixed well. To prepare 20 times diluted colostrum or lactoferrin, 2 mL of the 10 times diluted was added to 2 mL Milli-Q® H_2O in a 15 mL-falcon tube and mixed well. To prepare 40 times diluted colostrum or lactoferrin, 2 mL of the 20 times diluted was added to 2 mL Milli-Q® H_2O in a 15 mL-falcon tube and mixed well.

3.11.4 Sampling and measurement procedure

Two 96-well plates (Nunc™ 96-Well, Fisher Scientific, USA) were used in this analysis. A 12-channel auto pipette (10-100 µL, Eppendorf Research® Plus, Germany) was used to sample the plate. First, 265 µL of citrate-phosphate buffer (pH 3) was added to each well, then 20 µL H_2O or sample in duplicate was added to the designated well. The reaction was initiated by adding 15 µL DPPH solution to each well. The plate was immediately loaded to the plate reader (Synergy 2, BioTek, Vermont, USA 05404) to read the absorbance at 515 nm (recorded as As1). Citrate-phosphate buffer (pH 3) was used as a control. All plates were stored in the dark and read again after 2 h at 515 nm (recorded as As2). The radical scavenging ability was calculated as the percentage of loss of absorbance at 515 nm wavelength using **Equation 3.8**:

$$Radical\ Scanvenging\ Ability\ \% = \left[\frac{As1-As2}{As1} - \frac{Ac1-Ac2}{Ac1}\right] * 100\% \tag{3.8}$$

Where:

As1 and As2 are the absorbance of the sample at the beginning and after the 2-h incubation. Ac1 and Ac2 are the absorbances of the control at the beginning and after the 2-h incubation.

3.12 Antibacterial ability

In this research, the standardised EUCAST disc diffusion method (Matuschek *et al.*, 2014) was applied to evaluate the antimicrobial ability of lactoferrin and colostrum powder samples. The principle of this method is to place a sample-impregnated paper disc on agar previously inoculated with the tested microorganism, and the sample can diffuse radially from the disc into the agar medium.

When tested samples having antimicrobial ability, a clear zone or ring of inhibition around the paper disc is formed. This method provided reliable qualitative results to visualize the antimicrobial susceptibility of lactoferrin and colostrum samples (Rivera *et al.*, 2023). Two microorganisms selected to be tested on the lactoferrin and colostrum samples were *Escherichia coli* NCTC 8196 and *Staphylococcus aureus* NCTC 4163.

Preparation of 5% (w/v) sample stock solutions

Half a gram of sample powder was dissolved in Milli-Q® H_2O and made up to 10 mL in a volumetric flask. Solutions were firstly mixed by a vortex mixer and left to hydrate well at 4°C for about 10 h before use.

Preparation of media and inoculum

Mueller-Hinton (MH) agar (BD Difco, USA) was prepared according to the manufacturer's instructions. Thirty-eight grams of MH agar powder was suspended in 1 L of distilled H_2O, heated to 100 ± 2°C to dissolve completely and then sterilized by autoclaving at 121°C for 15 min. MH agar was then dispensed into Petri dishes and cooled down to 42-45°C, to achieve an even depth of 4.0 ± 0.5 mm. All plates were dried at 25°C in an incubator (Contherm, NZ) for about 8 h with lids partly ajar to evaporate the surface moisture.

The inoculum suspension was prepared by selecting several morphologically similar colonies of *E. coli* NCTC8196 and *S. aureus* NCTC 4163 from overnight growth (16–24 h of incubation) on a non-selective medium with a sterile loop or a cotton swab and suspending the colonies in sterile saline (0.85% NaCl w/v in H_2O) to the density of a McFarland 0.5 standard.

Inoculation of agar plates and paper discs application

A sterile cotton swab was dipped into the inoculum suspension and the excess fluid was removed by pressing and turning the swab against the inside of the tube to avoid over-inoculation of plates. Then the inoculum was spread evenly over the entire surface of the agar plate by moving the swab in three directions across the entire surface in a zigzag pattern. A swab was discarded after streaking three plates in a row and a new one was used for new plates. Five mm blank paper discs (BBL, USA) were used in this test. To each paper disc, 30 µL of the sample solution was pipetted onto the surface to wet the disc completely. On each plate, four discs were applied firmly on the agar surface within 15 min of inoculation.

Measurement of inhibition zone diameters and interpretation of results

After incubation, the inhibition zone diameters of a single sample on each plate were measured to the nearest millimetre with a calliper and photos were taken. The results were reported as the mean diameters of the inhibition zone from each sample.

3.13 Statistical analysis

All experiments in this study were carried out in at least duplicate. One-way analysis of variance (ANOVA) and followed by Tukey's test was performed to analyse data in SPSS statistical software (Version 27, IBM Corporation, Armonk NY, USA) to determine the significant difference between mean values. Confidence intervals were 95.0%. P-values less than 0.05 were considered significant.

Chapter 4. Results and Discussion

Results are presented and discussed in the same order of the methods described in Chapter 3.

4.1 Proximate analysis of liquid colostrum and lactoferrin

Proximate analysis of the liquid colostrum and lactoferrin starting material was completed in November 2022, by the Nutrition Laboratory, Massey University, Palmerston North, New Zealand. Moisture content, ash, crude protein, fat, carbohydrate and lactose were determined. Results are shown in **Table 4.1**.

Table 4.1 Proximate analysis of bovine colostrum and bovine lactoferrin liquid. Results presented are means ± standard deviation (for colostrum n=2, for lactoferrin n=1).

Ingredient	Moisture %	Ash %	Crude Protein %	Fat %	Carbohydrate %	Lactose g/100 g
Colostrum	73.0 ± 0.1	1.0 ± 0.1	18.0 ± 0.1	4.9 ± 0.1	3.1 ± 0.1	2.4 ± 0.1
Lactoferrin	82.9	0.1	16.4	0.2	0.4	ND

ND: Not detected

The lactoferrin had a total solids content of 17.1%, with 96% of this being protein, as it was purified from skim milk. In comparison, the colostrum had a moisture content of 73% and a total solids content of 27%, whereas mature milk typically has about 12.9% total solids (Playford & Weiser, 2021).

4.2 Freeze, spray and electrostatic spray drying results

4.2.1 Drying recovery yields

Drying yields of freeze, spray and electrostatic spray dried colostrum or lactoferrin were calculated as the mass of powder recovered by the original mass of total solids in the feed material (refer to Section 3.5). Results are reported in **Table 4.2**.

Table 4.2 Drying Yields% (w/w) of freeze, spray and electrostatic spray dried powders. Results presented are means ± standard deviation (n=1 or 2).

Material	Dried Powder	Drying recovery yield % (w/w)
Colostrum	FCL	98*
	SCL165	53 ± 1
	SCL180	59 ± 1
	ESCL75	60 ± 2
	ESCL150	63 ± 3
Lactoferrin	FLF	98*
	SLF165	46 ± 1
	SLF180	45 ± 1
	ESLF75	58 ± 18
	ESLF150	46 ± 1

*: Only one batch of drying was carried out (n=1)

The yield was calculated as the powder recovered based on the original total solids in the feed material. Freeze dried powders were found to have the highest drying recovery yields of 98% (w/w). Spray dried powders, conversely, had relatively much lower yields with about 50% powder loss in each run. SLF180 was seen to have lowest yield of 45 ± 1% (w/w) among the three different drying methods used. This low production yield can be common with conventional spray drying at the pilot plant scale (Garimano *et al.*, 2021) obtained spray dried colostrum powders with an average yield about 25% (w/w), Rodrigues *et al.* (2020) obtained spray dried milk powders with a yield of approximately 50% (w/w), and (de Oliveira *et al.*, 2021) obtained spray dried goat milk powder with yields ranging from 41 to 62% (w/w). In spray drying, the losses of materials can be attributed to the great loss of the product sticking to the drying chamber inner walls (Salama, 2020). In a commercial spray drying operation the yield is typically considerably higher.

4.2.2 Powder moisture content

The moisture content of freeze, spray, and electrostatic spray dried powders, for colostrum and lactoferrin were measured as described in Section 3.7.1 and the results are shown in **Tables 4.3 and 4.4**, respectively.

Table 4.3 Moisture content% (w/w) of freeze, spray and electrostatic spray dried colostrum powders. Different superscript letters (a, b, c) indicate significant difference across the different moisture contents of each powder. Results presented are means ± standard deviation (n=2 or 4).

Dried Powder	Moisture Content[1]% (w/w)
FCL	$2.6 \pm 0.1^{\alpha b}$
SCL165	4.4 ± 0.1^{c}
SCL180	4.0 ± 0.0^{c}
ESCL75	3.5 ± 0.7^{c}
ESCL150	1.4 ± 0.1^{a}

[α]: Only duplicates were carried out (n=2)
[1]: When standard error is 0.0, it is rounded to 0.1.

Table 4.4 Moisture content% (w/w) of freeze, spray and electrostatic spray dried lactoferrin powders. Different superscript letters (a, b, c, d) indicate significant difference across the different moisture contents of each powder. Results presented are means ± standard deviation (n=2 or 4).

Dried Powder	Moisture Content[1]% (w/w)
FLF	$1.3 \pm 0.1^{\alpha a}$
SLF165	4.2 ± 0.1^{d}
SLF180	3.1 ± 0.3^{c}
ESLF75	4.1 ± 0.1^{d}
ESLF150	2.3 ± 0.1^{b}

[α]: Only duplicates were carried out (n=2)
[1]: When standard error is 0.0, it is rounded to 0.1.

All powder samples recovered from different drying methods had a moisture content less than 4.5% (w/w). Of all tested, freeze dried lactoferrin showed the lowest moisture content at 1.3 ± 0.1%, closely followed by ESCL150 with 1.4 ± 0.1%. Meanwhile, the moisture content of samples SCL165, SLF165, ESLF75, and SCL180 was similar, averaging around 4%.

4.2.3 Powder water activity

The water activity (A_w) of freeze, spray, and electrostatic spray dried colostrum and lactoferrin powders were measured using a water activity meter as described in 3.7.2 and results are shown in **Tables 4.5 and 4.6,** respectively.

Table 4.5 Water activity of freeze, spray and electrostatic spray dried colostrum powders. Different superscript letters (a, b, c) indicate significant difference across the different water activity of each powder. Results presented are means ± standard error (n=4).

Dried Powder	Water Activity[1]
FCL	0.06 ± 0.01^{a}
SCL165	0.20 ± 0.01^{bc}
SCL180	0.19 ± 0.01^{bc}
ESCL75	0.26 ± 0.01^{c}
ESCL150	0.12 ± 0.01^{ab}

[1]: When standard error is 0.00, it is rounded to 0.01.

Table 4.6 Water activity of freeze, spray and electrostatic spray dried lactoferrin powders. Different superscript letters (a, b, c) indicate significant difference across the different water activity of each powder. Results presented are means ± standard error (n=4).

Dried Powder	Water Activity[1]
FLF	0.05 ± 0.01^{a}
SLF165	0.13 ± 0.01^{b}
SLF180	0.10 ± 0.01^{b}
ESLF75	0.19 ± 0.01^{c}
ESLF150	0.12 ± 0.01^{b}

[1]: When standard error is 0.00, it is rounded to 0.01.

All the powders analysed in this assay had a water activity below 0.35. Freeze dried colostrum and lactoferrin showed the lowest water activity, approximately 0.05. In contrast, colostrum and lactoferrin electrostatic spray dried at 75°C had the highest water activity, with values of 0.26 ± 0.01 and 0.19 ± 0.01, respectively, compared to their counterparts. Notably, colostrum samples were observed to have an overall higher water activity level than lactoferrin samples, this could be the bound crystallization water in the lactose of the colostrum powder (Reh *et al.*, 2004).

4.3 Compositional analysis-Kjeldahl protein content

The crude protein content of freeze, spray and electrostatic spray dried powders were determined by the Kjeldahl method as described in Section 3.6. Whole milk powder (WMP) (Anchor, Fonterra, New Zealand) was used as a control sample that had a true protein value of 24.36% (w/w). Results are shown in **Table 4.7**.

Table 4.7 Crude Protein Content% (w/w) of freeze, spray and electrostatic spray dried powders. Results presented are means ± standard deviation (n=2 or 4).

Material	Dried Powder	Crude Protein Content% (w/w)
Colostrum	FCL	$55 \pm 0.1^{\alpha}$
	SCL165	63 ± 0.8
	SCL180	61 ± 0.0
	ESCL75	61 ± 0.4
	ESCL150	60 ± 0.4
Lactoferrin	FLF	$96 \pm 0.1^{\alpha}$
	SLF165	95 ± 0.2
	SLF180	95 ± 0.2
	ESLF75	96 ± 0.3
	ESLF150	95 ± 0.4

$^{\alpha}$: Only replicates were carried out (n=2)

Kjeldahl results showed that dried colostrum powders had a crude protein content of approximately 60% (w/w), and lactoferrin powders had a crude protein of 95% (w/w), both values agree with the protein content (by the total solids) from the proximate analysis results in Section 4.1.

4.4 Physicochemical characterisation

4.4.1 Solubility

Solubility is an important feature in judging the physical characteristics of milk powder. Dissolution of powdered ingredients is particularly important to manufacturers and consumers as a benchmark of quality (Fang *et al.*, 2008). The solubility of milk powder is affected by the degree of heating in the drying process (Baldwin & Truong, 2007). Solubility results of colostrum and lactoferrin powders are shown in **Tables 4.8** and **4.9**, respectively.

Table 4.8 Solubility% (w/w) of colostrum powders at 23 ± 1°C. Different superscript letters (a, b) indicate significant difference across the different solubility of each powder. Results presented are means ± standard deviation (n=2).

Dried Powder	Solubility% (w/w)
FCL	72 ± 0.4^{a}
SCL165	88 ± 1.0^{b}
SCL180	85 ± 2.0^{b}
ESCL75	88 ± 2.0^{b}
ESCL150	87 ± 1.0^{b}

Table 4.9 Solubility% (w/w) of lactoferrin powders at 23 ± 1°C. Different superscript letters (a, b, c) indicate significant difference across the different solubility of each powder. Results presented are means ± standard deviation (n=2).

Dried Powder	Solubility% (w/w)
FLF	73 ± 4.0^{a}
SLF165	93 ± 1.0^{c}
SLF180	81 ± 1.0^{ab}
ESLF75	86 ± 1.0^{bc}
ESLF150	82 ± 0.4^{b}

All tested samples exhibited a solubility in water exceeding 70% at 23 ± 1°C. Freeze dried colostrum and lactoferrin had the lowest solubility, around 72% (w/w). Freeze drying typically results in a lower water content and water activity when compared to spray drying. This is then reflected in the lower solubility values of the freeze dried powders. Colostrum and lactoferrin spray dried at 165°C demonstrated better solubility compared to those dried at 180°C. Overall, colostrum and lactoferrin subjected to electrostatic spray drying at 75°C had better solubility compared to those dried at 150°C. Poor solubility may be attributed to high drying temperature. Fang *et al.* (2012) found that the solubility of milk protein concentrate (MPC) deteriorated with increasing drying temperature. Felfoul *et al.* (2022) reported that the solubility of spray dried skim dromedary milk (camel milk) increased significantly when dried at higher temperature, oppositely, the solubility of spray dried bovine milk decreased when dried at higher temperature.

4.4.2 Particle size distribution

Particle size distribution and the volumetric median particle size D_v50 were determined with a Mastersizer (Malvern, USA) using dry and wet methods as described in Section 3.7.4. Particle size distributions of the colostrum and lactoferrin powders measured by the dry method are shown in **Figures 4.1** and **4.2**, respectively.

The colostrum dried by electrostatic drying was not measured using the dry method. The colostrum and lactoferrin powders freeze dried were not measured as explained in Section 3.7.4. The particle size distribution is reported as a volume distribution, which is based on the volume occupied by the constituent particles.

The D_v50 value signifies that 50% of tested colostrum and lactoferrin samples are below this diameter, with the results from both the wet and dry methods presented in **Tables 4.10 and 4.11**, separately.

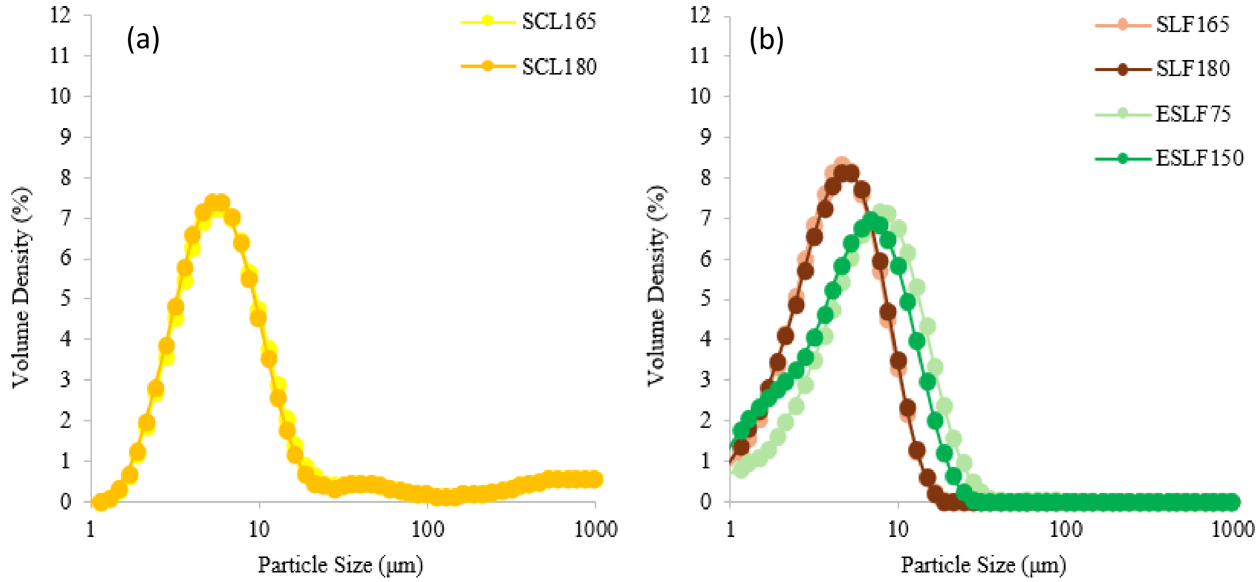

Figure 4.1 Particle size distribution of (a) colostrum powders (b) lactoferrin powders using the dry method (mean values were used to plot, n=2).

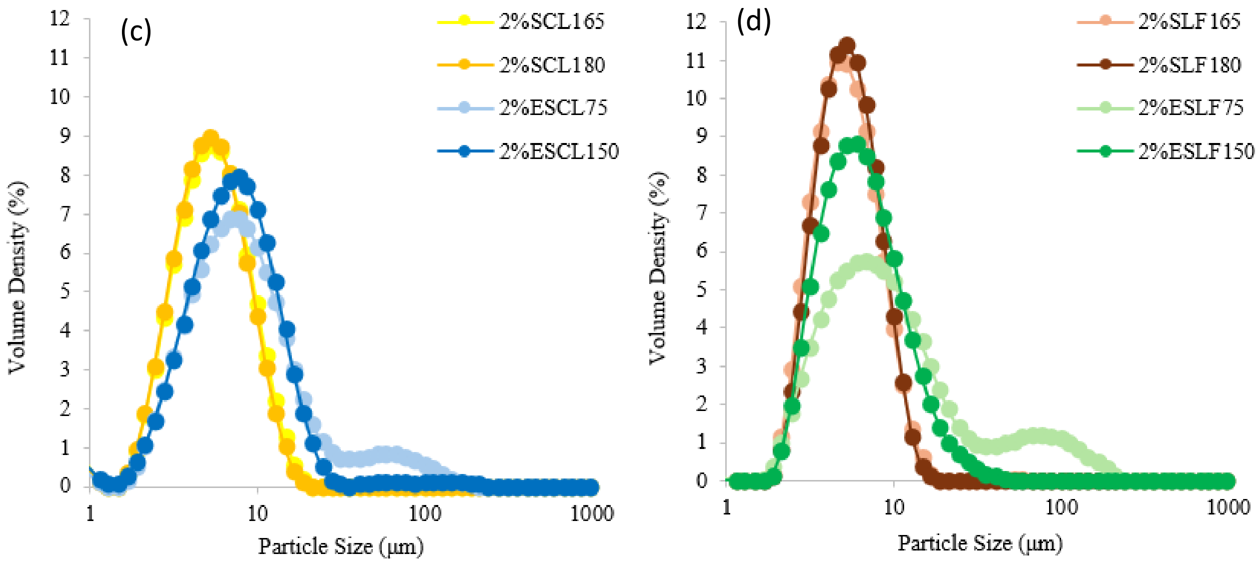

Figure 4.2 Particle distribution of (c) colostrum powders and (d) lactoferrin powders using the wet method. Powders were dispersed in isopropanol at 2% (w/v) (mean values were used to plot, n=2).

Table 4.10 Volumetric median particle size (D_v50) of tested colostrum powders. Different superscript letters (a, b) indicate significant difference across the particle size of each powder. Results presented are means ± standard deviation (n=2).

Colostrum Powder	D_v50[1] by Dry Method (μm)	D_v50 by Wet Method (μm)
SCL165	6.7 ± 0.2	5.3 ± 0.2[a]
SCL180	6.4 ± 0.2	5.2 ± 0.1[a]
ESCL75	-	7.9 ± 0.1[b]
ESCL150	-	7.2 ± 0.2[b]

[1]: When standard error is 0.0, it is rounded to 0.1.

Table 4.11 Volumetric median particle size (D_v50) of tested lactoferrin powders. Different superscript letters (a, b, c) indicate significant difference across the particle size of each powder. Results presented are means ± standard deviation (n=2).

Lactoferrin Powder	D_v50[1] by Dry Method (μm)	D_v50[1] by Wet Method (μm)
SLF165	5.0 ± 0.1[a]	5.4 ± 0.4[a]
SLF180	4.6 ± 0.1[a]	5.5 ± 0.1[a]
ESLF75	7.3 ± 0.2[c]	8.3 ± 0.7[ab]
ESLF150	5.8 ± 0.1[b]	6.6 ± 0.1[b]

[1]: When standard error is 0.0, it is rounded to 0.1.

The volumetric median particle size (D_v50) of electrostatic spray dried colostrum and lactoferrin were found to be larger than spray dried powders. It can be observed from the results that the particle size decreased as the drying temperature increased. The wet method tested sample ESCL75 had a particle size of 7.9 μm, and sample ESCL150 showed a reduced particle size of 7.2 μm, they were found not to be significantly different ($p>0.05$). This trend is more obvious with the electrostatic spray dried lactoferrin powders. The dry method tested ESLF75 had a particle size of 7.3 μm, significantly larger than that of ESLF150 ($p<0.05$) (5.8 μm). From the wet method, the particle size of ESLF75 was 8.3 μm whereas ESLF150 had a smaller particle size of 6.6 μm ($p >0.05$). Toro-Sierra *et al.* (2013) found that the particle size of spray dried whey proteins was not influenced by the drying temperatures. Wang *et al.* (2017a) reported different outlet drying temperatures had no significant effect on the particle size of lactoferrin.

4.4.3 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra can provide information about protein secondary structure. FTIR spectra were generated as per the method described in Section 3.7.5. Colostrum and lactoferrin spectra are shown in **Figures 4.3** and **4.4**, respectively.

Amide I band (1700-1600 cm^{-1}) is the most sensitive spectral region in the protein secondary structure because approximately 80% of the protein's secondary structures vibrations are due to C=O stretch. In the amide I region, each type of secondary structure gives rise to a different C=O stretching frequency due to the unique molecular geometry and hydrogen bonding pattern (Schulz *et al.*, 1979). Amide I components are important in detrmining the potential secondary structure changes and provides informtion on carbonyl stretching vibrations, and amide II components arise from NH bond vibrations (Kong & Yu, 2007). From the average assignment of amide I band positions (Barth, 2007), *a*-helix is observed between an average at 1654 cm^{-1}, *b*-sheet was observed at 1633 cm^{-1}, turns are observed at 1672 cm^{-1}. These band positions are circled and labeled in the enlargement of the amide I region (**Figures 4.5 and 4.6**, respectively).

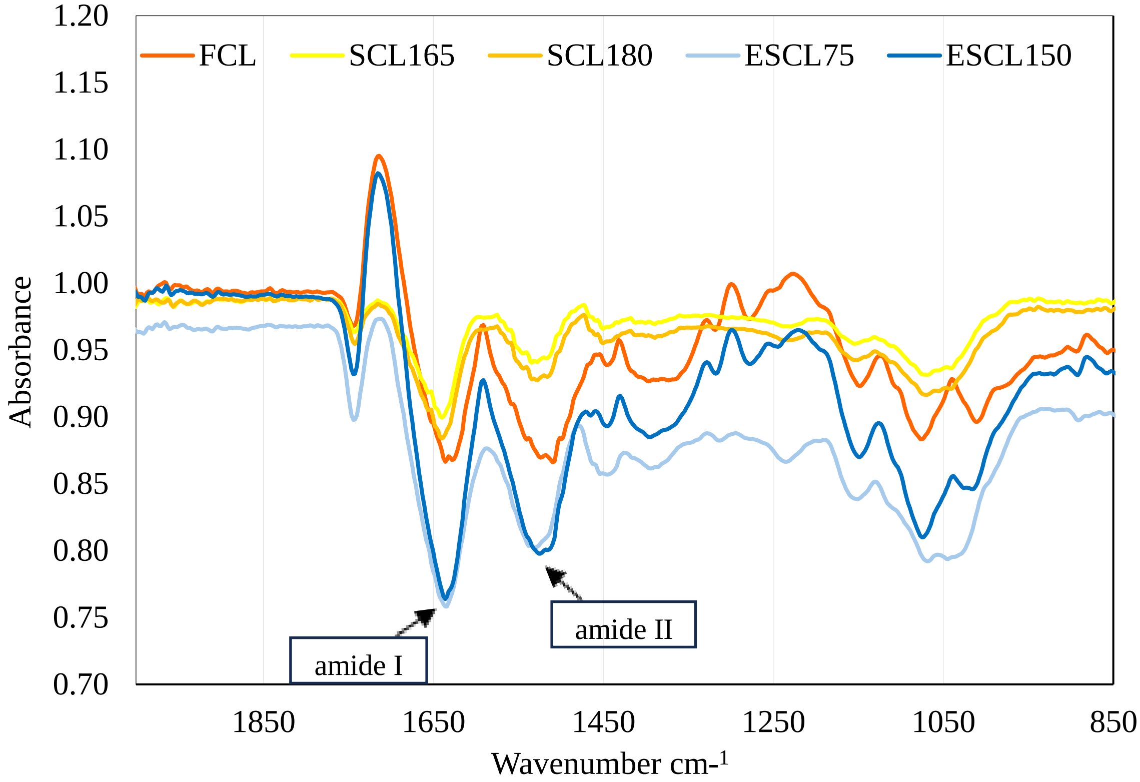

Figure 4.3 FTIR spectra of colostrum powders.

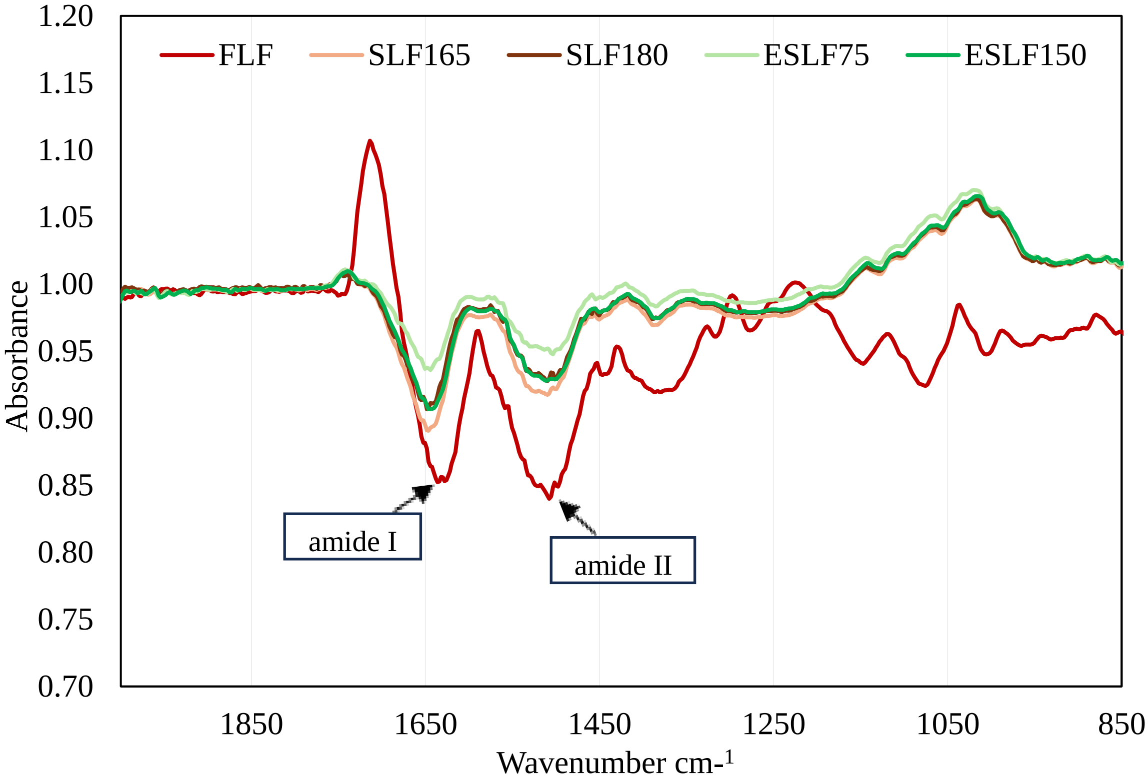

Figure 4.4 FTIR spectra of lactoferrin powders.

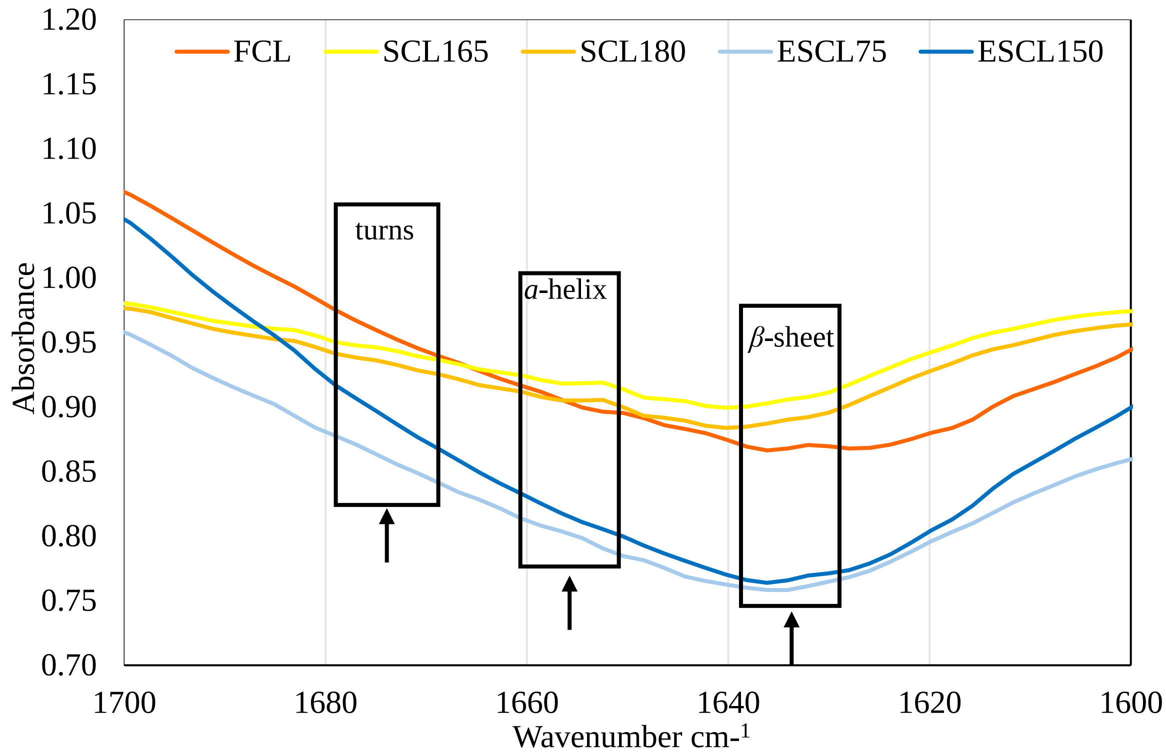

Figure 4.5 Enlarged FTIR amide I region of colostrum samples secondary derivative spectrum.

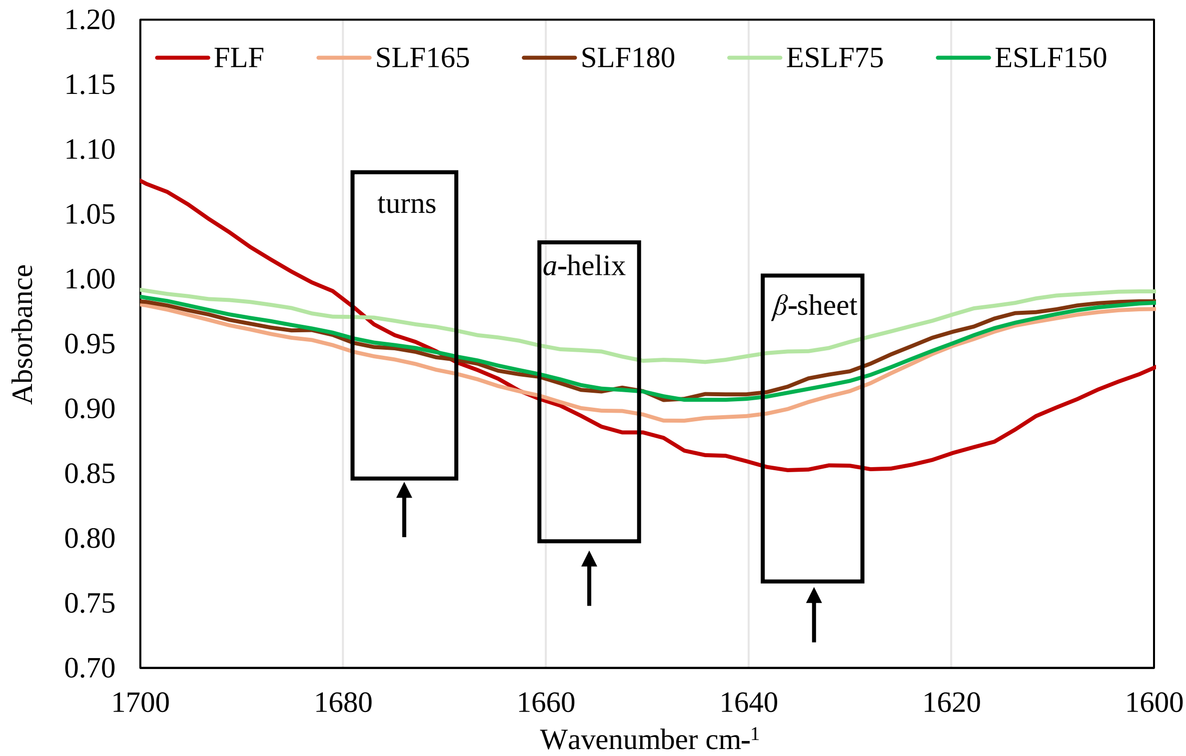

Figure 4.6 Enlarged FTIR amide I region of colostrum samples secondary derivative spectrum.

It can be seen in **Figure 4.3** that ESCL75 and ESCL150 have a lower intensity in the amide I and II regions, whereas SCL165 and SCL180 had higher intensities, indicating that samples ESCL75 and ESCL150 retained most of the band structures in the amide I and II regions. However SCL165 and SCL180 may have had more protein unfolding accuring due to protein denaturation during the high temperature drying process. **Figure 4.4** shows that FLF had the weakest absorbance at the amide I and II regions and likely retained more secondary structure than the other samples. Other spray dried and electrostatic spray dried lactoferrin powder showed similar vibrations at the amide I and II regions, suggesting that they had retained nearly the same amount of band structures. Specifically, in the enlarged amide I region, **Figure 4.5** showed that sample SCL165, SCL180 and FCL had similar absorbance at 1654 and 1672 cm^{-1} wavenumber, higher than that of ESCL75 and ESCL150, indicating that sample SCL165, SCL180 and FCL encountered more unfolding in the *a*-helix and in the turns. This was slightly different at 1633 cm^{-1} wavenumber where SCL165 and SCL180 had similar intensity but it was higher than FCL, ESCL75 and ESCL150 which were found to have the lowest intensity at 1633 cm^{-1} wavenumber, suggesting that they had the least unfolding of the *b*-sheet structure. **Figure 4.6** showed that all lactoferrin samples had similar absorption peaks in the *a*-helix and turns area, however, the *b*-sheet peak of FLF shifted down indicating FLF retained more *b*-sheet structure than other samples.

4.5 Bioactivity of lactoferrin and IgG protein

4.5.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) uses the catalytic properties of enzymes to detect and quantify immunological reactions in biological systems. It can be used to detect antigens that are present in very small quantities in a fluid sample (Stephanie *et al.*, 2013). This is because at a very low concentration, it is unlikely that an antibody can bind to a molecule other than its own antigen (Bethyl-E11-126/ Bethyl-E11-118 User Manual, Bethyl Laboratories, USA).

The ELISA kits used in this research are based on the sandwich ELISA, that the antigens are sandwiched between two layers of antibodies (capture and detection antibodies) (Sakamoto *et al.*, 2018). The bovine lactoferrin or IgG proteins tested are captured by anti-bovine lactoferrin or anti-bovine IgG antibodies that have been pre-adsorbed on the surface of the microtiter wells. Any unbound antigens are washed off following repeated incubation and washing. Then a streptavidin-conjugated horseradish peroxidase (SA-HRP) is added to catalyze a colorimetric reaction with the chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine). The colorimetric reaction produces a blue colour and it turns yellow when the reaction is terminated by the addition of sulphuric acid. The absorbance of the yellow product at 450 nm is proportional to the amount of lactoferrin or IgG analyte present in the samples.

The amount of lactoferrin and IgG in their active form were quantified from ELISA tests. By comparing the protein amount determined by ELISA, it was then possible to determine the amount of lactoferrin or IgG protein undenatured by the drying methods tested. If the drying methods damaged the proteins, then the results from ELISA will provide this information.

ELISA standard curves for bovine IgG and lactoferrin were produced with the standards supplied within the lactoferrin and IgG ELISA kits (replicated standard curves are presented in Appendix 1 and 2, respectively). The procedure followed was outlined in Section 3.8. Spray and electrostatic spray dried colostrum and lactoferrin powders were produced in duplicates and tested in triplicates, but freeze dried colostrum and lactoferrin powders were produced in one batch, tested in triplicates. Results are presented as mg (active lactoferrin or IgG) / g (powder) based on dry weight.

The results for the IgG protein in colostrum and lactoferrin powders are presented in **Figures 4.7** and **4.8**.

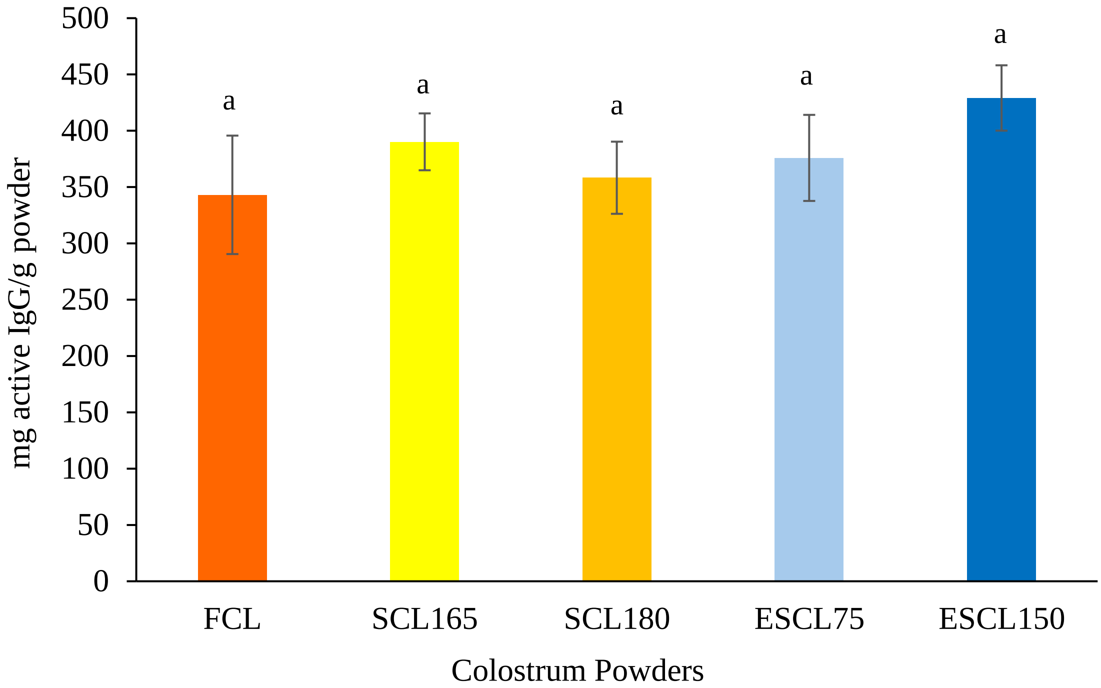

Figure 4.7 Concentration of IgG in colostrum powder samples (mg active IgG/g powder (based on dry weight)) (means ± standard error, n=5 to 10).

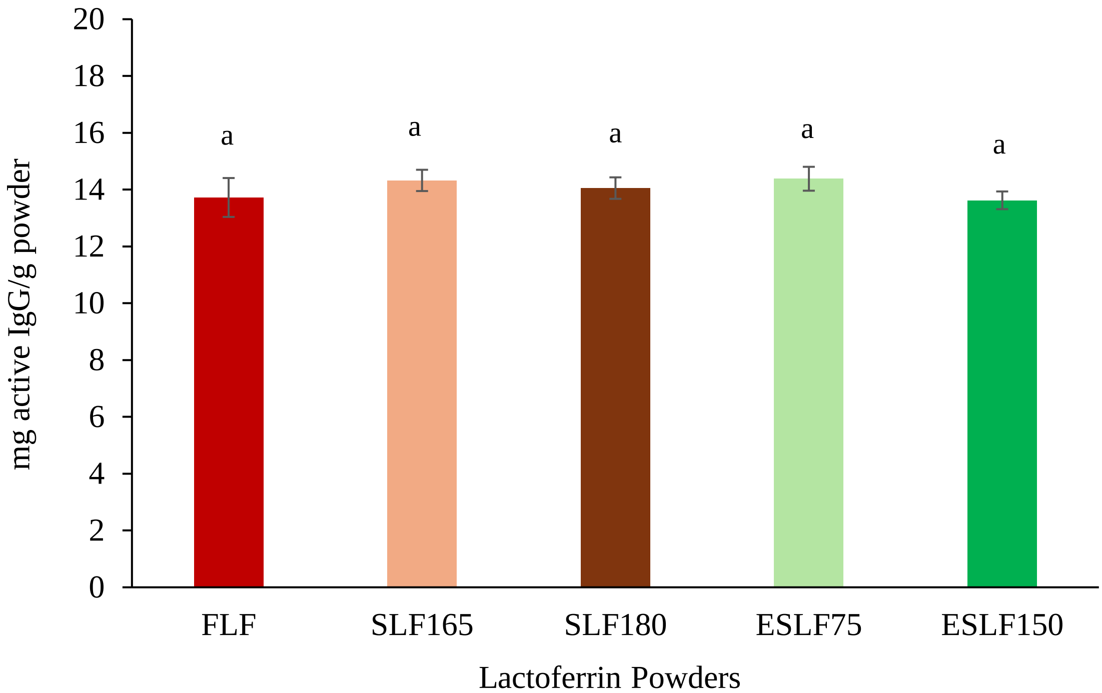

Figure 4.8 Concentration of IgG in lactoferrin powder samples (mg active IgG/g powder (based on dry weight)) (means ± standard error, n=5 to 12).

The ELISA results for the amount of lactoferrin protein in colostrum and lactoferrin powders is presented in **Figures 4.9** and **4.10.**

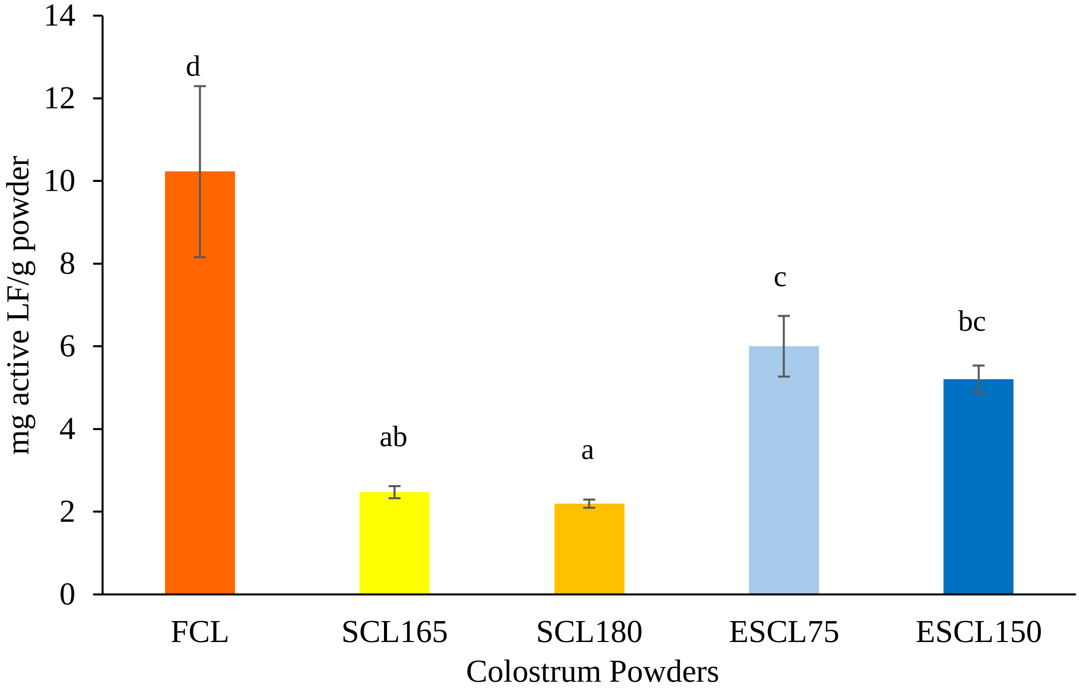

Figure 4.9 Concentration of lactoferrin in colostrum powder samples (mg active LF/g powder (based on dry weight)) (means ± standard error, n=3 to 6).

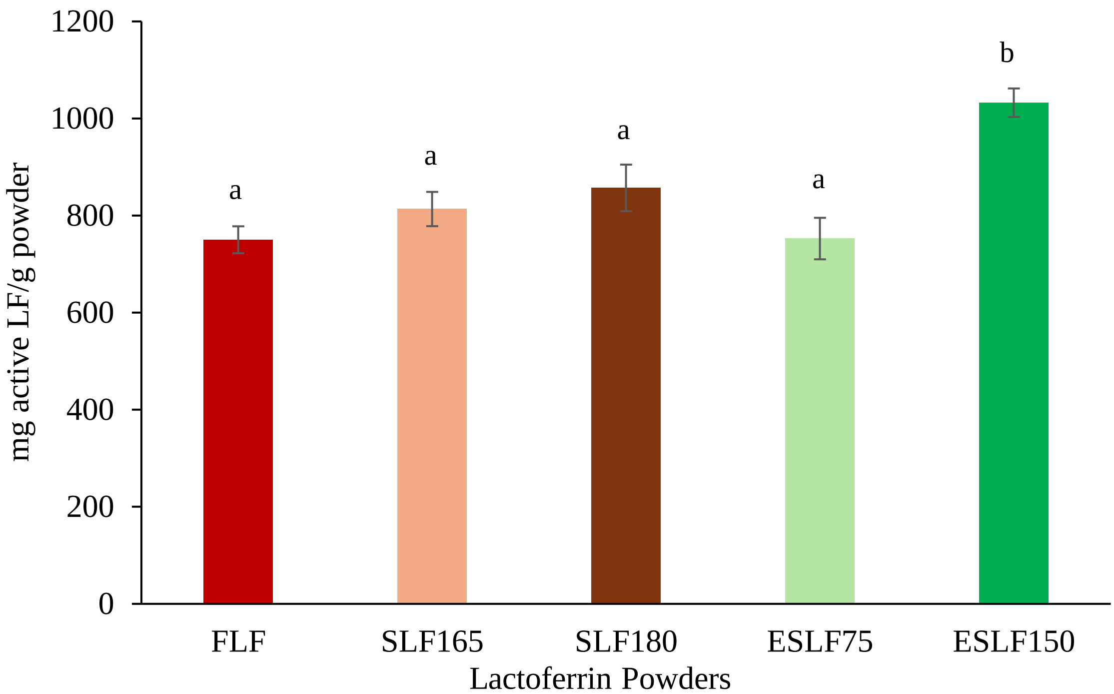

Figure 4.10 Concentration of lactoferrin in lactoferrin powder samples (mg active LF/g powder (based on dry weight) (means ± standard error, n=3 to 6).

Two commercial freeze dried lactoferrin powders (HG28, CG17) were also analysed for IgG and lactoferrin content. HG28 had an active IgG content of 15 ± 1 mg/g of powder (n=2) and an active lactoferrin content of 844 ± 16 mg/g of powder (n=2). CG17 had an active IgG content of 14 ± 1 mg/g of powder (n=2) and an active lactoferrin content of 926 ± 69 mg/g of powder (n=2).

Figure 4.7 showed that freeze dried colostrum had an active IgG protein content of 343 ± 53 mg/gram of powder. SCL165 and SCL180 had an active IgG protein content of 390 ± 25 and 358 ± 32 mg/g of powder, respectively. The ESCL75 electrostatic spray dried colostrum powder had an active IgG protein content of 376 ± 38 mg/g of powder, while that for ESCL150 colostrum powder was 429 ± 29 mg/g of powder. ANOVA analysis showed no significant difference between all values ($p>0.05$). **Figure 4.8** showed that all lactoferrin powders had comparable active IgG protein content of approximately 14 mg/g of powder. These values were small compared with both the active IgG protein values in the colostrum samples and the corresponding active lactoferrin content in the lactoferrin samples.

Figure 4.9 showed that freeze dried colostrum had a significantly higher active lactoferrin content of 10 ± 2mg/g of powder ($p<0.05$), followed by sample ESCL75 and ESCL150 with approximately 6 and 5mg/g of powder, respectively. These results indicated that freeze dried colostrum preserved the highest amount of active lactoferrin protein during the drying process. In contrast, SCL165 and SCL180 retained the least amount of active lactoferrin content, with only about 2mg/g of powder. **Figure 4.10** showed that FLF, SLF165, SLF180 and ESLF75 have a similar active lactoferrin content, each approximately 800mg/g of powder, with no significant difference from each other ($p>0.05$). Sample ESLF150 showed a significant higher ($p<0.05$) active lactoferrin content at about 1000mg/g of powder suggesting it retained near full amount of lactoferrin protein after drying.

4.5.2 Iron binding ability by relative colorimetric method

This method compares the relative iron binding capacity of spray dried powder with freeze dried powder assuming freeze drying results in minimal denaturation of lactoferrin protein and therefore does not affect its iron binding capability. The method used was based on the method of (Kawakami *et al.*, 1992) as described in Section 3.9. Results are reported as the percentage of iron binding ability for spray and electrostatic spray dried samples compared to freeze dried sample as a control. The iron binding ability results for colostrum and lactoferrin are shown in **Figures 4.11** and **4.12**, respectively.

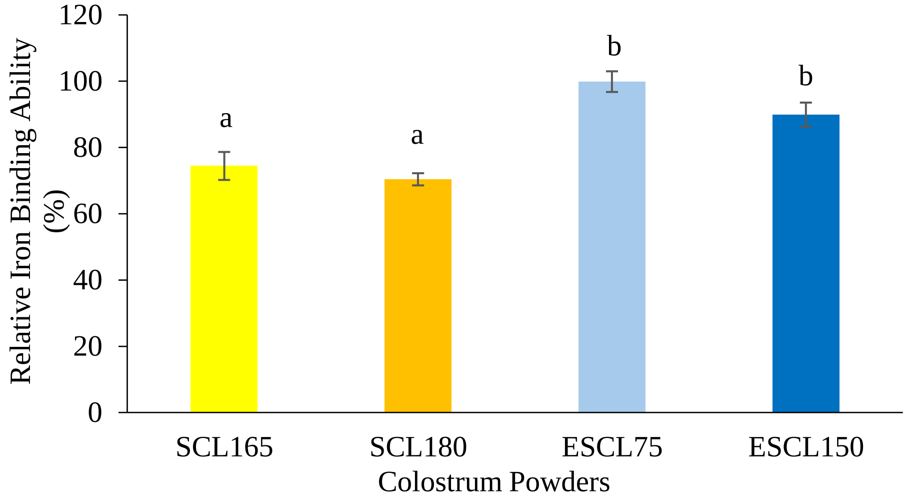

Figure 4.11 The relative iron binding ability of colostrum powders compared to freeze dried colostrum powder. Based on the method of (Kawakami *et al.*, 1992) (means ± standard deviation , n=2).

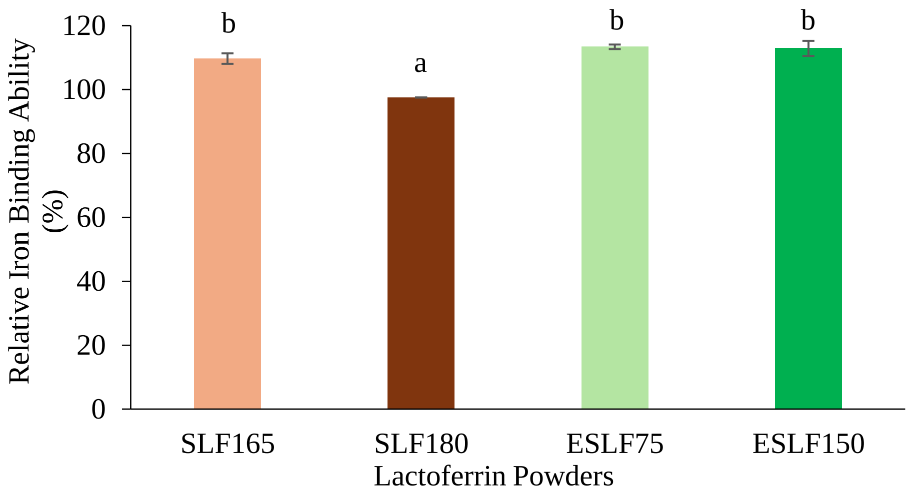

Figure 4.12 The relative iron binding ability of lactoferrin powders compared to freeze dried lactoferrin powder. Based on the method of (Kawakami *et al.*, 1992) (means ± standard deviation , n=2).

Two commercial freeze dried lactoferrin powders (HG28, CG17) were also analysed for iron binding ability using the colorimetric method described above. HG28 had an iron-binding ability of 82 ± 1% (n=2). CG17 had an iron-binding ability of 110 ± 1% (n=2).

Figure 4.11 showed that spray dried colostrum powders had an iron binding ability of approximnately 70% of the control. ESCL150 and ESCL75 had an iron binding ability of 90 ± 3 and 100 ± 3%, respectively, compared to the control. These results indicated that spray drying diminished the iron binding ability of colostrum, whereas electrostatic spray drying on the other hand, retained it much better and was significantly higher ($p<0.05$). **Figure 4.12** showed that the iron binding ability of spray and electrostatic spray dried lactoferrin powders were all above 100%, suggesting that they have higher iron binding capacity then the freeze dried control sample. SLF180 had an iron binding ability of 103 ± 1%, which was significantly lower than others ($p<0.05$). SLF165, ESLF75 and ESLF150 had comparable iron binding ability of approximately 115% compared to the freeze dried control. This may be due to less lactoferrin protein degradation during the low temperature drying process, therefore more iron was bound to the lactoferrin protein.

4.5.3 Iron-binding ability by MP-AES method

A modified four-step method to quantify the ratio of iron bound to lactoferrin protein was carried out based on Morel *et al*. (2022) in Section 3.10. The iron saturated lactoferrin protein was eluted out of a Sephadex™ G-25 desalting column (PD-10, Cytiva, New Zealand) and the eluate was subject to, firstly Bradford test for the concentration of protein, secondly MP-AES for the concentration of the iron. Therefore, a ratio of iron bound to protein was able to be determined. The Bradford method (Bradford, 1976) was used to determine the protein content in the lactoferrin and colostrum eluates collected from column separation, using a commercial lyophilised bovine lactoferrin standard (≥ 85%, Sigma-Aldrich, USA) as described in Section 3.10.5.

The Bradford assay standard curve is presented in Appendix 3 and the calculated protein content of colostrum and lactoferrin eluates were shown in Appendix 4. The MP-AES Fe concentration standard curve is presented in Appendix 5. The MP-AES method was validated by comparing determined iron content of the standard skim milk powder (ERM®-BD151, European Reference Materials, Belgium) with the product Certificate of Analysis (Appendix

6). The iron content of the standard skim milk powder was determined to be 46.6 ± 0.5 mg Fe/kg skim milk powder (means ± standard deviation, n=2), while the iron content provided from the product Certificate of Analysis (Appendix 6) was 53 mg Fe/kg skim milk powder (European Commission, Joint Research Centre, Consumers and Reference Materials, Belgium). Thus, the iron concentration in the standard skim milk powder (ERM®-BD151) measured by the MP-AES method agreed with the concentration reported on the product specification. The final iron binding results of colostrum and lactoferrin powders were reported as moles Fe per mole of protein (**Table 4.12** and **4.13**, respectively).

Table 4.12 Iron binding ratio of proteins present in colostrum powders. Different superscript letters (a) indicate significant difference across the iron binding ability of each powder. Results are presented as means ± standard deviation (n=20 or 40).

Sample	moles Fe/moles Protein
FCL	3.26 ± 0.69
SCL165	3.91 ± 0.19^{a}
SCL180	2.79 ± 1.15^{a}
ESCL75	4.32 ± 0.22^{a}
ESCL150	4.07 ± 0.15^{a}

*: Only one sample was tested, n=1

Table 4.13 Iron binding ratio of proteins present in lactoferrin powders. Different superscript letters (a) indicate significant difference across the iron binding ability of each powder. Results are presented as means ± standard deviation (n=20 or 40).

Sample	moles Fe/moles Protein
FLF	1.28 ± 0.02
SLF165	1.47 ± 0.19^{a}
SLF180	1.31 ± 0.05^{a}
ESLF75	1.47 ± 0.34^{a}
ESLF150	1.31 ± 0.01^{a}

*: Only one sample was tested, n=1

From **Table 4.12**, sample ESCL75 exhibited a high iron binding ratio of 4.32 ± 0.22, while ESCL150 had a slightly lower ratio of 4.07 ± 0.15. Both samples demonstrated better iron binding abilities compared to the other samples, though were not significantly higher (p>0.05). In contrast, SCL180 had the lowest iron binding ratio at 2.79 ± 1.15, similar to SCL165, which had a ratio of 3.91 ± 0.19. Freeze dried colostrum had an iron binding ratio of 3.26 ± 0.69. From **Table 4.13**, sample ESLF75 showed the highest iron binding ration of 1.47 ± 0.34, followed by SLF165 with a ratio of 1.47 ± 0.19. ESLF150 and SLF180 had very close results at approximately 1.30, all values were not significantly different to each other (p>0.05). Freeze dried lactoferrin was tested the lowest iron binding ratio of 1.28 ± 0.02.

The observed decrease in iron binding capacity may be attributed to protein damage during high-temperature processing. Both electrostatic spray dried colostrum and lactoferrin exhibited better iron binding abilities compared to their respective others. Overall, colostrum powders demonstrated showed a better iron binding ability than lactoferrin. This could be related to other components in colostrum that also have affinity to iron. Previous studies have reported casein proteins present in milk also bind iron (Baumy *et al.*, 1988; Delshadian *et al.*, 2018; Kibangou *et al.*, 2005).

4.6 Physicochemical properties

4.6.1 DPPH antioxidant properties

The antioxidant ability of colostrum and lactoferrin samples were determined as described in Section 3.11 based on DPPH radical scavenging ability in a citrate-phosphate buffer system. Samples were loaded on a 96-well plate and the absorbance read at 515 nm wavelength. Each sample was analysed in dupliacte. The DPPH radical scavenging ability was calculated as the percentage of loss of absorbance (**Equation 3.8**). The results obtained for colostrum and lactoferrin are shown in **Figures 4.13** and **4.14**, respectively.

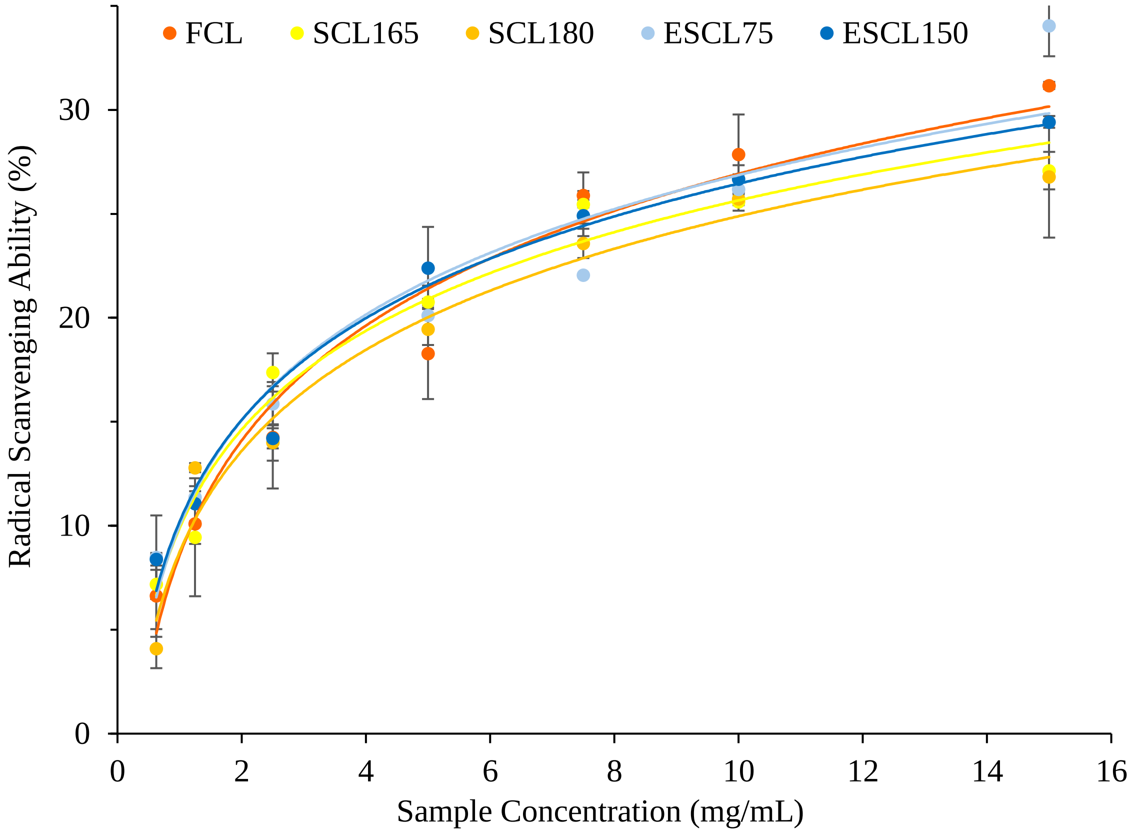

Figure 4.13 The DPPH free radical scavenging ability (%) of colostrum powders (means ± standard deviation, n=2).

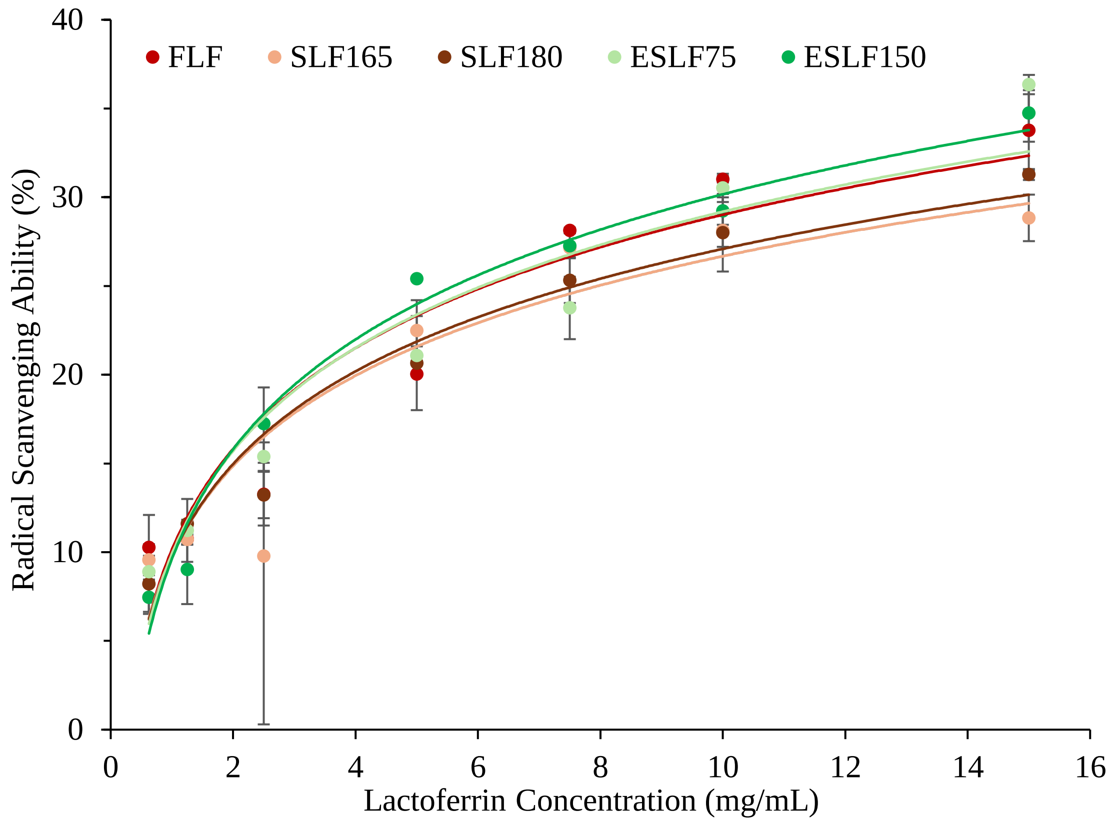

Figure 4.14 The DPPH free radical scavenging ability (%) of lactoferrin powders (means ± standard deviation, n=2).

For the raw materials, liquid colostrum and lactoferrin, they were diluted 10, 20 and 40 times and measured for the DPPH radical scavenging ability following the same procedure as for the powders. The results are presented in **Figure 4.15**.

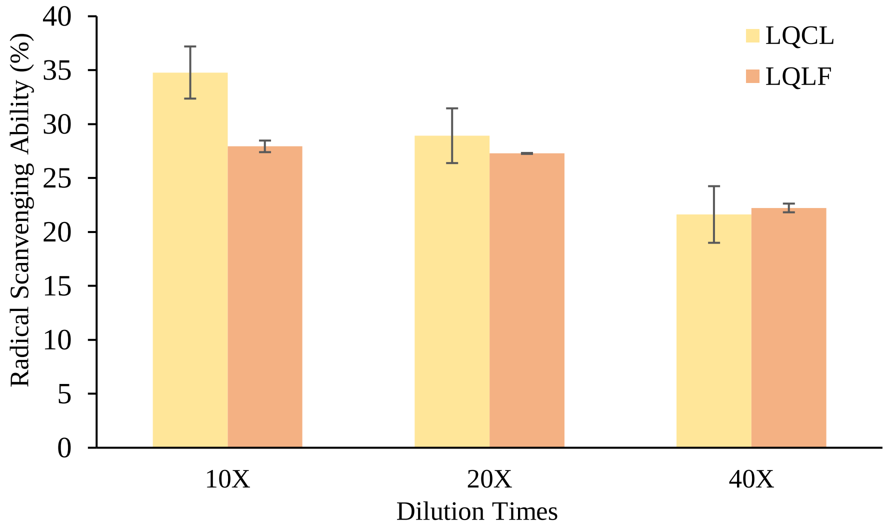

Figure 4.15 The DPPH free radical scavenging ability (%) of diluted colostrum and lactoferrin raw materials (means ± standard deviation, n=2).

Two commercial freeze dried lactoferrin powders (HG28, CG17) were also analysed for DPPH radical scavenging ability. HG28 had a DPPH radical scavenging ability of 40 ± 2%(n=3) at a concentration of 15 mg/mL. CG17 had an iron-binding ability of 35 ± 2% (n=3) at a concentration of 15 mg/mL.

Figure 4.13 showed that the two electrostatic spray dried colostrum samples had very similar DPPH radical scavenging ability to the freeze dried colostrum and these values were greater than for the spray dried colostrum samples. ESCL75 had a DPPH radical scavenging ability of approximately 34% at a concentration of 15 mg/mL. FCL and SCL180 exhibited around 31% scavenging ability at the highest concentration tested (15 mg/mL), with ESCL150 a little lower at about 29%. SCL165 had a slightly higher scavenging ability than SCL180, both around 27% at 15 mg/mL, which was lower than other colostrum samples. From **Figure 4.14**, ESLF75 and ESLF150 displayed similar DPPH radical scavenging abilities of about 36%. Freeze dried lactoferrin showed a DPPH radical scavenging ability of around 33% at 15 mg/mL. Similar to colostrum samples, SCL165 and SCL180 showed lower DPPH radical scavenging ability at approximately 29% at the same concentration. Overall, the electrostatic spray dried powders exhibited slightly higher DPPH radical scavenging ability compared to all the other powders.

Figure 4.15 showed that the raw liquid colostrum at ten- and twenty-times dilutions showed better DPPH radical scavenging ability than lactoferrin of approximately 35 and 29%, respectively, compared to lactoferrin with a result of 29 and 27%, respectively. Forty-times diluted colostrum and lactoferrin showed a similar DPPH radical scavenging ability of about 22%.

4.7 Antimicrobial activity

Antimicrobial activity was carried out using the disc diffusion method described in 3.12. Gram-negative *Escherichia coli* NCTC 8196 and Gram-positive *Staphylococcus aureus* NCTC 4163 were used in this test. Five millimetre blank paper discs (BBL, USA) were used in this test. Each petri dish contained two samples with each duplicate placed diagonally. The zones of inhibition were observed after an incubation period of 18 h at 37°C. Results of inhibition in **Table 4.14** are presented as means and their standard deviations (n=2 to 5). Zone of potential inhibitions was shown in **Figure 4.16-4.19.**

Table 4.14 Zone of diameters of colostrum and lactoferrin against bacterial species. Results are presented as means ± standard deviation (n=2 to 5).

Sample (5% w/v)	*E. coli* (mm)	*S. aureus* (mm)
LQCL	2.4 ± 0.1 (n=3)	N
FCL	2.7 ± 0.9 (n=5)	N
SCL165	2.3 ± 0.1 (n=2)	3.2 ± 0.1 (n=2)
SCL180	2.9 ± 0.4 (n=5)	2.7 ± 0.1 (n=2)
ESCL75	3.6 ± 0.1 (n=5)	4.3 ± 0.1 (n=2)
ESCL150	2.2 ± 0.1 (n=4)	N
LQLF	3.5 ± 0.6 (n=3)	4.2 ± 1.1 (n=3)
FLF	3.9 ± 0.1 (n=5)	3.9 ± 0.2 (n=5)
SLF165	2.9 ± 0.1 (n=2)	3.8 ± 0.9 (n=2)
SLF180	3.7 ± 0.6 (n=5)	3.6 ± 1.2 (n=5)
ESLF75	3.6 ± 0.6 (n=5)	3.8 ± 0.9 (n=5)
ESLF150	3.3 ± 0.6 (n=4)	3.5 ± 0.4 (n=4)
Westland FLF HG28	3.7 ± 1.1 (n=2)	N
Westland FLF CG17	2.5 ± 0.1 (n=2)	N

N: No inhibition zone formed

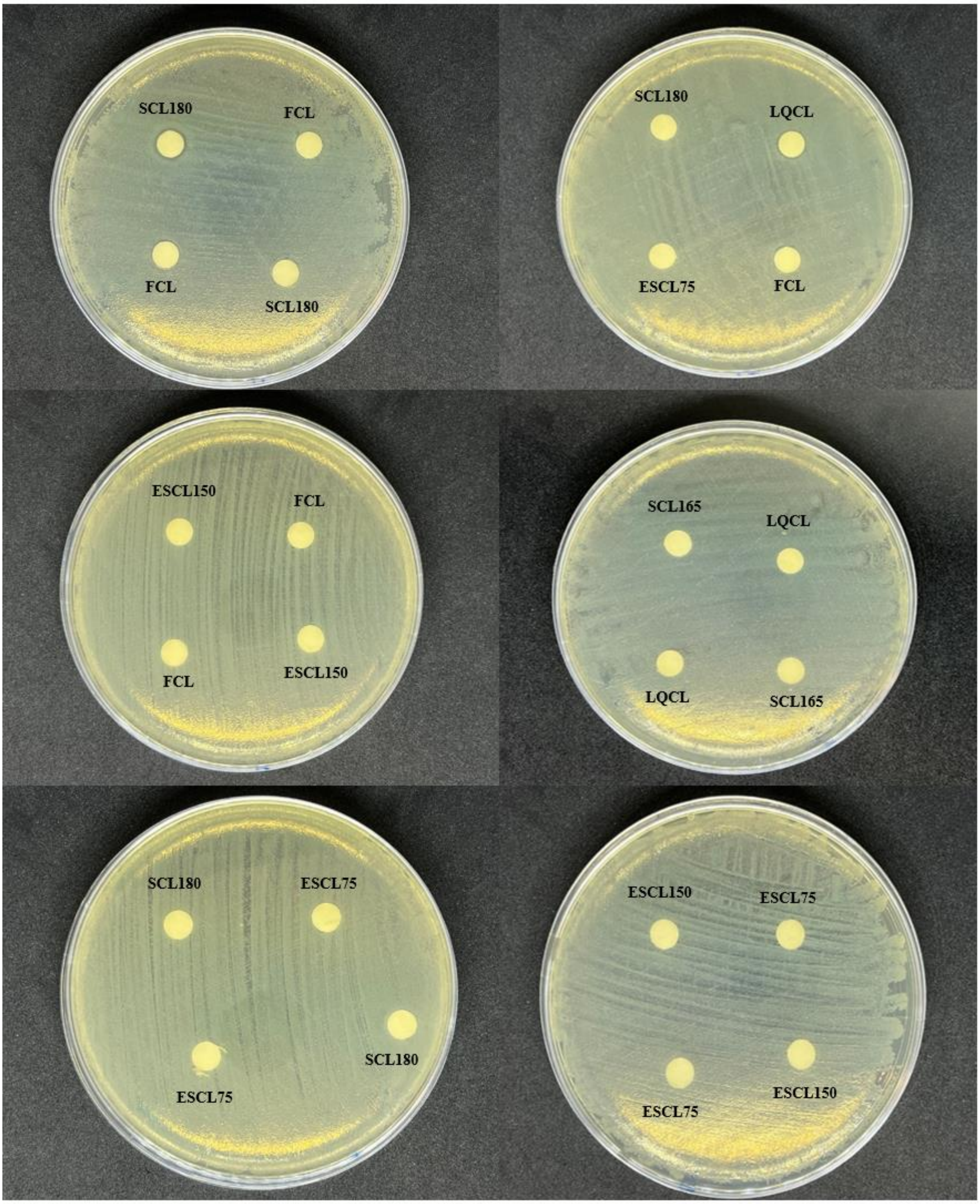

Figure 4.16 Zone of inhibitions of colostrum samples against *E. coli.*

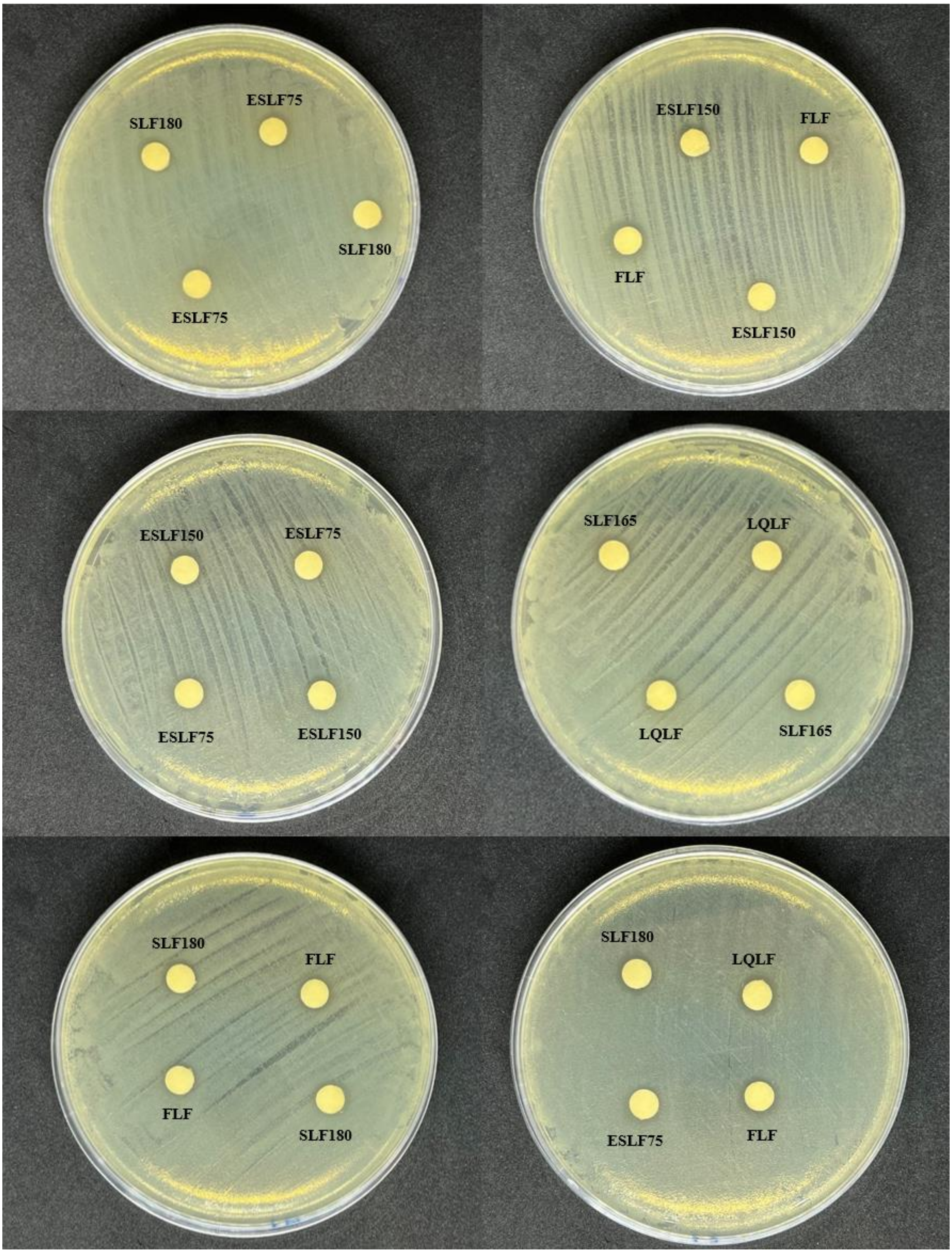

Figure 4.17 Zone of inhibitions of lactoferrin samples against *E. coli.*

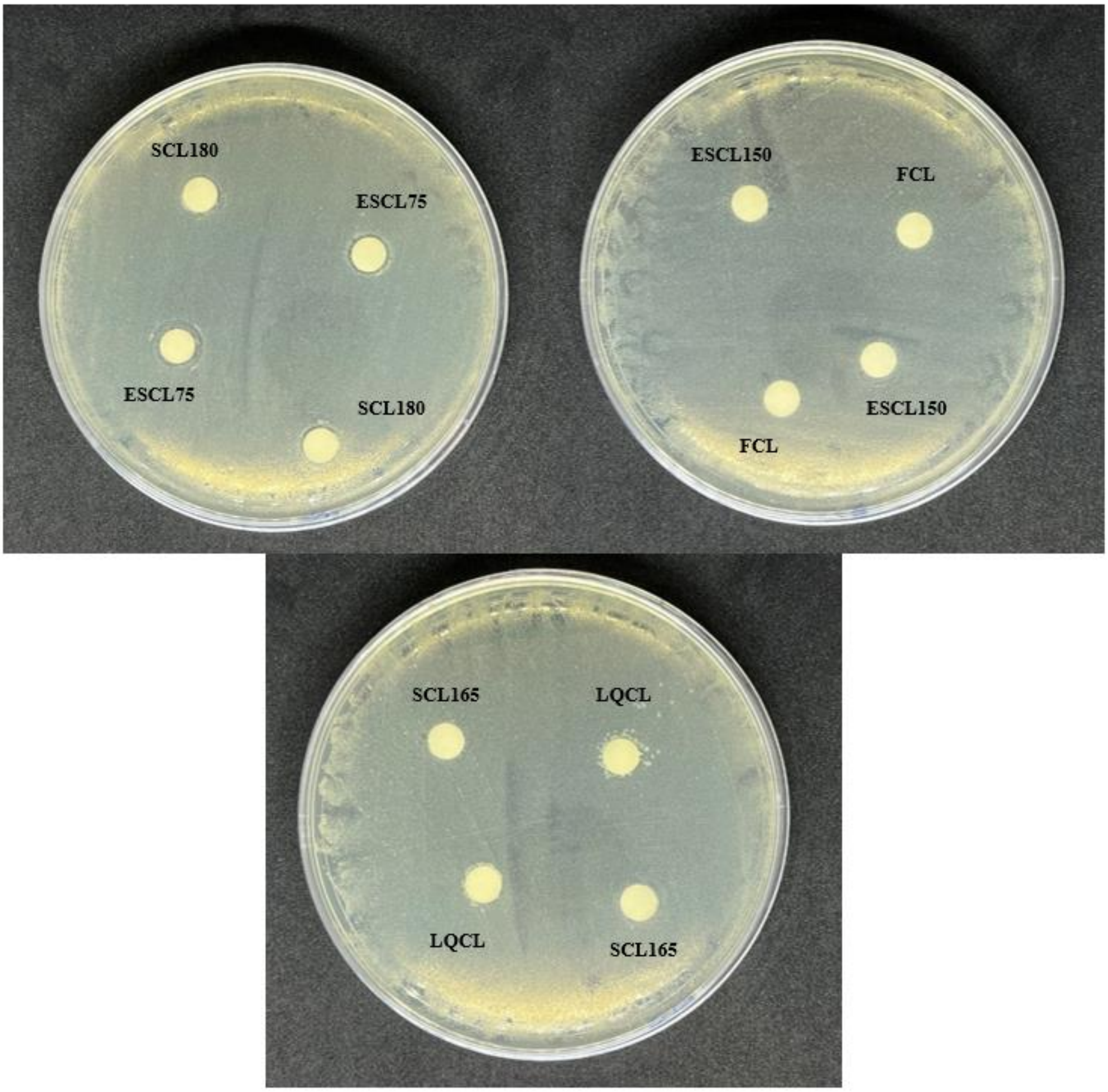

Figure 4.18 Zone of inhibitions of colostrum samples against *S. aureus*.

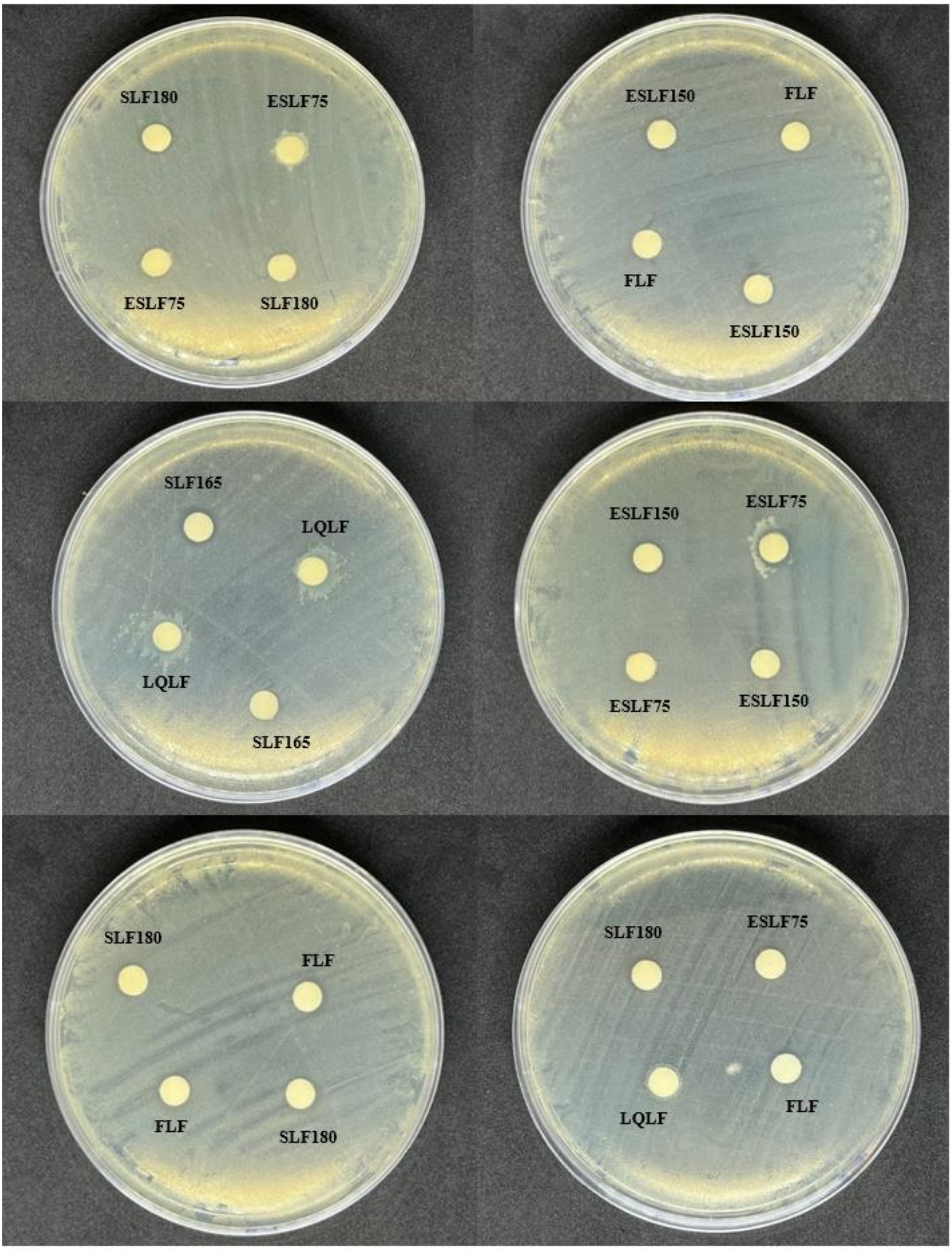

Figure 4.19 Zone of inhibitions of lactoferrin samples against *S. aureus*.

Samples LQCL, FCL, SCL165 and SCL180 showed similar inhibition zones of approximately 2.5 mm to *E. coli*. No inhibition zones to *S. aureus* were observed for samples LQCL and FCL. ESCL75 had an inhibition zone of approximately 4 mm to both bacteria. ESCL150 had an approximately 2 mm inhibition zone to *E. coli* while nothing was observed to *S. aureus.* LQLF had an inhibition zone of 3.5 ± 0.6 and 4.2 ± 1.1 mm with *E. coli* and *S. aureus*, respectively. FLF had similar inhibition zones of 3.9 ± 0.1 and 3.9 ± 0.2 mm to both bacteria. SLF165, SLF180, ESLF75 and ESLF150 all observed similar inhibition zones in the range of 3 to 4 mm to *E. coli* and *S. aureus*, respectively. Two commercial freeze dried lactoferrin powders (HG28, CG17) were observed to have no inhibition zone to *S. aureus.* HG28 and CG17 had an inhibition zone of 3.7 ± 1.1 and 2.5 ± 0.1 mm to *E. coli,* respectively (Appendix 7).

Overall, lactoferrin samples were observed to have larger inhibition zones than colostrum samples to both bacteria. Moreover, colostrum and lactoferrin samples showed better anti-microbial ability to *E. coli* than to *S. aureus*. Lactoferrin samples were seen to possess more effective antimicrobial activity to *S. aureus* when compared with the colostrum samples. This agrees with the findings of (Jahani et al., 2015) that lactoferrin is more effective on the Gram-positive bacteria such as *Staphylococcus epidermidis* and *Bacillus cereus*.

Chapter 5. Overall Discussion

The objectives of this project were to investigate the effects of freeze drying, spray drying and electrostatic spray drying on the physicochemical and bioactivities of dried bovine lactoferrin and colostrum powders.

The produced colostrum and lactoferrin powders using the different drying technologies had relatively good production yields (based on the dry weight) compared to literature results (de Oliveira *et al.,* 2021; Fatima *et al.,* 2024; Garimano *et al.,* 2021). All spray drying methods provided a powder yield of greater than 45% (w/w). Lactoferrin was found to be stickier in the dryer hence there was more build up on the chamber wall and in the cyclone.

The moisture content and water activity of colostrum and lactoferrin powders was seen to decrease in vale with increasing drying temperatures.

It was however the other way around for the powder solubility and particle size profile. Spray dried (165°C) or electrostatic spray dried (75°C) powders exhibited better solubility in water and larger particle size when compared to the other powders. Alamilla-Beltran *et al.* (2005) reported an increase in the particle size by over 20 µm when spray dried at higher temperature at inlet 170/ outlet 145 and inlet 200/ outlet 173°C).

The correlation between increasing solubility with increasing drying temperatures has also been reported in other studies. Fang *et al.* (2012) reported a decrease in milk protein concentrate (MPC) powder solubility as a result of increasing inlet air temperature (107, 155 and 178°C) with increasing protein denaturation. Anandharamakrishnan *et al.* (2007) reported higher spray drying temperature at 120°C increased insolubility of whey proteins. Felfoul *et al.* (2022) reported that the solubility of spray dried bovine milk at 85°C was lower than when they dried at 75°C.

The FTIR results showed that high temperature spray dried colostrum displayed more unfolded structure in the amide I region than freeze dried and electrostatic spray dried powders. Notably, electrostatic spray dried colostrum powders retained more *a*-helix, turns and *b*-sheet. However, with lactoferrin powders, all the samples had similar degree of protein unfolding with minor differences, e.g., freeze dried lactoferrin retained more *a*-helix and *b*-sheet than the others. High temperature spray dried colostrum (180°C) was observed to have more heat-induced protein unfolding than electrostatic spray dried colostrum that retained more secondary structure. Freeze dried lactoferrin denatured the least compared to other samples but with no major difference regarding the *a*-helix and turns structures.

The ELISA results showed that the three different drying techniques did not lead to significantly differences ($p>0.05$) in the IgG protein bioactivity. Spray dried colostrum samples (at 165 and 180°C) retained less lactoferrin protein content by approximately 8 and 4 mg per gram of powder than freeze dried and electrostatic spray dried samples, respectively. Electrostatic spray drying at 150°C was seen as being significantly effective in retaining the lactoferrin protein in lactoferrin powders.

Lactoferrin is known for its iron binding properties. The iron binding ability of the colostrum and lactoferrin samples quantitively analysed and compared to freeze dried sampled provided a comparison of iron binding ability of lactoferrin protein. A relative colorimetric method described in Section 3.9 showed a relative iron binding capacity of colostrum and lactoferrin powders, and Section 3.10 described a quantitative method to investigate the molar ratio binding ability of tested samples. From the relative method, high temperature spray dried colostrum powders had significantly lower iron binding ability than electrostatic spray dried ($p<0.05$). Spray dried lactoferrin (at 180°C) showed significantly lower iron binding ability compared to the SLF165 spray dried and electrostatic spray dried lactoferrin powders ($p<0.05$). These results from the relative comparison were in agreement with second method which quantitively presented the molar ratios of iron to protein. The results indicated that higher drying temperature deteriorated the iron binding ability of the proteins. Electrostatic spray dried colostrum (at 75°C) had an iron binding ratio of 4.32 ± 0.22, while that for spray dried colostrum at 180°C was 2.79 ± 1.15, although one way ANOVA showed they were not significantly different ($p>0.05$). The iron binding ratios of lactoferrin powders were similar in the range of 1.3 to 1.5. These results agreed with Morel *et al.* (2022), they reported a molar iron binding ratio of approximately 1.2 of the spray dried lactoferrin.

The antioxidant activity results of colostrum and lactoferrin showed that 180°C spray dried colostrum and lactoferrin powders achieved approximately 27 and 31% DPPH radical scavenging ability at highest tested concentration (15 mg/mL). Electrostatic spray dried (at 75°C) colostrum and lactoferrin showed overlapping radical scavenging ability of approximately 34%. Freeze dried colostrum and lactoferrin showed slightly better DPPH radical scavenging ability than high temperature spray dried powders. This trend agrees with Wang *et al.* (2017a) who reported that 180°C spray dried lactoferrin exhibited a lower DPPH radical scavenging ability than freeze dried lactoferrin.

All colostrum and lactoferrin samples were effective on both the Gram-positive and Gram-negative bacteria. Electrostatic spray dried (at 75°C) colostrum had an inhibition zone on the *E. coli* and *S. aureus* plate of 3.6 ± 0.1 and 4.3± 0.1 mm, respectively. The inhibition zones were larger than the other colostrum samples. Liquid lactoferrin had the largest inhibition zone of 4.2 ± 1.1 mm on the *S. aureus* plate. Freeze dried lactoferrin had the largest inhibition zone of 3.9 ± 0.1 mm on the *E. coli* plate. Lactoferrin samples showed better antimicrobial activity on the Gram-positive *S. aureus*.

Chapter 6. Conclusions and Future Recommendations

6.1 Conclusions

This study showed that different drying techniques can affect the physicochemical and functional properties of bovine colostrum and lactoferrin. High temperature spray dried colostrum and lactoferrin had lower solubility in water and smaller volumetric median particle size (D_v50) than low temperature electrostatic-spry-dried. Also the high temperatures involved in the spraydrying degraded the protein more than freeze or electrostatic spray dried colostrum and lactoferrin.

ELISA results showed higher bioactive IgG and lactoferrin protein content was retained in electrostatic spray dried samples, they also had better DPPH radical scanvenging ability compared to freeze and high temperature spray dried. This might due to the protein denaturation induced by heat during high temperature drying. The iron-bind ability of colostrum or lactoferrin showed very small difference between the different drying techniques. Lactoferrin samples showed better antimicrobial activity than colostrum, most probably due to the lower lactoferrin protein content in colostrum.

6.2 Future recommendations

The determination of secondary structure changes of colostrum and lactoferrin samples was analysed by FTIR, it would be recommended to also investigate their thermal stability using Differential Scanning Calorimetry (DSC) to determine their thermal denaturation temperatures.

From the quantitative iron-binding method's results, notably colostrum samples were found to bind more iron than lactoferrin samples. The literature has reported that the casein proteins present in milk are able to bind iron (Baumy *et al.*, 1988; Delshadian *et al.*, 2018; Kibangou *et al.*, 2005). Therefore the higher iron-binding ratio of the analysed colostrum samples could be related to the casein proteins present in colostrum that also bound iron. It is also recommended to investigate the iron-binding ability of other components except for lactoferrin protein solely in colostrum.

References

Abd El-Fattah, A. M., Abd Rabo, F. H. R., El-Dieb, S. M., & El-Kashef, H. A. S. (2014). Preservation methods of buffalo and bovine colostrum as a source of bioactive components. *International Dairy Journal*, *39*(1), 24-27.

Abe, H., Saito, H., Miyakawa, H., Tamura, Y., Shimamura, S., Nagao, E., & Tomita, M. (1991). Heat stability of bovine lactoferrin at acidic pH. *Journal of Dairy Science*, *74*(1), 65-71.

Alamilla-Beltran, L., Chanona-Perez, J. J., Jimenez-Aparicio, A. R., & Gutierrez-Lopez, G. F. (2005). Description of morphological changes of particles along spray drying. *Journal of Food Engineering*, *67*(1-2), 179-184.

Ali, A., Lee, S.-J., & Rutherfurd-Markwick, K. J. (2019). Sports and exercise supplements. In *Whey Proteins* (pp. 579-635). Elsevier.

Aly, E., Ros, G., & Frontela, C. (2013). Structure and functions of lactoferrin as ingredient in infant formulas. *Journal of Food Research*, *2*(4), 25.

Anandharamakrishnan, C., Rielly, C., & Stapley, A. (2007). Effects of process variables on the denaturation of whey proteins during spray drying. *Drying Technology*, *25*(5), 799-807.

Andersen, J. H., Osbakk, S. A., Vorland, L. H., Traavik, T., & Gutteberg, T. J. (2001). Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. *Antiviral research*, *51*(2), 141-149.

Anema, S., Pinder, D., Hunter, R., & Hemar, Y. (2006). Effects of storage temperature on the solubility of milk protein concentrate (MPC85). *Food Hydrocolloids*, *20*(2-3), 386-393.

Anon. (2024a). Colostrum Market Size, Share & Trends Analysis Report By Nature (Organic, Conventional), By Source, By Form, By Distribution Channel, By Region, And Segment Forecasts, 2024 – 2030. Grand View Research. August 2024. https://www.grandviewresearch.com/industry-analysis/colostrum-market

Anon. (2024b). Bovine Lactoferrin Market Size, Share & Industry Analysis, By Product (Spray dried and Freeze dried), By Application (Infant Formula, Pharmaceuticals, Dietary Supplements, Food, and Others), and Regional Forecast, 2024-2032.Fortune Business Insights. August 2024. https://www.fortunebusinessinsights.com/industry-reports/bovine-lactoferrin-market-101656

Anon. (2024c). Electrostatic Spray Drying. Spray Drying Overview. Fluid Air, Spraying Systems Co. USA. https://www.fluidairinc.com/en-us/processes/spray drying

AOAC Official Method 930.29, Kjeldahl method. *Official Methods of Analysis of AOAC INTERNATIONAL*, 22nd Edition (New York, 2023; online edn, AOAC Publications, 4 Jan. 2023).

Arnold, R., Russell, J., Champion, W., & Gauthier, J. (1981). Bactericidal activity of human lactoferrin: influence of physical conditions and metabolic state of the target microorganism. *Infection and immunity*, *32*(2), 655-660.

Auldist, M. J., Walsh, B. J., & THOMSON, N. A. (1998). Seasonal and lactational influences on bovine milk composition in New Zealand. *Journal of dairy research*, *65*(3), 401-411.

Badal Tejedor, M., Fransson, J., & Millqvist-Fureby, A. (2020). Freeze dried cake structural and physical heterogeneity in relation to freeze-drying cycle parameters. *International Journal of Pharmaceutics*, *590*, 119891.

Baker, E., & Baker, H. (2005). Lactoferrin: Molecular structure, binding properties and dynamics of lactoferrin. *Cellular and Molecular Life Sciences*, *62*, 2531-2539.

Baker, H. M., & Baker, E. N. (2004). Lactoferrin and iron: structural and dynamic aspects of binding and release. *Biometals*, *17*(3), 209-216.

Baker, H. M., & Baker, E. N. (2012). A structural perspective on lactoferrin function. *Biochemistry and Cell Biology*, *90*(3), 320-328.

Baldwin, A., & Truong, G. (2007). Development of insolubility in dehydration of dairy milk powders. *Food and Bioproducts Processing*, *85*(3), 202-208.

Bar, E., Tiris, I., Sarbu, D., Iridon, C., Ochea, I., & Bratu, I. (2010). Full characterization of bovine colostrum, raw material for dietary supplements. His beneficial effect on the human immune system. *AUCFT*, *14*(2), 33-40.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1767*(9), 1073-1101.

Basmadjian, D. (2007). *Mass transfer and separation processes: principles and applications*. CRC Press.

Baumy, J. J., & Brule, G. (1988). Effect of pH and ionic strength on the binding of bivalent cations to β-casein. *Le lait*, *68*(4), 409-417.

Beeckman, D., Van Droogenbroeck, C., De Cock, B., Van Oostveldt, P., & Vanrompay, D. (2007). Effect of ovotransferrin and lactoferrins on Chlamydophila psittaci adhesion and invasion in HD11 chicken macrophages. *Veterinary research*, *38*(5), 729-739.

Bhandari, B., Senoussi, A., Dumoulin, E., & Lebert, A. (1993). Spray drying of concentrated fruit juices. *Drying Technology*, *11*(5), 1081-1092.

Bhandari, B. R., Patel, K. C., & Chen, X. D. (2008). Spray drying of food materials-process and product characteristics. *Drying technologies in food processing*, *4*, 113-157.

Bhimani, R., Vendrov, Y., & Furmanski, P. (1999). Influence of lactoferrin feeding and injection against systemic staphylococcal infections in mice. *Journal of applied microbiology*, *86*(1), 135-144.

Borad, S., Singh, A., Kapila, S., Behare, P., Arora, S., & Sabikhi, L. (2019). Influence of unit operations on immunoglobulins and thermal stability of colostrum fractions. *International Dairy Journal*, *93*, 85-91.

Boss, E. A., Maciel Filho, R., & de Toledo, E. C. V. (2004). Freeze drying process: real time model and optimization. *Chemical Engineering and Processing: Process Intensification*, *43*(12), 1475-1485.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254.

Brisson, G., Britten, M., & Pouliot, Y. (2007). Heat-induced aggregation of bovine lactoferrin at neutral pH: Effect of iron saturation. *International Dairy Journal*, *17*(6), 617-624.

Bullen, J., Rogers, H. J., & Leigh, L. (1972). Iron-binding proteins in milk and resistance to Escherichia coli infection in infants. *Br med J*, *1*(5792), 69-75.

Bush, L., & Staley, T. (1980). Absorption of colostral immunoglobulins in newborn calves. *Journal of Dairy Science*, *63*(4), 672-680.

Cal, K., & Sollohub, K. (2010). Spray drying technique. I: Hardware and process parameters. *Journal of Pharmaceutical Sciences*, *99*(2), 575-586.

Çalışkan Koç, G., Tekgül, Y., Yüksel, A. N., Khanashyam, A. C., Kothakota, A., & Pandiselvam, R. (2022). Recent development in foam-mat drying process: Influence of foaming agents and foam properties on powder properties. *Journal of Surfactants and Detergents*, *25*(5), 539-557.

Caric, M., & Kalab, M. (1987). Effects of drying techniques on milk powders quality and microstructure: A review. *Food Structure*, *6*(2), 9.

Ceniti, C., Costanzo, N., Morittu, V. M., Tilocca, B., Roncada, P., & Britti, D. (2023). Colostrum as an emerging food: Nutraceutical properties and food supplement. *Food Reviews International*, *39*(7), 4636-4664.

Chandwe, K., & Kelly, P. (2021). Colostrum therapy for human gastrointestinal health and disease. *Nutrients*, *13*(6), 1956.

Chiang, S.-H., & Chang, C.-Y. (2005). Antioxidant properties of caseins and whey proteins from colostrums. *Journal of food and drug analysis*, *13*(1), 6.

Ciurzynska, A., & Lenart, A. (2011). Freeze-drying-application in food processing and biotechnology-a review. *Polish Journal of Food and Nutrition Sciences*, *61*(3).

Copestake, D. E., Indyk, H. E., & Otter, D. E. (2006). Affinity liquid chromatography method for the quantification of immunoglobulin G in bovine colostrum powders. *Journal of AOAC International*, *89*(5), 1249-1256.

Costa, A., Sneddon, N., Goi, A., Visentin, G., Mammi, L., Savarino, E., Zingone, F., Formigoni, A., Penasa, M., & De Marchi, M. (2023). Invited review: Bovine colostrum, a promising ingredient for humans and animals—Properties, processing technologies, and uses. *Journal of Dairy Science*, *106*(8), 5197-5217.

Damodaran, S., Parkin, K. L., & Fennema, O. R. (2007). *Fennema's food chemistry*. CRC press.

Delshadian, Z., Mortazavian, A. M., Tabarzad, M., Hosseini, S. M., Mohammadi, R., Rouhi, M., & Khosravi‐Darani, K. (2018). Optimisation of experimental conditions for binding of divalent iron to bioactive casein phosphopeptides. *International Journal of Food Science & Technology, 53*(3), 784-793.

de Oliveira, A. H., Mata, M. E. R. C., Fortes, M., Duarte, M. E. M., Pasquali, M., & Lisboa, H. M. (2021). Influence of spray drying conditions on the properties of whole goat milk. *Drying Technology*, *39*(6), 726-737.

DePeters, E., & Cant, J. (1992). Nutritional factors influencing the nitrogen composition of bovine milk: a review. *Journal of Dairy Science*, *75*(8), 2043-2070.

Devahastin, S., & Jinorose, M. (2020). A concise history of drying. *Drying Technologies for Biotechnology and Pharmaceutical Applications*, 9-21.

Duran, R., Deschler, C., Precigou, S., & Goulas, P. (2002). Degradation of chlorophenols by Phanerochaete chrysosporium: effect of 3, 4-dichlorophenol on extracellular peroxidase activities. *Applied microbiology and biotechnology*, *59*, 284-288.

Egerton, T. A., & Mattinson, J. A. (2007). Comparison of photooxidation and photoreduction reactions on TiO2 nanoparticles. *Journal of Photochemistry and Photobiology A: Chemistry*, *186*(2-3), 115-120.

Elfstrand, L., Lindmark-Månsson, H., Paulsson, M., Nyberg, L., & Åkesson, B. (2002). Immunoglobulins, growth factors and growth hormone in bovine colostrum and the effects of processing. *International Dairy Journal*, *12*(11), 879-887.

Ellison, R., 3rd, & Giehl, T. J. (1991). Killing of gram-negative bacteria by lactoferrin and lysozyme. *The Journal of clinical investigation*, *88*(4), 1080-1091.

Fang, Y., Rogers, S., Selomulya, C., & Chen, X. D. (2012). Functionality of milk protein concentrate: Effect of spray drying temperature. *Biochemical Engineering Journal*, *62*, 101-105.

Farnaud, S., & Evans, R. W. (2003). Lactoferrin—a multifunctional protein with antimicrobial properties. *Molecular immunology*, *40*(7), 395-405.

Fatima, M., Sajid, M., Saima, H., Nadeem, M. T., Ahmad, R. S., Mehmood, S., Zubair Khalid, M., Ali Alharbi, S., Ansari, M. J., & Kasongo, E. L. M. (2024). In vitro evaluation of spray and freeze dried bovine colostrum powder and their effects on the nutritional and functional properties. *International Journal of Food Properties*, *27*(1), 985-1002.

Felfoul, I., Burgain, J., Perroud, C., Gaiani, C., Scher, J., Attia, H., & Petit, J. (2022). Impact of spray drying conditions on physicochemical properties and rehydration ability of skim dromedary and cow's milk powders. *Drying Technology*, *40*(3), 665-677.

Foroutan, A., Guo, A. C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., Badran, H., Budinski, Z., Mandal, R., & Ametaj, B. N. (2019). Chemical composition of commercial cow's milk. *Journal of agricultural and food chemistry*, *67*(17), 4897-4914.

Franks, F. (1998). Freeze-drying of bioproducts: putting principles into practice. *European journal of pharmaceutics and biopharmaceutics*, *45*(3), 221-229.

Fujihara, T., & Hayashi, K. (1995). Lactoferrin inhibits herpes simplex virus type-1 (HSV-1) infection to mouse cornea. *Archives of Virology*, *140*, 1469-1472.

Gallagher, W. (2009). FTIR analysis of protein structure. *Course manual Chem*, *455*.

García-Carrasco, M., Valdez-Baro, O., Cabanillas-Bojórquez, L. A., Bernal-Millán, M. J., Rivera-Salas, M. M., Gutiérrez-Grijalva, E. P., & Heredia, J. B. (2023). Potential agricultural uses of micro/nano encapsulated chitosan: a review. *Macromol*, *3*(3), 614-635.

García-Montoya, I. A., Cendón, T. S., Arévalo-Gallegos, S., & Rascón-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: an overview. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1820*(3), 226-236.

Garimano, N., Vergara, L. D., Kim, A., Badin, E. E., Sodero, S., Bernal, A. M., Gonzalez, D., Amaral, M. M., Lespinard, A. R., & Porporatto, C. (2021). Preservation of protective capacity of hyperimmune anti-Stx2 bovine colostrum against enterohemorrhagic Escherichia coli O157: H7 pathogenicity after pasteurization and spray drying processes. *Journal of Dairy Science*, *104*(5), 5229-5238.

GEA. (2024). Analytical Methods for Dry Milk Products.

Giansanti, F., Rossi, P., Massucci, M. T., Botti, D., Antonini, G., Valenti, P., & Seganti, L. (2002). Antiviral activity of ovotransferrin discloses an evolutionary strategy for the defensive activities of lactoferrin. *Biochemistry and Cell Biology*, *80*(1), 125-130.

Godden, S., Smith, S., Feirtag, J., Green, L., Wells, S., & Fetrow, J. (2003). Effect of on-farm commercial batch pasteurization of colostrum on colostrum and serum immunoglobulin concentrations in dairy calves. *Journal of Dairy Science*, *86*(4), 1503-1512.

Goldoni, P., Sinibaldi, L., Valenti, P., & Orsi, N. (2000). Metal complexes of lactoferrin and their effect on the intracellular multiplication of Legionella pneumophila. *Biometals*, *13*(1), 15-22.

Gomori, G. (1955). [16] Preparation of buffers for use in enzyme studies.

González-Chávez, S. A., Arévalo-Gallegos, S., & Rascón-Cruz, Q. (2009). Lactoferrin: structure, function and applications. *International journal of antimicrobial agents*, *33*(4), 301. e301-301. e308.

Goula, A. M., Adamopoulos, K. G., & Kazakis, N. A. (2004). Influence of spray drying conditions on tomato powder properties. *Drying Technology*, *22*(5), 1129-1151.

Groves, M. L. (1960). The isolation of a red protein from Milk2. *Journal of the American Chemical Society*, *82*(13), 3345-3350.

Gu, W., Heil, P. E., Choi, H., & Kim, K. (2007). Comprehensive model for fine Coulomb fission of liquid droplets charged to Rayleigh limit. *Applied physics letters*, *91*(6).

Guberti, M., Botti, S., Capuzzo, M. T., Nardozi, S., Fusco, A., Cera, A., Dugo, L., Piredda, M., & De Marinis, M. G. (2021). Bovine colostrum applications in sick and healthy people: a systematic review. *Nutrients*, *13*(7), 2194.

Hao, L., Shan, Q., Wei, J., Ma, F., & Sun, P. (2019). Lactoferrin: major physiological functions and applications. *Current Protein and Peptide Science*, *20*(2), 139-144.

Hardy, Z., & Jideani, V. (2017). Foam-mat drying technology: A review. *Critical Reviews in Food Science and Nutrition*, *57*(12), 2560-2572.

Harper, J., & Tappel, A. (1957). Freeze-drying of food products. *Advances in Food Research*, *7*, 171-234.

Haug, A., Høstmark, A. T., & Harstad, O. M. (2007). Bovine milk in human nutrition–a review. *Lipids in health and disease*, *6*, 1-16.

Hendsch, Z. S., & Tidor, B. (1994). Do salt bridges stabilize proteins? A continuum electrostatic analysis. *Protein Science*, *3*(2), 211-226.

Huntington, D. H. (2004). The influence of the spray drying process on product properties. *Drying Technology*, *22*(6), 1261-1287.

Ishwarya, S. P., Anandharamakrishnan, C., & Stapley, A. G. (2015). Spray-freeze-drying: A novel process for the drying of foods and bioproducts. *Trends in Food Science & Technology*, *41*(2), 161-181.

Jahani, S., Shakiba, A., & Jahani, L. (2015). The Antimicrobial effect of lactoferrin on Gram-negative and Gram-positive bacteria. *International Journal of Infection*, *2*(3).

Jenssen, H., & Hancock, R. E. (2009). Antimicrobial properties of lactoferrin. *Biochimie*, *91*(1), 19-29.

Kaplan, M., Arslan, A., Duman, H., Karyelioğlu, M., Baydemir, B., Günar, B. B., Alkan, M., Bayraktar, A., Tosun, H. İ., & Ertürk, M. (2022). Production of bovine colostrum for human consumption to improve health. *Frontiers in Pharmacology*, *12*, 796824.

Kasper, J. C., & Friess, W. (2011). The freezing step in lyophilization: Physicochemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals. *European journal of pharmaceutics and biopharmaceutics*, *78*(2), 248-263.

Kawakami, H., Tanaka, M., Tatsumi, K., & Dosako, S. I. (1992). Effects of ionic strength and pH on the thermostability of lactoferrin. *International Dairy Journal*, *2*(5), 287-298.

Kehoe, S., Jayarao, B., & Heinrichs, A. (2007). A survey of bovine colostrum composition and colostrum management practices on Pennsylvania dairy farms. *Journal of Dairy Science*, *90*(9), 4108-4116.

Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R., Wyckoff, H., & Phillips, D. C. (1958). A three-dimensional model of the myoglobin molecule obtained by x-ray analysis. *Nature*, *181*(4610), 662-666.

Keshani, S., Daud, W. R. W., Nourouzi, M., Namvar, F., & Ghasemi, M. (2015). Spray drying: An overview on wall deposition, process and modeling. *Journal of Food Engineering*, *146*, 152-162.

Kibangou, I. B., Bouhallab, S., Henry, G., Bureau, F., Allouche, S., Blais, A., & Bouglé, D. L. (2005). Milk proteins and iron absorption: contrasting effects of different caseinophosphopeptides. *Pediatric research*, *58*(4), 731-734.

King Dean, C. J. (1987). Spray Drying Handbook, Fourth Edition By K. Masters, Halstead Press, New York, 1985, 696 pp. *AIChE Journal*, *33*(1), 172-173.

Kong, J., & Yu, S. (2007). Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta biochimica et biophysica Sinica*, *39*(8), 549-559.

Kowalski, S. J. (2012). *Thermomechanics of drying processes* (Vol. 8). Springer Science & Business Media.

Król, J., Brodziak, A., Litwińczuk, Z., & Barlowska, J. (2012). Selected factors determining the content of lactoferrin, lysozyme and immunoglobulins G in bovine milk. *A search for antibacterial agents. InTech*, 107-124.

Krolitzki, E., Schwaminger, S. P., Pagel, M., Ostertag, F., Hinrichs, J., & Berensmeier, S. (2022). Current practices with commercial scale bovine lactoferrin production and alternative approaches. *International Dairy Journal*, *126*, 105263.

Kudra, T., & Ratti, C. (2006). Foam-mat drying: Energy and cost analyses. *Canadian Biosystems Engineering*, *48*, 3.

Launder, B. E. (1978). Heat and mass transport. In P. Bradshaw (Ed.), *Turbulence* (pp. 231-287). Springer Berlin Heidelberg.

Le, T. T., Deeth, H. C., & Larsen, L. B. (2017). Proteomics of major bovine milk proteins: Novel insights. *International Dairy Journal*, *67*, 2-15.

Legrand, D. (2016). Overview of lactoferrin as a natural immune modulator. *The Journal of pediatrics*, *173*, S10-S15.

Legrand, D., Elass, E., Carpentier, M., & Mazurier, J. (2005). Lactoferrin: Lactoferrin: a modulator of immune and inflammatory responses. *Cellular and Molecular Life Sciences*, *62*, 2549-2559.

Levay, P. F., & Viljoen, M. (1995). Lactoferrin: a general review. *Haematologica*, *80*(3), 252-267.

Li, Q., Hu, W., Zhao, J., Wang, J., Dai, Y., Zhao, Y., Meng, Q., & Li, N. (2014). Supplementation transgenic cow's milk containing recombinant human lactoferrin enhances systematic and intestinal immune responses in piglets.*Molecular biology reports*, *41*, 2119-2128.

Li, W., Liu, B., Lin, Y., Xue, P., Lu, Y., Song, S., Li, Y., Szeto, I. M.-Y., Ren, F., & Guo, H. (2024a). The application of lactoferrin in infant formula: The past, present and future. *Critical Reviews in Food Science and Nutrition*, *64*(17), 5748-5767.

Li, X., Al-Asady, R. B., Sanders, C., & Salman, A. D. (2024b). Comparing different single milk droplet drying process with spray drying process. *Chemical Engineering Research and Design*, *205*, 47-56.

Liapis, A. I., & Bruttini, R. (2020). Freeze drying. In *Handbook of industrial drying* (pp. 309-343). CRC press.

Lopez, A., & Heinrichs, A. (2022). Invited review: The importance of colostrum in the newborn dairy calf. *Journal of Dairy Science*, *105*(4), 2733-2749.

MAINER, G., DOMÍNGUEZ, E., RANDRUP, M., SÁNCHEZ, L., & CALVO, M. (1999). Effect of heat treatment on anti-rotavirus activity of bovine colostrum. *Journal of dairy research*, *66*(1), 131-137.

Makino, Y., & Nishimura, S. (1992). High-performance liquid chromatographic separation of human apolactoferrin and monoferric and diferric lactoferrins. *Journal of Chromatography B: Biomedical Sciences and Applications*, *579*(2), 346-349.

Malumba, P., Vanderghem, C., Deroanne, C., & Béra, F. (2008). Influence of drying temperature on the solubility, the purity of isolates and the electrophoretic patterns of corn proteins. *Food Chemistry*, *111*(3), 564-572.

Marchetti, M., Longhi, C., Conte, M. P., Pisani, S., Valenti, P., & Seganti, L. (1996). Lactoferrin inhibits herpes simplex virus type 1 adsorption to Vero cells. *Antiviral research*, *29*(2-3), 221-231.

Marchetti, M., Superti, F., Ammendolia, M. G., Rossi, P., Valenti, P., & Seganti, L. (1999). Inhibition of poliovirus type 1 infection by iron-, manganese-and zinc-saturated lactoferrin. *Medical microbiology and immunology*, *187*, 199-204.

Masum, A., Saxena, J., & Zisu, B. (2022). Electrostatic spray drying of high oil load emulsions, milk and heat sensitive biomaterials. In *Food Engineering Innovations Across the Food Supply Chain* (pp. 237-246). Elsevier.

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*(10), 3964-3970.

Matuschek, E., Brown, D. F., & Kahlmeter, G. (2014). Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical microbiology and infection*, *20*(4), O255-O266.

Maury, M., Murphy, K., Kumar, S., Shi, L., & Lee, G. (2005). Effects of process variables on the powder yield of spray dried trehalose on a laboratory spray-dryer. *European journal of pharmaceutics and biopharmaceutics*, *59*(3), 565-573.

McGrath, B. A., Fox, P. F., McSweeney, P. L., & Kelly, A. L. (2016). Composition and properties of bovine colostrum: a review. *Dairy Science & Technology*, *96*, 133-158.

Mohamed, W. A., & Schaalan, M. F. (2018). Antidiabetic efficacy of lactoferrin in type 2 diabetic pediatrics; controlling impact on PPAR-γ, SIRT-1, and TLR4 downstream signaling pathway. *Diabetology & metabolic syndrome*, *10*, 1-12.

Moore, S. A., Anderson, B. F., Groom, C. R., Haridas, M., & Baker, E. N. (1997). Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *Journal of molecular biology*, *274*(2), 222-236.

Morel, J., Zain, S. N. M., & Archer, R. (2022). Comparison of drying techniques for bovine lactoferrin: Iron binding and antimicrobial properties of dried lactoferrin. *International Dairy Journal*, *124*, 105142.

Mulder, A. M., Connellan, P. A., Oliver, C. J., Morris, C. A., & Stevenson, L. M. (2008). Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males. *Nutrition Research*, *28*(9), 583-589.

Mutukuri, T. T., Maa, Y.-F., Gikanga, B., Sakhnovsky, R., & Zhou, Q. T. (2021). Electrostatic spray drying for monoclonal antibody formulation. *International Journal of Pharmaceutics*, *607*, 120942. https://doi.org/https://doi.org/10.1016/j.ijpharm.2021.120942

Nagy, D. W. (2009). Resuscitation and critical care of neonatal calves. *Veterinary Clinics of North America: Food Animal Practice*, *25*(1), 1-11.

Nail, S. L., Jiang, S., Chongprasert, S., & Knopp, S. A. (2002). Fundamentals of freeze-drying. *Development and manufacture of protein pharmaceuticals*, 281-360.

Nguyen, D. N., Clasen, C., & Van den Mooter, G. (2016). Pharmaceutical applications of electrospraying. *Journal of Pharmaceutical Sciences*, *105*(9), 2601-2620.

Niaz, B., Saeed, F., Ahmed, A., Imran, M., Maan, A. A., Khan, M. K. I., Tufail, T., Anjum, F. M., Hussain, S., & Suleria, H. A. R. (2019). Lactoferrin (LF): A natural antimicrobial protein. *International Journal of Food Properties*, *22*(1), 1626-1641.

Nugroho, R. W. N., Outinen, M., Toikkanen, O., & Rojas, O. J. (2021). Particle size and fat encapsulation define the colloidal dispersibility and reconstitution of growing-up milk powder. *Powder Technology*, *391*, 133-141.

O'Leary, J., & Busta, F. (1974). Effect of food components on growth of Bacillus stearothermophilus. *Journal of Food Science*, *39*(6), 1157-1160.

O'Sullivan, J. J., Norwood, E.-A., O'Mahony, J. A., & Kelly, A. L. (2019). Atomisation technologies used in spray drying in the dairy industry: A review. *Journal of Food Engineering*, *243*, 57-69.

Pakkanen, R., & Aalto, J. (1997). Growth factors and antimicrobial factors of bovine colostrum. *International Dairy Journal*, *7*(5), 285-297.

Patel, R., Patel, M., & Suthar, A. (2009). Spray drying technology: an overview. *Indian Journal of Science and Technology*, *2*(10), 44-47.

Pelton, J. T., & McLean, L. R. (2000). Spectroscopic methods for analysis of protein secondary structure. *Analytical biochemistry*, *277*(2), 167-176.

Perré, P., & May, B. (2007). The existence of a first drying stage for potato proved by two independent methods. *Journal of Food Engineering*, *78*(4), 1134-1140.

Petschow, B. W., Talbott, R. D., & Batema, R. P. (1999). Ability of lactoferrin to promote the growth of Bifidobacterium spp. in vitro is independent of receptor binding capacity and iron saturation level. *Journal of medical microbiology*, *48*(6), 541-549.

Pitts, J., Schuck, E., & Wan, J. (1964). Photoreduction of 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) in hydrocarbons. *Journal of the American Chemical Society*, *86*(2), 296-297.

Pizarro, F., Yip, R., Dallman, P. R., Olivares, M., Hertrampf, E., & Walter, T. (1991). Iron status with different infant feeding regimens: relevance to screening and prevention of iron deficiency. *The Journal of pediatrics*, *118*(5), 687-692.

Playford, R. J., Macdonald, C. E., Calnan, D. P., Floyd, D. N., Podas, T., Johnson, W., Wicks, A. C., Bashir, O., & Marchbank, T. (2001). Co-administration of the health food supplement, bovine colostrum, reduces the acute non-steroidal anti-inflammatory drug-induced increase in intestinal permeability. *Clinical Science*, *100*(6), 627-633.

Playford, R. J., & Weiser, M. J. (2021). Bovine colostrum: Its constituents and uses. *Nutrients*, *13*(1), 265.

Puppel, K., Gołębiewski, M., Grodkowski, G., Slósarz, J., Kunowska-Slósarz, M., Solarczyk, P., Łukasiewicz, M., Balcerak, M., & Przysucha, T. (2019). Composition and factors affecting quality of bovine colostrum: A review. *Animals*, *9*(12), 1070.

Qureshi, T. M., Yaseen, M., Nadeem, M., Murtaza, M. A., & Munir, M. (2020). Physico–chemical composition and antioxidant potential of buffalo colostrum, transition milk, and mature milk. *Journal of Food Processing and Preservation*, *44*(10), e14763.

Rathe, M., Müller, K., Sangild, P. T., & Husby, S. (2014). Clinical applications of bovine colostrum therapy: a systematic review. *Nutrition reviews*, *72*(4), 237-254.

Ratti, C. (2012). Freeze-Drying Process Design. *Handbook of food process design*, 621-647.

Ratti, C., & Kudra, T. (2006). Drying of foamed biological materials: opportunities and challenges. *Drying Technology*, *24*(9), 1101-1108.

Reh, C., Bhat, S. N., & Berrut, S. (2004). Determination of water content in powdered milk. *Food Chemistry, 86*(3), 457-464.

Rivera, A., Viñado, B., Benito, N., Docobo-Pérez, F., Fernández-Cuenca, F., Fernández-Domínguez, J., Guinea, J., López-Navas, A., Moreno, M. Á., & Larrosa, M. N. (2023). Recommendations of the Spanish Antibiogram Committee (COESANT) for in vitro susceptibility testing of antimicrobial agents by disk diffusion. Enfermedades Infecciosas y Microbiología Clínica, 41(9), 571-576.

Rodrigues, L., Paiva, G., Lisboa, H. M., Pasquali, M., Gusmão, R., Duarte, M. E., Cavalcanti-Mata, M. E., & Abrantes, T. (2020). Impact of spray drying parameters on lactose-free milk powder properties and composition. *J. Agric. Stud*, *8*(3), 32.

Rodríguez-Franco, D. A., Vázquez-Moreno, L., & Montfort, G. R.-C. (2005). Antimicrobial mechanisms and potential clinical applications of lactoferrin. *Revista latinoamericana de microbiologia*, *47*(3-4), 102-111.

Roseanu, A., Florian, P., Condei, M., Cristea, D., & Damian, M. (2010). Antibacterial activity of lactoferrin and lactoferricin against oral streptococci. *Romanian Biotechnological Letters*, *15*(6), 5788-5792.

Sadikoglu, H., & Liapis, A. I. (1997). Mathematical modelling of the primary and secondary drying stages of bulk solution freeze-drying in trays: Parameter estimation and model discrimination by comparison of theoretical results with experimental data. *Drying Technology*, *15*(3-4), 791-810.

Salama, A. H. (2020). Spray drying as an advantageous strategy for enhancing pharmaceuticals bioavailability. *Drug Delivery and Translational Research*, *10*(1), 1-12.

Samantha, S. C., Bruna, A. S. M., Adriana, R. M., Fabio, B., Sandro, A. R., & Aline, R. C. A. (2015). Drying by spray drying in the food industry: Micro-encapsulation, process parameters and main carriers used. *African Journal of Food Science*, *9*(9), 462-470.

Sánchez, L., Calvo, M., & Brock, J. H. (1992a). Biological role of lactoferrin. *Archives of disease in childhood*, *67*(5), 657.

Sánchez, L., Peiró, J., Castillo, H., Perez, M., Ena, J., & Calvo, M. (1992b). Kinetic parameters for denaturation of bovine milk lactoferrin. *Journal of Food Science*, *57*(4), 873-879.

Schanbacher, F., Goodman, R., & Talhouk, R. (1993). Bovine mammary lactoferrin: implications from messenger ribonucleic acid (mRNA) sequence and regulation contrary to other milk proteins. *Journal of Dairy Science*, *76*(12), 3812-3831.

Schuck, P. (2002). Spray drying of dairy products: state of the art. *Le Lait*, *82*(4), 375-382.

Schuck, P., Jeantet, R., Bhandari, B., Chen, X. D., Perrone, Í. T., de Carvalho, A. F., Fenelon, M., & Kelly, P. (2016). Recent advances in spray drying relevant to the dairy industry: A comprehensive critical review. *Drying Technology*, *34*(15), 1773-1790.

Schulz, G. E., Schirmer, R. H., Schulz, G. E., & Schirmer, R. H. (1979). Patterns of folding and association of polypeptide chains. *Principles of Protein Structure*, 66-107.

Sharma, S., Sinha, M., Kaushik, S., Kaur, P., & Singh, T. P. (2013). C-Lobe of Lactoferrin: The Whole Story of the Half-Molecule. *Biochemistry Research International*, *2013*(1), 271641.

Sherman, M. P., Bennett, S. H., Hwang, F. F., & Yu, C. (2004). Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and Lactobacillus GG. *Biometals*, *17*, 285-289.

Siciliano, R., Rega, B., Marchetti, M., Seganti, L., Antonini, G., & Valenti, P. (1999). Bovine lactoferrin peptidic fragments involved in inhibition of herpes simplex virus type 1 infection. *Biochemical and Biophysical Research Communications*, *264*(1), 19-23.

Singh, A., Pandita, S., Vaidya, M., Singh, S., Chandra, G., Pampoori, Z., Huozha, R., Pathan, M., Kushwaha, R., & Sharma, V. (2011). Bovine colostrum and neonate immunity-a review. *Agricultural Reviews*, *32*(2), 79-90.

Singh, R. P. and D. R. Heldman (2014). Chapter 12 - Dehydration. Introduction to Food Engineering (Fifth Edition). R. P. Singh and D. R. Heldman. San Diego, Academic Press: 675-710.

Sorensen, M., & Sorensen, S. (1939). The proteins in whey.

Sosnik, A., & Seremeta, K. P. (2015). Advantages and challenges of the spray drying technology for the production of pure drug particles and drug-loaded polymeric carriers. *Advances in colloid and interface science*, *223*, 40-54.

Stähle, P., Gaukel, V., & Schuchmann, H. P. (2015). Investigation on the applicability of the effervescent atomizer in spray drying of foods: Influence of liquid viscosity on nozzle internal two-phase flow and spray characteristics. *Journal of Food Process Engineering*, *38*(5), 474-487.

Stanciuc, N., Aprodu, I., Râpeanu, G., van der Plancken, I., Bahrim, G., & Hendrickx, M. (2013). Analysis of the thermally induced structural changes of bovine lactoferrin. *Journal of agricultural and food chemistry*, *61*(9), 2234-2243.

Steijns, J. M., & Van Hooijdonk, A. (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition*, *84*(S1), 11-17.

Stelwagen, K., Carpenter, E., Haigh, B., Hodgkinson, A., & Wheeler, T. (2009). Immune components of bovine colostrum and milk. *Journal of animal science*, *87*(13suppl), 3-9.

Stobiecka, M., Król, J., & Brodziak, A. (2022). Antioxidant activity of milk and dairy products. *Animals*, *12*(3), 245.

Stott, G., Marx, D., Menefee, B., & Nightengale, G. (1979). Colostral immunoglobulin transfer in calves I. Period of absorption. *Journal of Dairy Science*, *62*(10), 1632-1638.

Thakkar, S., & Misra, M. (2020). Electrospray drying of docetaxel nanosuspension: A study on particle formation and evaluation of nanocrystals thereof. *Journal of Drug Delivery Science and Technology*, *60*, 102009.

Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S., & Hayasawa, H. (2002). Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochemistry and Cell Biology*, *80*(1), 109-112.

Toro-Sierra, J., Schumann, J., & Kulozik, U. (2013). Impact of spray drying conditions on the particle size of microparticulated whey protein fractions. *Dairy Science & Technology*, *93*(4), 487-503.

Umar, M., Ruktanonchai, U. R., Makararpong, D., & Anal, A. K. (2023). Compositional and functional analysis of freeze dried bovine skim colostrum powders. *Journal of Food Measurement and Characterization*, *17*(5), 4294-4304.

Valentina, V., Pratiwi, R. A., Hsiao, P., Tseng, H., Hsieh, J., & Chen, C. (2016). Sensorial characterization of foods before and after freeze-drying. *Sensorial Characterization of Foods Before and After Freeze-drying*, *1*(6), 1-5.

Van der Strate, B., Beljaars, L., Molema, G., Harmsen, M., & Meijer, D. (2001). Antiviral activities of lactoferrin. *Antiviral research*, *52*(3), 225-239.

Vogel, H. J. (2012). Lactoferrin, a bird's eye view. *Biochemistry and Cell Biology*, *90*(3), 233-244.

Wakabayashi, H., Yamauchi, K., & Takase, M. (2006). Lactoferrin research, technology and applications. *International Dairy Journal*, *16*(11), 1241-1251.

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*(5), 606-615.

Wang, B., Timilsena, Y. P., Blanch, E., & Adhikari, B. (2019). Lactoferrin: Structure, function, denaturation and digestion. *Critical Reviews in Food Science and Nutrition*, *59*(4), 580-596.

Wang, X., Hirmo, S., Willen, R., & Wadstrom, T. (2001). Inhibition of Helicobacter pylori infection by bovine milk glycoconjugates in a BAlb/cA mouse model. *Journal of medical microbiology*, *50*(5), 430-435.

Wang, Z., Finlay, W., Peppler, M., & Sweeney, L. (2006). Powder formation by atmospheric spray-freeze-drying. *Powder Technology*, *170*(1), 45-52.

Ward, P., Paz, E., & Conneely, O. (2005). Lactoferrin: Multifunctional roles of lactoferrin: A critical overview. *Cellular and Molecular Life Sciences*, *62*, 2540-2548.

Wąsowska, E., & Puppel, K. (2018). Changes in the content of immunostimulating components of colostrum obtained from dairy cows at different levels of production. *Journal of the Science of Food and Agriculture*, *98*(13), 5062-5068.

Wu, W. D., Liu, W., Gengenbach, T., Woo, M. W., Selomulya, C., Chen, X. D., & Weeks, M. (2014). Towards spray drying of high solids dairy liquid: Effects of feed solid content on particle structure and functionality. *Journal of Food Engineering*, *123*, 130-135.

Yamauchi, K., Wakabayashi, H., Shin, K., & Takase, M. (2006). Bovine lactoferrin: benefits and mechanism of action against infections. *Biochemistry and Cell Biology*, *84*(3), 291-296.

Yegorov, D. Y., Kozlov, A. V., Azizova, O. A., & Vladimirov, Y. A. (1993). Simultaneous determination of Fe (III) and Fe (II) in water solutions and tissue homogenates using desferal and 1, 10-phenanthroline. *Free Radical Biology and Medicine, 15*(6), 565-574.

Yen, C.-C., Shen, C.-J., Hsu, W.-H., Chang, Y.-H., Lin, H.-T., Chen, H.-L., & Chen, C.-M. (2011). Lactoferrin: an iron-binding antimicrobial protein against Escherichia coli infection. *Biometals*, *24*, 585-594.

Zang, D., Tarafdar, S., Tarasevich, Y. Y., Choudhury, M. D., & Dutta, T. (2019). Evaporation of a Droplet: From physics to applications. Physics Reports, 804, 1-56.

Zarcula, S., Cernescu, H., Mircu, C., Tulcan, C., Morvay, A., Baul, S., & Popovici, D. (2010). Influence of breed, parity and food intake on chemical composition of first colostrum in cow. *Scientific Papers Animal Science and Biotechnologies*, *43*(1), 154-154.

Zhou, Z., & Langrish, T. (2021). A review of Maillard reactions in spray dryers. *Journal of Food Engineering*, *305*, 110615.

Appendices

Appendix 1-ELISA bovine IgG standard curve A, B

Bovine IgG ELISA standard curve was generated from bovine IgG standards supplied by the kit. Protein standards of eight different concentrations (0.69 to 500 ng/mL) were used and measured in triplicates on the 96-well plate. Non-linear standard curve was generated from software SigmaPlot (SigmaPlot 1.45, Grafiti LLC).

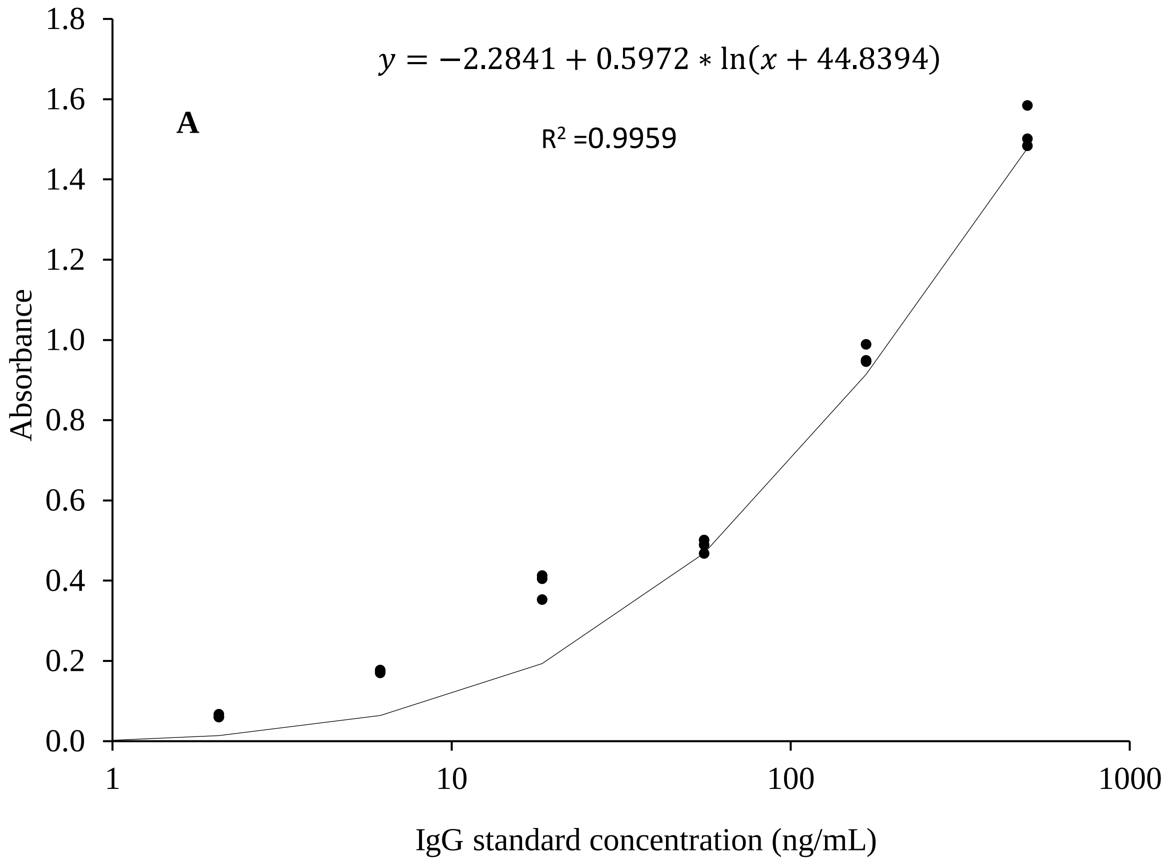

Figure A1. Bovine IgG ELISA standard curve A. Points plotted were individual data, and model was generated from means (n=3).

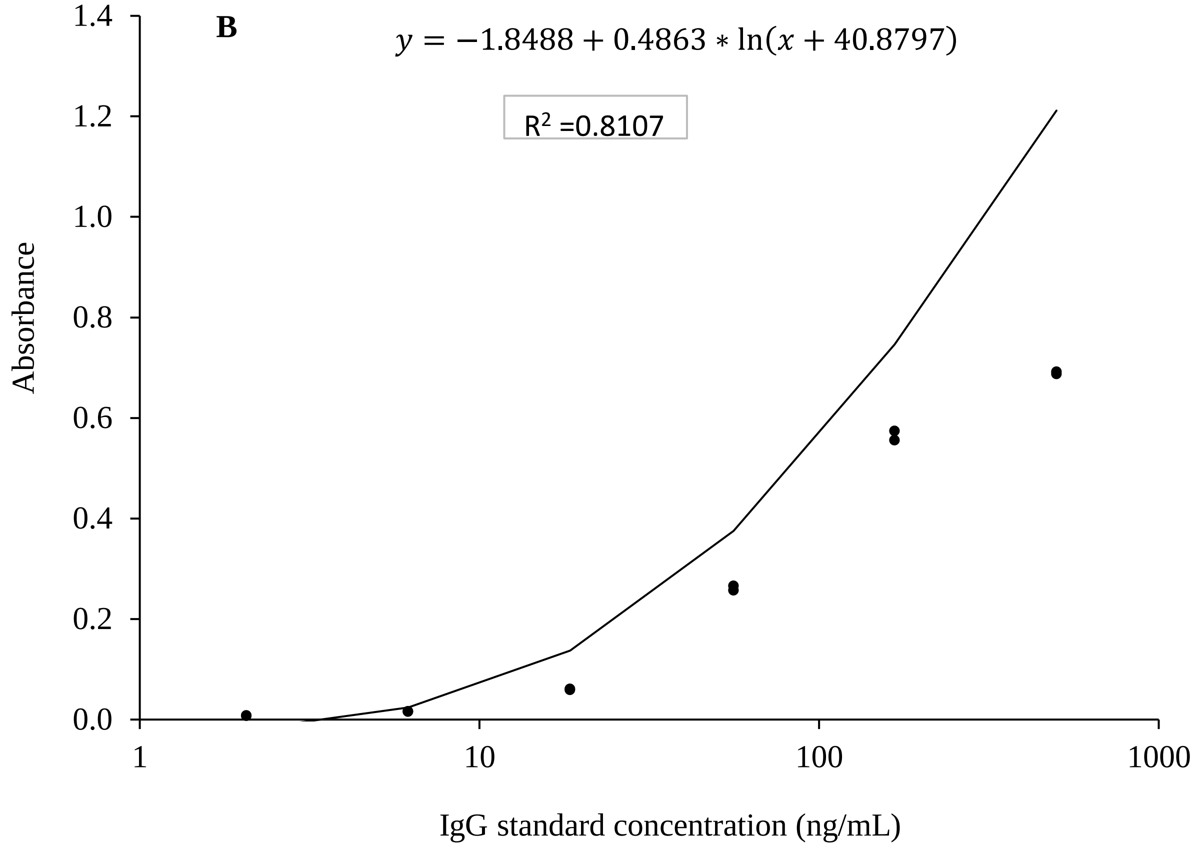

Figure A2. Bovine IgG ELISA standard curve B. Points plotted were individual data, and model was generated from means (n=2).

Appendix 2-ELISA bovine lactoferrin standard curve A, B

Bovine lactoferrin ELISA standard curve was generated from bovine lactoferrin standards supplied by the kit. Protein standards of eight different concentrations (0.69 to 500 ng/mL) were used and measured in triplicates on the plate. Non-linear standard curve was generated from software SigmaPlot (SigmaPlot 1.45, Grafiti LLC).

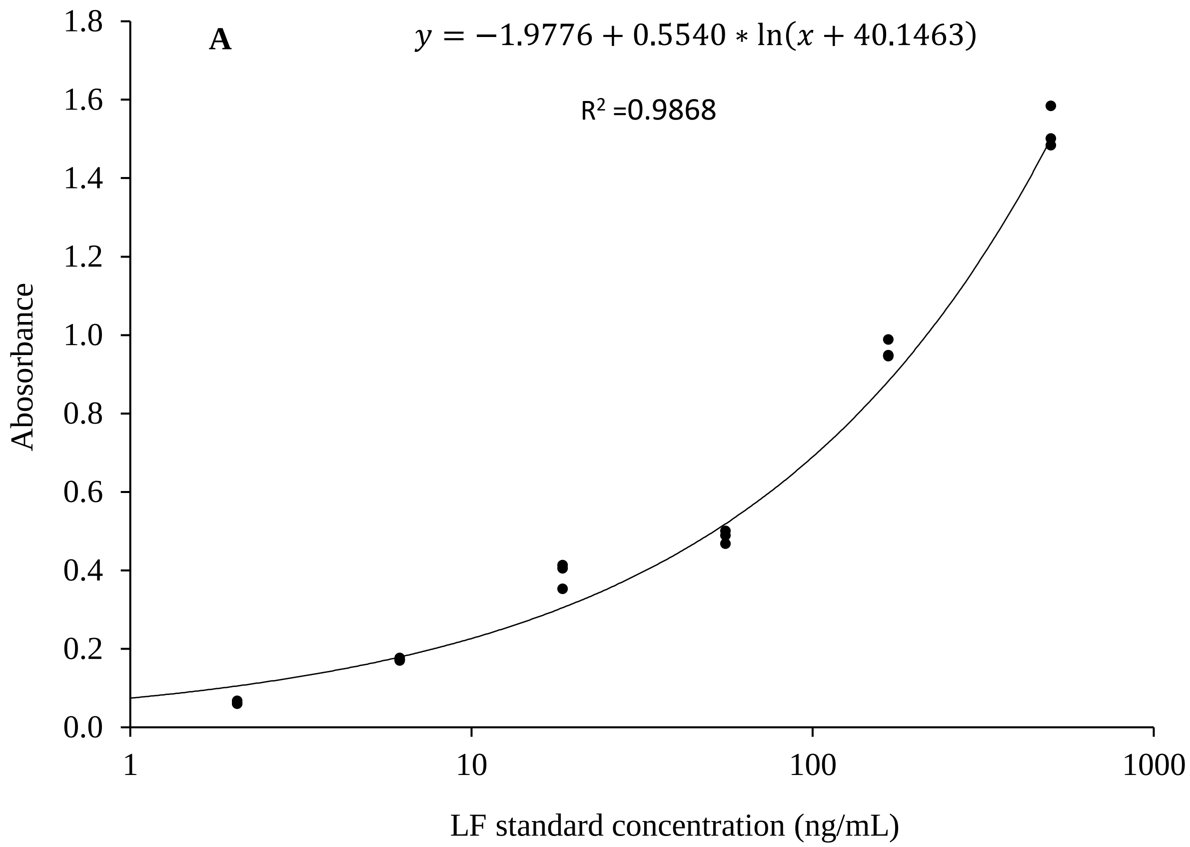

Figure A3. Bovine lactoferrin ELISA standard curve A. Points plotted were individual data, and model was generated from means (n=3).

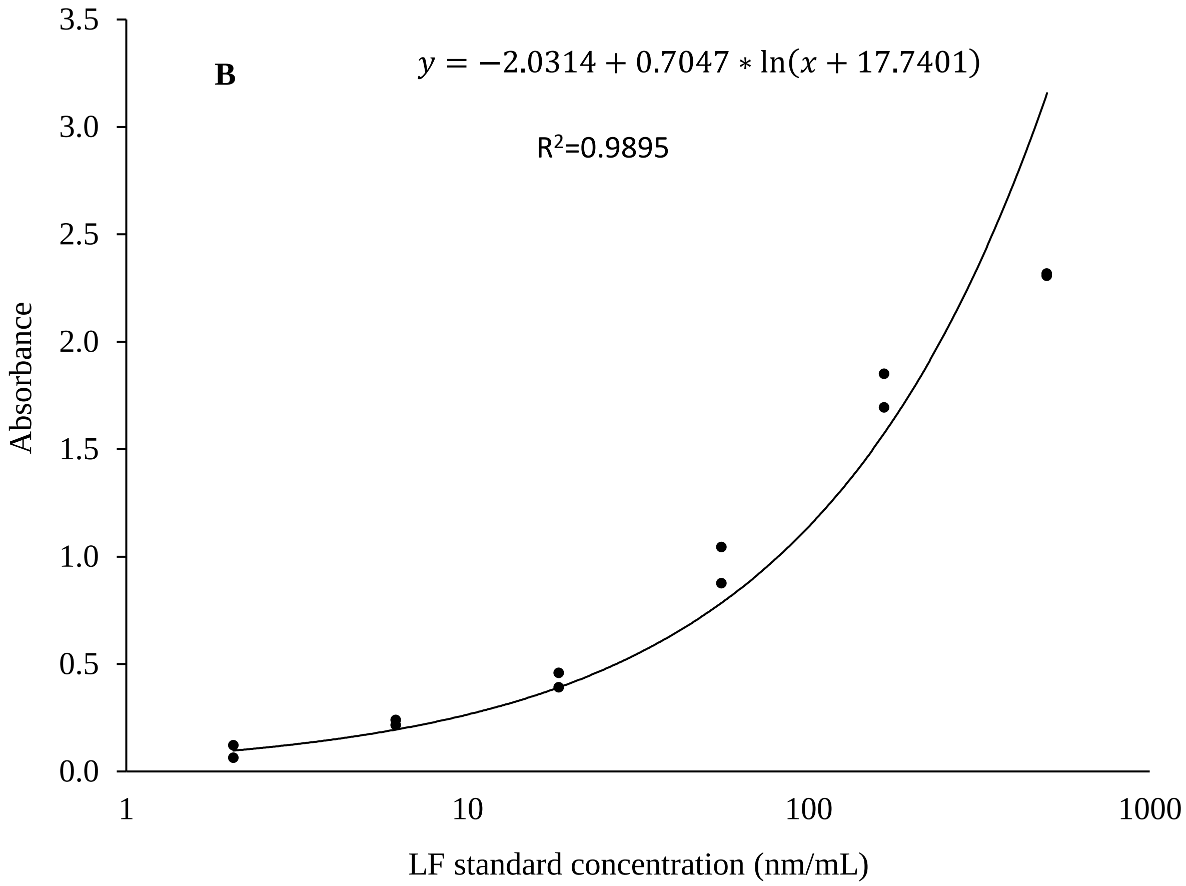

Figure A4. Bovine lactoferrin ELISA standard curve B. Points plotted were individual data, and model was generated from means (n=2).

Appendix 3-Bradford standard curve of lyophilized bovine lactoferrin in different concentrations

The standard curve was generated from eight different concentrations (0 to 1.5 mg/mL) of lyophilized bovine lactoferrin (≥ 85%, Sigma-Aldrich, USA).

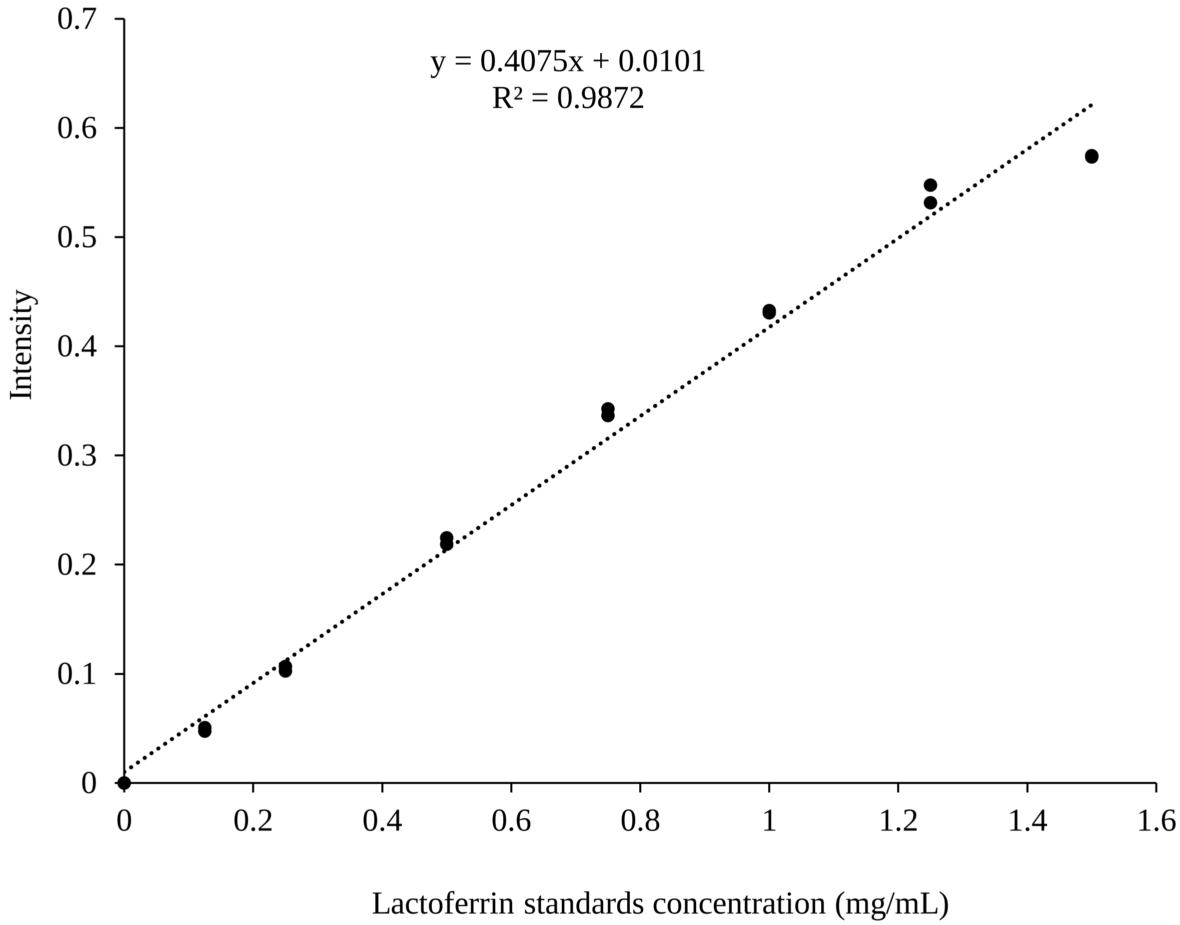

Figure A5. Bovine lyophilized bovine lactoferrin Bradford standard curve. Points plotted were individual data, and model was generated from means (n=2).

Appendix 4-Bradford results of colostrum and lactoferrin eluates protein concentration (mg/mL)

Table A1. Protein concentration (mg/mL) of colostrum and lactoferrin eluates collected from column.

Material	Dried Powder	Protein Concentration (mg/mL)
Colostrum	FCL	9.65 ± 0.21
	SCL165	10.78 ± 0.27
	SCL180	11.85 ± 0.06
	ESCL75	11.96 ± 0.12
	ESCL150	12.46 ± 0.17
Lactoferrin	FLF	30.86 ± 0.42
	SLF165	28.24 ± 0.75
	SLF180	28.68 ± 1.77
	ESLF75	27.94 ± 3.00
	ESLF150	29.88 ± 1.02

Appendix 5–MP-AES Fe concentration standard curve

The standard curve was generated from five different concentrations (0 to 0.8 mg/mL) of Fe standard (Agilent Technologies, DE, USA).

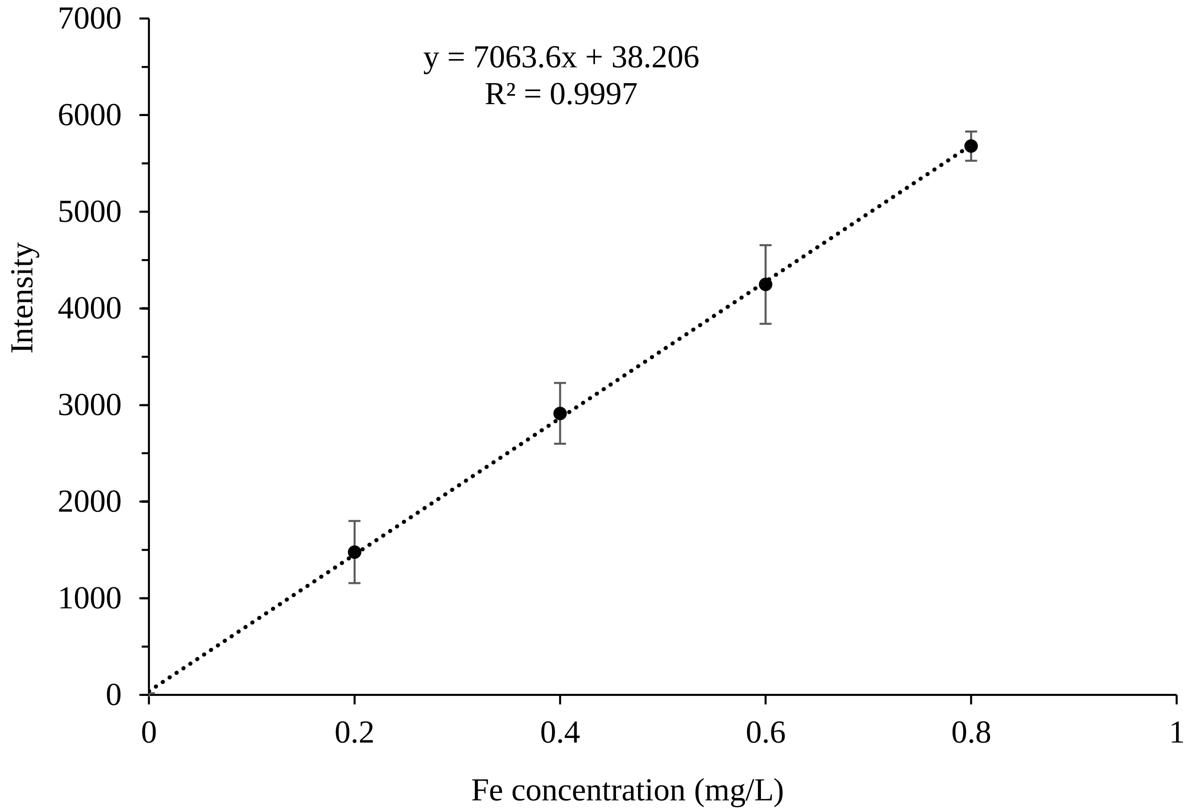

Figure A6. MP-AES Fe standard curve. The points plotted are means ± standard deviation (n=20).

Appendix 6-Certificate of analysis for the standard skim milk powder (ERM® - BD151)

JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials

CERTIFICATE OF ANALYSIS

ERM®- BD151

SKIMMED MILK POWDER		
	Mass Fraction	
	Certified value [1,2)] [g/kg]	Uncertainty [2,3)] [g/kg]
Ca	13.9	0.7
Cl	9.8	1.2
K	17.0	0.8
Mg	1.26	0.07
Na	4.19	0.23
P	11.0	0.6
	Certified value [1,2)] [mg/kg]	Uncertainty [2,3)] [mg/kg]
Cd	0.106	0.013
Cu	5.00	0.23
Fe	53	4
Hg	0.52	0.04
I	1.78	0.17
Mn	0.29	0.03
Pb	0.207	0.014
Se	0.19	0.04
Zn	44.9	2.3

1) Unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value and its uncertainty are traceable to the International System of Units (SI).

2) Certified mass fractions are corrected for the water content of the material (and expressed as dry mass), determined as described in the section "Instructions for use and intended use".

3) The uncertainty is expanded with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

This certificate is valid for one year after purchase.

Sales date:

The minimum amount of sample to be used is 500 mg for Fe and 200 mg for all other elements.

Accepted as an ERM®, Geel, August 2013
Latest revision: January 2017

Signed: D. Flo

Dr Doris Florian
European Commission, Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Retieseweg 111
B-2440 Geel, Belgium

Registration No. 268-RM
ISO Guide 34 for the
production of reference materials

All following pages are an integral part of the certificate.

Page 1 of 3

NOTE

European Reference Material ERM®-BD151 was produced and certified under the responsibility of the of the European Commission's Joint Research Centre according to the principles laid down in the technical guidelines of the European Reference Materials® co-operation agreement between BAM-IRMM-LGC. Information on these guidelines is available on the internet (http://www.erm-crm.org).

DESCRIPTION OF THE MATERIAL

The sample consists of about 20 g of skimmed milk powder in a brown glass bottle with a plastic neck insert and screw cap.

ANALYTICAL METHODS USED FOR CERTIFICATION

Cold-Vapour Atomic Absorption Spectrometry
Electro-thermal Atomic Absorption Spectrometry
Flame Atomic Absorption Spectrometry
Hydride-Generation Atomic Absorption Spectrometry
High-pressure Liquid Chromatography Inductively Coupled Plasma Quadrupole Mass Spectrometry
Inductively Coupled Plasma Optical Emission Spectrometry
Inductively Coupled Plasma Quadrupole Mass Spectrometry
Ion chromatography
Isotope-Dilution Inductively Coupled Plasma Mass Spectrometry
Neutron Activation Analysis (radiochemical and k0)
Pyrolysis Atomic Absorption Spectrometry (Mercury)
Sector-Field Inductively Coupled Plasma Mass Spectrometry
Titrimetry

PARTICIPANTS

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(Accredited to ISO Guide 34 for production of certified reference materials; BELAC No. 268-RM)
(Measurements performed under ISO/IEC 17025 accreditation; BELAC No. 268-TEST)

Australian Nuclear Science and Technology Organisation, Kirawee (AU)

ALS Scandinavia AB, Luleå (SE)
(Measurements performed under ISO/IEC 17025 accreditation; SWEDAC 1087)

Ceinal, S.A. (Silliker), Área Análisis Físico-Químicos, Barcelona (ES)
(Measurements performed under ISO/IEC 17025 accreditation; ENAC 257/LE413)

The Food and Environment Research Agency, York (UK)
(Measurements performed under ISO/IEC 17025 accreditation; UKAS 1642)

Helmholtz Zentrum München - Deutsches Forschungszentrum für Gesundheit und Umwelt GmbH, München (DE)

Institut "Jozef Stefan", Ljubljana, (SI)
(Measurements performed under ISO/IEC 17025 accreditation; Slovenska Akreditacija LP-090)

Laboratoire national de métrologie et d'Essais, Paris (FR)
(Measurements performed under ISO/IEC 17025 accreditation; COFRAC 22)

LGC Ltd., Teddington (UK)
(Measurements performed under ISO/IEC 17025 accreditation; UKAS 0003)

muva kempten, Kempten (DE)
(Measurements performed under ISO/IEC 17025 accreditation; DAkkS D-PL-14429-01)

SCK-CEN, Mol (BE)
(Measurements performed under ISO/IEC 17025 accreditation; BELAC 015-TEST)

Umweltbundesamt GmbH, Wien (AT)
(Measurements performed under ISO/IEC 17025 accreditation; Wirtschaftsministerium 92714/499-IV/9/01)

SAFETY INFORMATION

The usual laboratory safety precautions apply.

INSTRUCTIONS FOR USE AND INTENDED USE

This material is intended for quality control and assessment of method performance. As any reference material, it can also be used for control charts or validation studies.

Certified mass fractions are corrected for the water content of the material (dry mass): To determine dry mass, accurately weigh an aliquot of at least 1 g on an analytical balance and dry the sample in an oven at atmospheric pressure, at 102 °C ± 1 °C, until constant mass is attained. Weighing of the samples for dry mass determination and weighing for the analysis shall be done at the same time to avoid differences due to possible take up of moisture by the material.

Dispose in accordance with good laboratory practice.

STORAGE

The materials shall be stored at -20 °C ± 5 °C in the dark. Care shall be taken to avoid change of the moisture content once the units are open.

However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

LEGAL NOTICE

NOTE

A detailed technical report is available https://crm.jrc.ec.europa.eu. A paper copy can be obtained from the Joint Research Centre Directorate F – Health, Consumers and Reference Materials on request.

European Commission – Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Retieseweg 111, B - 2440 Geel (Belgium)
Telephone: +32-(0)14-571.705 - Fax: +32-(0)14-590.406

Appendix 7-Inhibitions zones of commercial freeze dried lactoferrin (HG28, CG17)

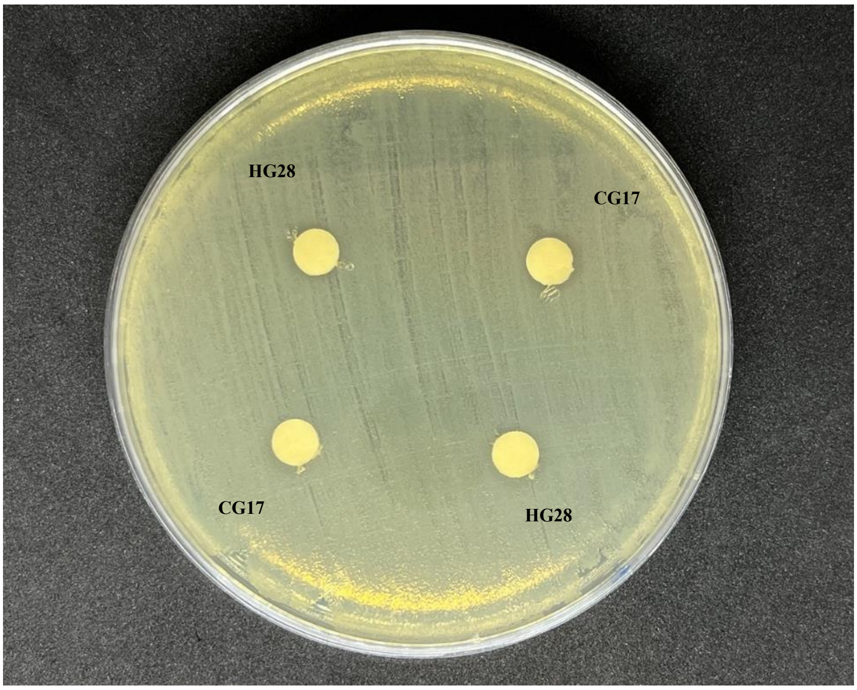

Figure A7. Zone of inhibitions of commercial freeze dried lactoferrin samples *Escherichia coli* NCTC 8196.

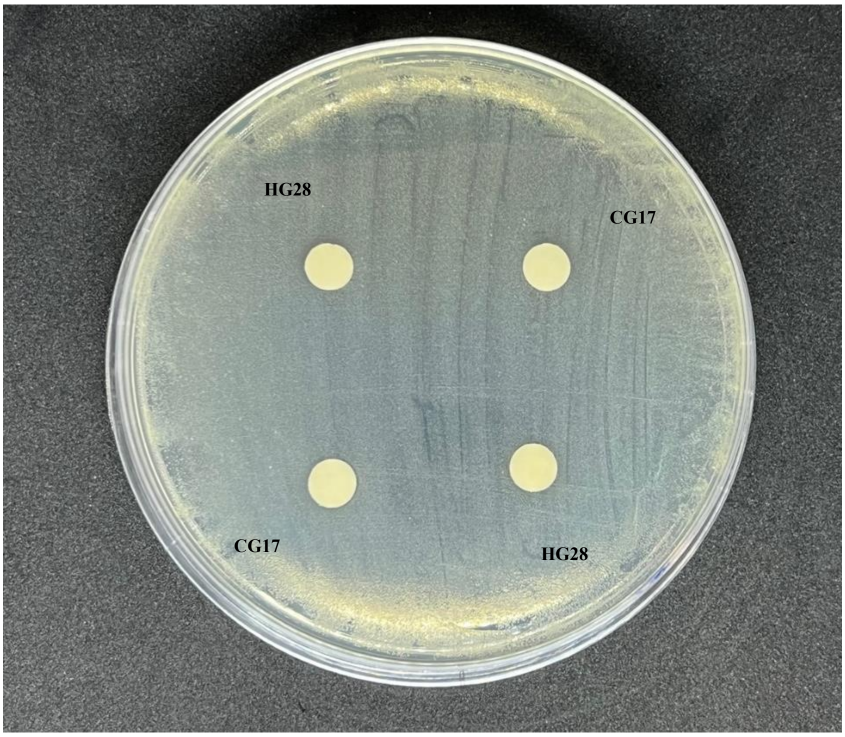

Figure A8. Zone of inhibitions of commercial freeze dried lactoferrin samples *Staphylococcus aureus* NCTC 4163.