Investigating The Therapeutic Potential Of Whey Derived Bioactive Peptides As Antiviral Agents In The Prevention Of Viral Infections

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By

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Declaration

I, Kelly Murphy, hereby declare that the material included in this thesis submitted for assessment for the programme of MSc Food Business Management and Technology is entirely my own work, under the guidance of my supervisor, Mary O'Connor M.Sc. This work has not been submitted for any academic award at this University, or any other University or Higher Education Institute. Any use of the work of others has been fully acknowledged by reference in both text and bibliography.

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Abstract

The impact of infectious diseases have always been a concern globally. Now more than ever do we understand the devastating impacts that they can have on every aspect of our lives as we begin to emerge from the catastrophic impacts, at both a mortality and economical level, from the SARS-CoV-2 pandemic. As we gain greater insights into the threat that emerging viruses have to human health, their mechanism of adaptation, combined with external factors that facilitate their proliferation around the globe, do we appreciate and understand the need for a wide range of strategies and therapies in order to counteract the spread of infection and their associated symptoms that can lead to increased mortality rates globally.

While there are a number of strategies such as vaccines and antiviral medications already well established in order to reduce the devastating impacts of infectious diseases, there is a need for alternative, and more affordable approaches in order to better counteract their spread and devastating impacts of harmful viral infections; especially for those who find themselves to be immunocompromised or at risk of suffering from the side-effects and complications associated with traditional treatments. Thus, there is a need for a wide range of alternative therapies and more affordable approaches in order to better control and counteract the spread of harmful viral infections.

With global volumes of bovine milk in the region of 714 billion kg per annum, the subsequent whey volumes generated from the cheese manufacturing industry offers an abundant source of whey derived bioactive peptides that warrant further investigation to assess their antiviral potential. Although whey was historically regarded as a problematic waste stream generated from the cheese manufacturing process, it has, over the past number of decades been valorised into a key nutritional ingredient within the food and beverage industry thanks to advances in both processing and analytical technologies within the dairy industry.

The ability to identify, purify and concentrate the native protein fragments β-lactoglobulin, α-lactalbumin, Bovine Serum Albumin, Immunoglobulins and Lactoferrin has allowed researchers to conduct vast amounts of both in-vitro and in-vivo studies in recent years, which have displayed that these bioactive peptides have the potential to

impart a wide variety of health benefits, which are increasingly gaining ground in clinical practice.

In conclusion, the antiviral potential of the whey derived bioactive peptides: β-lactoglobulin, α-lactalbumin, and Lactoferrin in particular, is one such benefit that this study's findings have displayed a growing body of evidence is supporting, thus showcasing them as a viable, widely available and more cost-effective option to work in conjunction with or as an alternative to traditional vaccines and anti-viral medication.

Abbreviations

α-LA	α-lactalbumin
AA	Amino Acids
bLf	Bovine Lactoferrin
BLF	Bovine Lactoferrin
BOD	Biological Oxygen Demand
β-LG	β-lactoglobulin
BSA	Bovine Serum Albumin
COD	Chemical Oxygen Demand
DI	Diafiltration
DWP	Demineralized Whey Powder
EAA	Essential Amino Acids
FSMP	Foods for Special Medical Purposes
γ-Globulin	Gamma Globulin
GMP	Glycomacropeptide
HA	Hemagglutination Activity
HCL	Hydrochloric Acid
HI	Hemagglutination Inhibition Assay
HIV	Human Immunodeficiency Virus
HLF	Human Lactoferrin
hpi	Hours Post Infection
HPV	Human Papillomavirus
HSV-1	Herpes Simplex virus type 1
H2SO4	Sulphuric Acid
kDa	Kilodaltons
LAB	Lactic Acid Bacteria
LF	Lactoferrin
MDCK	Madin-Darby Canine Kidney
MF	Micro-filtration
MHC Class I	Class I major histocompatibility complex proteins
mRNA	

N	Number
NA	Neuraminidase
NK Cells	Natural Killer Cells
NEAA	Non-essential Amino Acids
pH	Potential of Hydrogen
pI	Isoelectric Point
pM	Picometers
rT-PCR	Real-time Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SOC	Standard Of Care
SWP	Sweet Whey Powder
TCR	T Cell Receptors
UF	Ultra-filtration
WPC	Whey Protein Concentrate
WPE	Whey Protein Extract
WPH	Whey Protein Hydrolysate
WPI	Whey Protein Isolate

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Chapter 1

Introduction

The focus of this thesis is to investigate the bioactive peptides present in whey proteins derived from bovine milk and assess their potential as antiviral agents in the prevention of viral infections. The thesis will discuss the bioactive structure of these peptides, as well as their proposed mechanism of action in modulating immune response to viral infections. The introductory chapter will provide insights into whey protein ingredients derived from bovine milk, the technological advances that have made their isolation possible, their composition as well as their commercial relevance in the market today. The chapter will also explore the definition of viral infections, their mechanism of action in the human body, as well as their instances of occurrence and global impact, in terms of both economic impact and mortality rates.

1.1 Background of Whey Proteins

For generations, milk has been regarded as an excellent source of nutrients, and it is the first source of nutrition for most mammalian infants to ensure optimal growth and development (Givens, 2020). The term "Milk", as defined by Regulation (EU) No 1308/2013, is the normal mammary secretion obtained from one or more milking's, with nothing added and nothing taken away. (Regulation (EU) No 1308/2013). In terms of its composition, milk is described as a colloidal suspension comprising of fat globules and casein micelles suspended in a serum (also referred to as whey) phase, which consists of whey proteins, lactose and minerals (Guo, 2019). **Figure 1.1** below, depicts a schematic depiction of the colloidal suspension of globules of fat and casein micelles in the serum phase that is typically observed in milk.

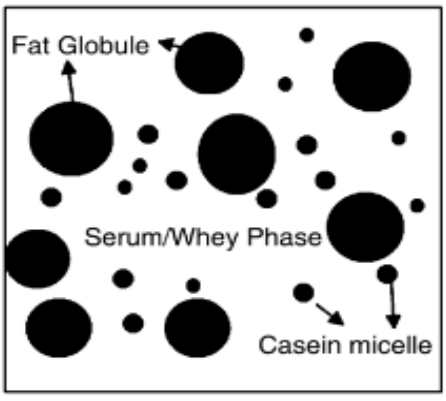


Figure 1.1 Schematic diagram of milk, displaying the colloidal suspension of fat globules and casein micelles in the serum phase

*** Taken from:** (Guo, 2019).

The composition of milk can be influenced by a number of various factors including: the animal species, environmental factors, the stage of lactation, and the overall nutritional status of the animal. Bovine milk has an approximate composition within the rages of: 87% water, 4 - 5% lactose, ~ 3% protein (including both casein and whey), 3 - 4% fat, and ~ 1% of vitamins and mineral combined (Tetra Pak, Dairy Processing Handbook. 2015). An overview of the chemical composition of milk derived from different species can be observed and compared in **Table 1.1**. When comparing these, there are some notable differences that can be observed between the different species. For example, buffalo and sheep milks have a notably higher fat content than other milks. Human milk has significantly lower levels of casein and ash, while having higher levels of lactose (Tetra Pak, Dairy Processing Handbook. 2015).

Species	**Water** (g/100g)	**Fat** (g/100g)	**Casein** (g/100g)	**Lactose** (g/100g)	**Ash** (g/100g)	**Whey Protein** (g/100g)
Cow	87.3	4.4	2.8	4.6	0.7	0.6
Buffalo	82.2	7.8	3.2	4.9	0.8	0.6
Sheep	82.0	7.6	3.9	4.8	0.9	0.7
Goat	86.7	4.5	2.6	4.4	0.8	0.6
Human	87.1	4.6	0.4	6.8	0.2	0.7

Table 1.1 Average Composition of milk (g/100g) from different species

***Adapted from:** (Tetra Pak, Dairy Processing Handbook. 2015).

Globally, bovine milk is the most frequently consumed source, due to its availability at an industrial scale. **Figure 1.2** looks at the Global milk volumes produced by different species year on year for the period between 2010 – 2019. It is noted that cow milk volumes reached over 700 million tonnes in 2019, while other sources of milk available globally are small in comparison (International Dairy Federation. 2019).

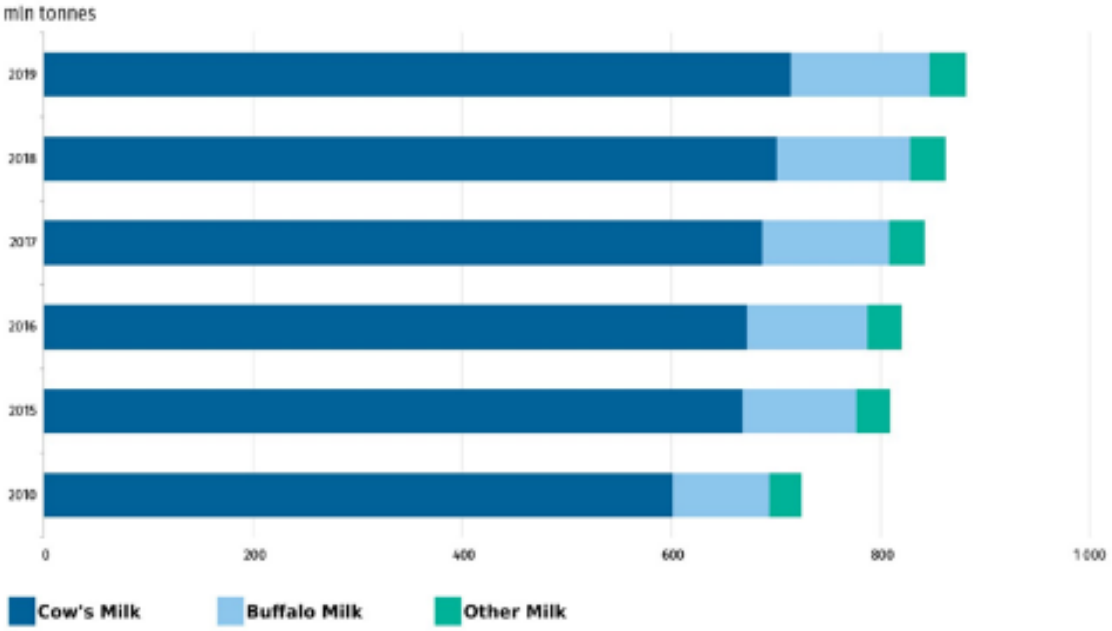


Figure 1.2 Summary of global milk production by species, for the period 2010 – 2019

*** Taken from:** (International Dairy Federation. 2019).

The main protein constituents present in bovine milk are that of casein and whey proteins. The whey portion represents up to 85% of the total volume of milk and accounts for in the region of 20% of the total proteins present, with the casein portion contributing to the remaining 80% of the proteins (Ryan and Walsh, 2016). The CODEX definition for whey describes it as the fluid milk product which is generated during the manufacturing of cheese, casein or other similar products, through the separation of the liquid whey portion from the casein curd, as a result of the coagulation of milk (Codex Alimentarius 289 - 1995). This coagulation is typically achieved by the action of acid or the enzyme action of rennet (Horne, *et al.* 2004).

When looking back on the history of cheese making and subsequently the consumption of whey, it can be dated back to more than 4,000 years ago, coinciding with the domestication of milk producing animals, which occurred sometime between 8,000 and

10,000 years ago. The transforming of milk into curds and whey is thought to have first occurred when an Arabian merchant used a pouch made out of a young sheep's stomach to carry milk. The lining of the stomach pouch still contained traces of rennet – the enzyme found in the stomachs of ruminant animals - this combined with the heat from the sun while he was travelling across the dessert, resulted in the milk separating into curds and whey (International Dairy Foods Association. Unknown).

This accidental discovery of forming curds and whey from milk in the presence of the enzyme rennet (present in the lining of stomachs from ruminant animals), soon gave rise to the process of cheese making being practiced throughout the Roman Empire, Middle East and Europe (Guo, 2019) (National Historic Cheesemaking Center. Unknown). It was extremely common for both the cheese curds and the liquid whey were utilised during these times. In fact, there is evidence from as early as 460 BC, referencing Greek philosophers recommending the consumption of whey for its health benefits of boosting the immune system, healing gastrointestinal issues and skin irritations (Guo, 2019), (Smithers, 2008).

The craft of cheese making continued to flourish across Europe, and ultimately made its way to the New World of America. During this time, cheese making was only ever a local level, with variations in the way in which it was produced. A review published by Smithers in 2008, has even referenced evidence from the 17th century, of whey being prescribed for wound healing and sepsis (Smithers, 2008). By the 19th century, the industrialisation of the cheese making process had commenced (National Historic Cheesemaking Center. unknown). As cheese production began to expand, so too did the volumes of whey that were being produced. With such large volumes of whey being generated, and no outlet capable of utilizing it, it was common place for whey to be directly disposed of into rivers and streams nearby to production plants (Smithers, 2008) (Guo, 2019).

Within the dairy processing industry, whey was, and still is an unavoidable by-product that was generated in order to manufacture cheese. For the most part, it was an undesirable waste stream and proved to be particularly difficult to dispose of due to environmental concerns regarding its high biochemical (BOD) and chemical oxygen demands (COD) (Guo, 2019). As sustainability has come to the forefront in recent years, the industry has had to adapt and evolve, embracing new technologies such as membrane processing and

spray drying, which resulted in the generation of powders with high protein contents and extended shelf-life's, meaning they are suitable for various nutritional applications across the infant, sports and adult nutrition industries (Smithers, 2008) (Ryan & Walsh, 2016). This application of membrane processing technologies combined with an appreciation for the nutritional profile that whey offers, the dairy industry has been able to turn a waste product into nutritional ingredients at an industrial and commercial scale (Ryan & Walsh, 2016).

Applications for liquid whey that is derived from the cheese making process spans across an array of uses and applications, ranging from a nutrient rich biofertilizer and animal feed, to more purified, concentrated ingredients in the form of nutritional whey powders, which have applicational uses in the sports, infant and adult nutrition industries (Ryan & Walsh, 2016). The types of whey powders that can be generated from whey can vary in nutritional composition, depending on the processing technologies applied to them before the concentration and spray drying processes. Such whey powders include:
Sweet Whey Powder (SWP), Neutralized Acid Whey, Demineralized Whey Powder (DWP), Whey Protein Concentrates (WPC), Whey Protein Isolates (WPI) and Whey Protein Hydrolysates (WPH) (Tetra Pak, Dairy Processing Handbook. 2015).

1.2 Production of Whey Protein Ingredients

Whey can be put into two categories depending on the processed utilised in order to generate it: Sweet whey and Acid whey. Sub-sections **1.2.1** and **1.2.2** below, provide a brief overview of the processes by which each of these two whey streams can be generated; typically, via hard cheese, rennet casein or acid casein processes. When it comes to the processing of liquid whey that has been derived from the cheese and casein making processes, there are numerous processing steps that can be applied in order to achieve the desired ingredient. It is imperative that whey is process in some manner as soon as possible, due to its composition of high lactose and a source of protein favouring bacterial growth and lactic acid formation. First and foremost, the whey is clarified to remove fines, whey cream is separated off, and the remaining whey is then pasteurised and cooled to below 5 degrees Celsius in order to retard bacterial growth before the next processing step commences (Tetra Pak, Dairy Processing Handbook. 2015).

1.2.1 Sweet Whey

Sweet whey is the by-product generated from the production of most hard cheese making processes. For this, the addition of the enzyme rennet is utilised in order to cause the milk to coagulate, separating out into different phases of curds and whey. This mixture of curds and whey is passed through a centrifugal separator, which allows for the whey to be drained away and the remaining curd before it is pressed and moulded in order to form blocks of cheese (Guo, 2019). **Figure 1.0.3** below, extracted from the Tetrapak dairy processing handbook, provides a simplistic overview of the basic processing steps that occur during the cheese production process, which ultimately generates the associated large volumes of liquid sweet whey (Tetra Pak, Dairy Processing Handbook. 2015).

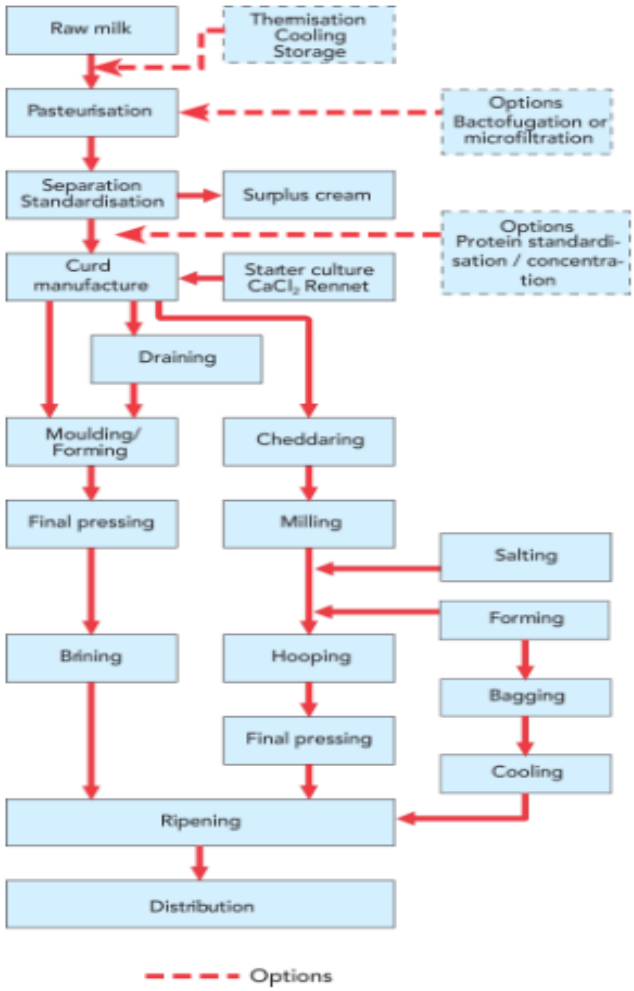


Figure 1.0.3 General process flow outlining the production of hard and semi-hard cheese which generates sweet whey.

*** Taken from:** (Tetra Pak, Dairy Processing Handbook. 2015).

1.2.2 Acid Whey

The action of lactic acid bacteria (LAB) or the direct addition of food grade acids such as hydrochloric acid (HCL) or sulphuric acid (H2SO4), are the mechanisms of choice within the dairy industry in order to acidify milk, resulting in the coagulation of the casein proteins. This process is typically applied for the production of Greek style yoghurt, soft cheeses and acid casein (Ryan & Walsh, 2016). All of the fore mentioned production processes generate large volumes of acid whey, which has a characteristically lower pH than that of sweet whey – typically pH 4.7 or lower. Acid whey also has a much higher mineral loading that sweet whey, which can be observed and compared in **Error! Reference source not found.** (Pires, et al. 2021). The process flow depicted below (**Figure 1.0.4**) shows the typical production steps applied for the generation of a soft cheese product, and ultimately acid whey (Tetra Pak, Dairy Processing Handbook. 2015).

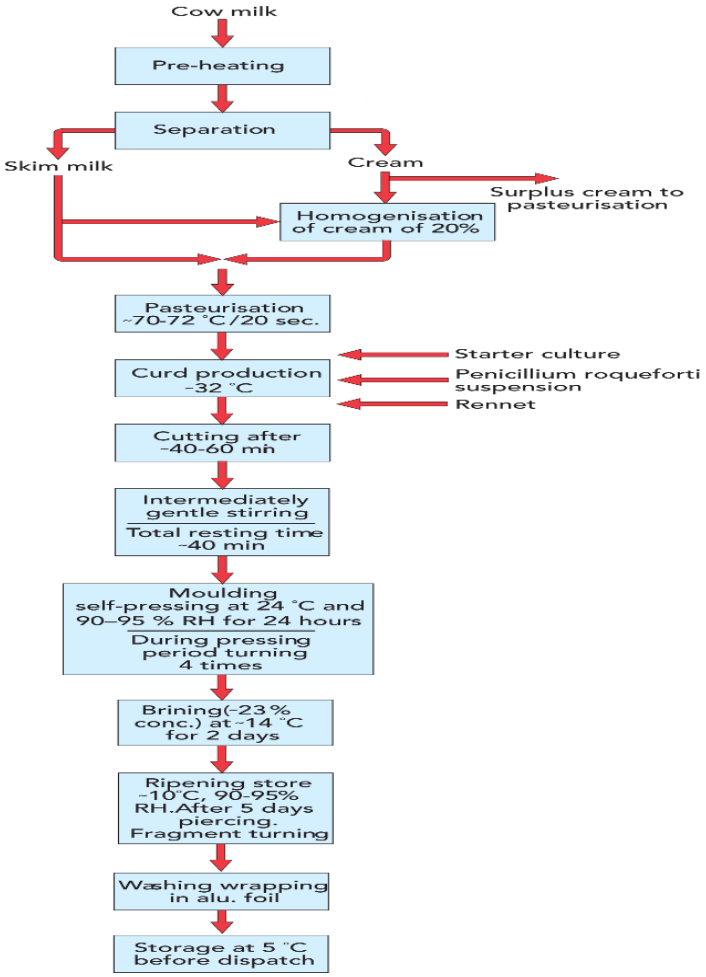


Figure 1.0.4 General process flow outlining the production of soft cheese, which generates acid whey.

*** Taken from:** (Tetra Pak, Dairy Processing Handbook. 2015).

1.2.3 Technologies for the valorisation of whey

At an industrial scale, there are a number of processing steps that can be implemented in order to recover and concentrate the protein fractions present within the liquid whey streams. Depending on the desired product to be generated, the technologies, or combination thereof as outlined in **Figure 1.5**, can be utilized in order to valorise the whey, generating nutritional ingredients (Akpinar, et al. 2009).The processes outlined below are typically carried out before whey is concentrated and dried via evaporation and spray drying in order to generate powdered ingredients (de Wit. 2001) (Tetra Pak, Dairy Processing Handbook. 2015) (Achmadi. 2021).

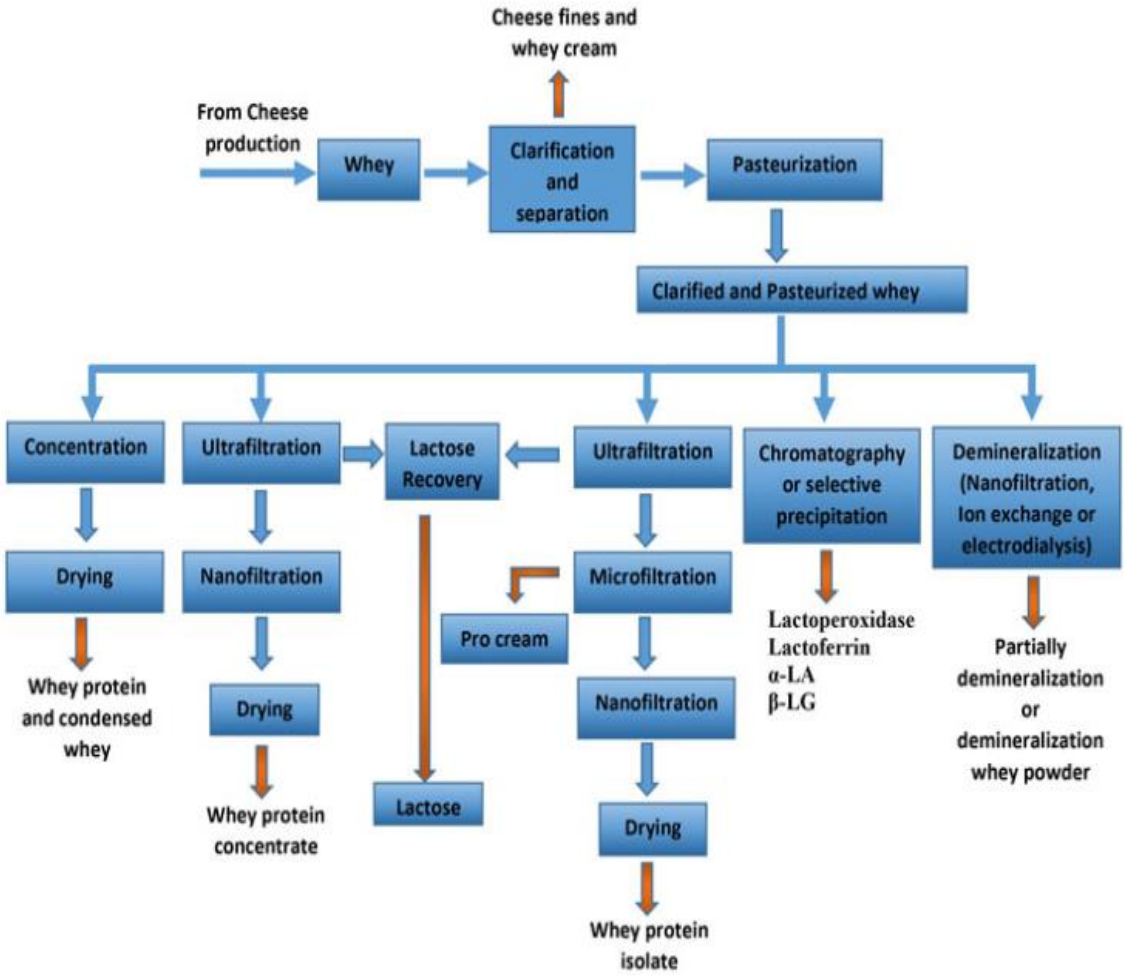


Figure 1.5 Examples of industrial processes utilised for whey recovery and concentration

*** Taken from:** (Achmadi. 2021).

1.2.3.1 Whey Protein Concentrate - WPC

Whey protein concentrate, typically referred to by its acronym, WPC, results from the utilisation of membrane separation technology. The membrane separation technology of ultrafiltration (UF) is employed to fractionate, purify and concentrate whey protein (Akpinar, et al. 2009). It can be categorised based on its finished powder protein content. low protein WPC is typically referred to as WPC-35, a medium level protein WPC is referred to as WPC-60, while a WPC with a high protein percentage is referred to as WPC-80. WPC-80 is produced through a combination of ultrafiltration (UF) and diafiltration (DI). Using these technologies in combination, a protein content of about 80% is the maximum that can be achieved. A basic explanation of how whey can be processed through membranes is depicted below in

Figure 1.6. Put simply, the whey passes through the semipermeable membrane whilst being held under a hydrostatic pressure gradient. Depending on the pose size of the membrane being used, various constituents in the whey are essentially filtered out based upon their particle size. The remaining retentate has an increased protein content, while the permeate stream is a lactose rich effluent, of about 6% total solids that is a problematic waste stream within the industry which is typically directed to biodigesters and waste water treatment plants (de Wit. 2001) (Tetra Pak, Dairy Processing Handbook. 2015) (Achmadi. 2021).

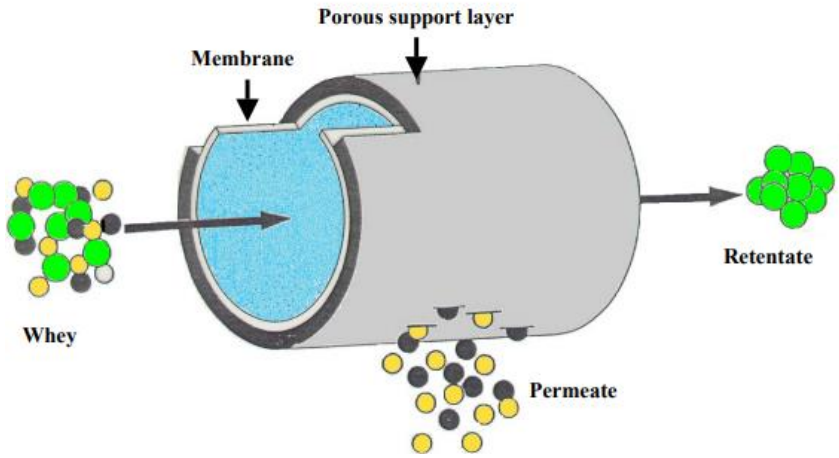


Figure 1.6 Basic principle of membrane filtration of whey

*** Taken from:** (de Wit. 2001).

1.2.3.2 Whey Protein Isolate – WPI

Whey protein isolate (WPI), has a notably higher protein content than that of WPC's. As membrane technology advanced, a process referred to as microfiltration (MF) allowed for the production of Whey Protein Isolates. WPI's are prepared from defatted whey, which is obtained by firstly passing through ultrafiltration (UF), before following up with the treatments of microfiltration (MF) and nanofiltration (NF) are applied in order to achieve the higher protein content of up to 90% (de Wit. 2001) (Tetra Pak, Dairy Processing Handbook. 2015).

1.2.3.3 Demineralised Whey Protein - DWP

Whey has quite a significant mineral loading, as noted in **Table 1.3**. This results in whey being quite salty to taste – which can prove to be unfavourable when it is incorporated into various food applications. It can also restrict the use of whey in nutritional applications such as infant formula, as the mineral content is simply too high for FSMP Foods for Special Medical Purposes (Božanić, *et al*. 2014). This is where the utilisation of electrodialysis comes in, in order to demineralise the whey stream, improving its compositional profile and taste. The basic principle of electrodialysis, which works by ionic exchange as displayed in **Figure 1.7**.

This process involves an ED unit that contains a number of alternating cationic and anionic compartments, to which direct current electrodes are applied to. As the whey passes through the ED unit, the negative ions (chloride, iodide and nitride) can pass through the positively charged anionic membrane and are then stopped when it passes through the negatively charged cationic membrane. Equally, the positive ions (calcium, magnesium, potassium and sodium) can pass through the cationic membrane, but not through the anionic compartments. As the whey flows through and is circulated in each compartment, it becomes more demineralised as the minerals migrate out of the whey and into the by-product brine solution (de Wit. 2001) (Tetra Pak, Dairy Processing Handbook. 2015).

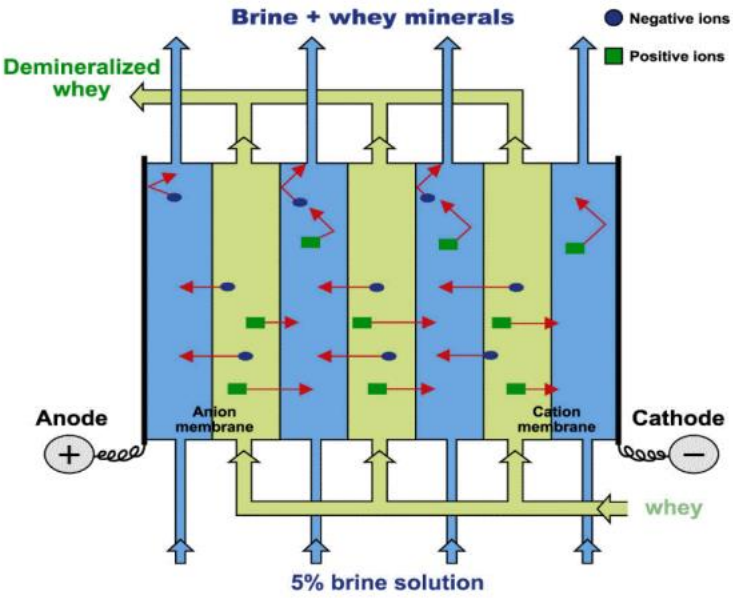


Figure 1.7 Principle of whey demineralisation via electrodialysis

*** Taken from:** (de Wit. 2001).

1.2.3.4 Hydrolysed Whey

When using whey proteins in nutritional formulations, particularly those targeted for infants and patients requiring formulations that meet the requirements for Foods for Special Medical Purposes (FSMP), there is often a need for the whey to be broken down, or partially digested in order to allow for sufficient absorption and increased digestive comfort. This is where the role of enzymatic hydrolysis of whey proteins comes into play, whereby whey is exposed to specially selected proteolytic enzymes under controlled conditions. **Figure 1.8** below provides a very basic overview of how a proteolytic enzyme can break down the long amino acid chain of a protein into smaller fragments of di- and tri- peptides.

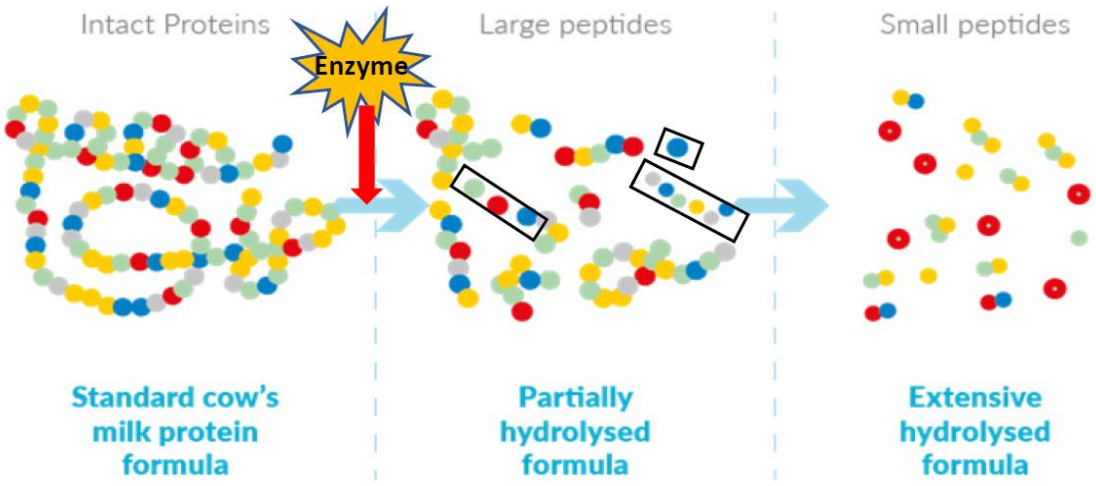


Figure 1.8 Schematic diagram outlining how enzyme can be utilised to partially digest whey proteins long chain of amino acids into smaller protein fragments – referred to as peptides.

1.2.3.5 Whey Derived Peptides

The various proteins constituents outlined in **Table 1.7** from section **1.4** below, can be isolated and fractionated from the whey protein stream through the utilisation of technologies such as chromatographic separation. There are a number of various chromatography techniques such as Ion exchange chromatography (IEC) and Affinity chromatography that can be applied in order to isolate specific proteins from whey, depending on their charge or by their affinity to interact with other biological substances via covalent bonds (de Wit. 2001).

In the case of the peptides such as β-Lactoglobulin, α-Lactalbumin and Lactoferrin, ion exchange chromatography is utilised in order to isolate the positively charged peptide by causing it to bind to beads in a negatively charged ion exchange column. In order to elute the positively charged Lf from the negatively charged beads, various salt solutions are applied. Further membrane processing of ultrafiltration and diafiltration is then applied to yield lactoferrin with a purity of up to 95%, followed by either freeze drying or spray drying (de Wit. 2001) (Wu, et al. 2009).

1.3 Composition Of Whey Powders

It has been well established by research that whey protein derived from bovine milk provides an excellent nutritional profile, with it being a source of all of the essential amino

acids which are needed for optimum growth and repair within the human body (Ryan & Walsh, 2016) (Gorissen, et al. 2018).

Table *1.2* below provides an outline of the amino acid content (including both essential and non-essential amino acids) present in whey derived from bovine milk, expressed as g/100g of whey powder (Gorissen, et al 2018).

Essential Amino Acids	**g/100g**
Threonine	5.4
Methionine	1.8
Phenylalanine	2.5
Histidine	1.4
Lysine	7.1
Valine	3.5
Isoleucine	3.8
Leucine	8.6
ΣEAA	**34.1**
Non-essential Amino Acids	
Serine	4.0
Glycine	1.5
Glutamic acid	15.5
Proline	4.8
Cysteine	0.8
Alanine	4.2
Tyrosine	2.4
Arginine	1.7
ΣNEAA	**34.9**

Table 1.2 Outline of the Amino Acid Composition of Bovine Whey

***Taken from:** (Gorissen, et al 2018).

The chemical composition of whey, whether it be that of sweet whey or acid whey can vary due to a number of different factors which relate directly back to the source of milk that it is derived from, including: animal breed, feed and seasonality e.g. grass fed, as well

as the type of manufacturing process that is utilised in order to generate it (Arona, *et al*. 2021). Compositional differences that are worth noting between sweet whey and acid whey include their mineral profiles and pH, which are noted below in **Table 1.3**. Acid whey is regarded as the more problematic of the two streams from a production and processing point of view, due to its very high levels of calcium and ash, which leads to the generation of an extremely sticky and difficult to handle powder when spray dried (Tetra Pak, Dairy Processing Handbook. 2015) (Guo, 2019).

Constitute	Sweet Whey	Acid Whey
Protein, Nx6.38 (%)	12.9	12.2
Non-protein nitrogen (%)	0.5	0.58
Fat (%)	1.1	0.5
Lactose (%)	74.5	63
Total Ash (%)	8.3	10.7
pH	5.6 – 6.1	4.7
Vitamins		
Vitamin A (IU)	44	107
Vitamin C (mg)	1.5	0.3
Vitamin E (mg)	0.03	0.05
Thiamin (B1) (mg)	0.5	0.5
Riboflavin (B2) (mg)	2.2	11.8
Pyridoxine (B6) (mg)	0.6	0.6
Vitamin B12 (mcg)	2.4	2.5
Pantothehenic Acid (mg)	5.6	1.8
Niacin (mg)	1.3	1
Folate (mcg)	11.6	0.03
Minerals		
Calcium (mg)	796	2279
Phosphorus (mg)	931.7	1516
Sodium (mg)	1079	1022
Potassium (mg)	2080	1885
Magnesium (mg)	176	247
Zinc (mg)	1.97	7.7
Iron (mg)	0.9	1.4
Copper (mg)	0.07	5.3
Selenium (mcg)	0.06	27.3

Table 1.3 Compositional comparison of sweet whey and acid whey

* Based on 100g of dry whey powder.

*** Taken from**: The Handbook of Dairy Foods & Nutrition 2ND Edition (Miller *et al*, 2000) Source: USDA, ARS, USDA Nutrient Database for Standard Reference

1.3.1 WPC

As previously mentioned, whey protein concentrates can be categorised based on their finished powder protein content. Low protein WPC is typically referred to as WPC-35, a medium level protein WPC is referred to as WPC-60, while a WPC with a high protein percentage is referred to as WPC-80. **Table 1.4** provides a comparison of the various WPC options. As the protein content is increased through membrane processing, the lactose content reduces, as it is filtered out through the pores in the membrane, into the permeate stream.

Component %	WPC-35	WPC-60	WPC-80
Total Protein	36.2	63.0	81.0
True Protein	29.7	59.4	75.0
NPN	6.5	3.6	6.0
Lactose	46.5	21.1	3.5
Ash	7.8	3.9	3.1
Lipids	2.1	5.6	7.2
Lactic Acid	2.8	2.2	1.2
Moisture	4.6	4.2	4.0

Table 1.4 Composition of powders from WPC's generated via UF

***Adapted from:** (de Wit. 2001).

1.3.2 WPI

A comparison of a typical whey protein isolate (WPI) powder compared to its precursors of whey protein concentrate (WPC) and ultimately sweet whey (SW) can be observed in **Table 1.5**. By applying the various membrane processing applications of ultrafiltration (UF), before following up with the treatments of microfiltration (MF) and nanofiltration (NF), a higher protein content in the region of 90% can be achieved.

Component %	Sweet Whey	WPC-80	WPI
Total Protein	12.9	80	87.88
Lactose	74.5	3.5	
Fat	1.1	5.55	0.5
Ash	8.3	2.27	2.43
Moisture		4.02	3.37
pH	5.6 - 6.1	6.60	6.22
Calcium (mg/100g)	796	548.0	525.0
Magnesium (mg/100g)	176	57.80	122.5
Potassium (mg/100g)	2080	519.0	422.0
Sodium (mg/100g)	1079	151.0	231.5
Phosphate (mg/100g)	931.7	330.5	219.0

Table 1.5 Mean compositional analysis of WPC-80 VS. WPI from a manufacturer processing cheddar whey in the West Coast of the United States.

***Adapted from:** (Carunchia *et al*. 2005)

1.3.3 DWP

As mentioned in section **1.2.3.3** above, whey has quite a significant mineral loading. The demineralisation process reduces this loading. **Table 1.6** below displays a comparison of the composition of whey before and after a demineralisation process has been applied is compared below in. The level of demineralisation can be controlled, depending on the desired composition. Available varieties of demineralised whey commonly produced in the dairy industry range from D50 (meaning that the whey is demineralised by 50%), D70 and D90.

Component %	Sweet Whey Protein	Demineralised Whey Protein
Total Protein	13	14.5
Lactose	73	77
Fat	1	1
Ash	8	4.5
Moisture	3	3
Calcium	0.6	0.5
Magnesium	0.2	0.1
Phosphorus	0.6	0.5

Table 1.6 Typical composition of sweet whey versus demineralised whey

***Adapted from:** (de Wit. 2001).

1.4 Whey Derived Bioactive Peptides

Proteins are molecules that are made up of long chains of amino acids, connected together via peptide bonds. When these long chains are broken down into shorter chains of amino acids, they are referred to as peptides. Peptides typically contain two or more amino acids (The University of Queensland - Institute of Molecular Bioscience. 2017).

Bioactive peptides are those that are derived from the whey portion of bovine milk are best described as specific protein fragments that are made up of amino acids held together by covalent bonds. These protein fragments have, in a variety of studies, been shown to exert a range of beneficial effects on human health such as anti-bacterial, anti-microbial, immune modulating and anti-viral. These bioactive peptides are not just derived from bovine whey protein but have also been derived from other sources including but not limited to various plants, animals, microorganisms, and fermented food which have the potential to be consumed as nutraceuticals (Mahgoub, *et al*. 2021).

The whey derived from bovine milk has been found to contain a number of proteins which act as bioactive peptides, upon liberation from the long peptide chain of amino acids in whey. The liberation of the bioactive peptides, mentioned in

Table 1.7, is achieved naturally in our bodies through the digestion process. However, in order to manufacture at both an industrial and commercial scale, processing applications such as enzymatic hydrolysis and microbial fermentation, followed by chromatography and membrane filtration steps in order to isolate and concentrate them (Pires, *et al*. 2021).

Protein	%
β-Lactoglobulin	53.3–66.0
α-Lactalbumin	15.0–20.0
Serum Albumin (SA)	6.0–7.0
Immunoglobulins	11.0–13.3
Lactoferrin	0.7-3.3
Lactoperoxidase	0.5–1.0
Enzymes	0.5

Table 1.7 Proteins present in the whey fraction of bovine milk

*** Taken from:** (Pires, et al. 2021).

It is through these various technological advances that the dairy industry has had the ability to commercialise a number of these peptides. This has been achieved in conjunction with scientists having the ability to investigate the therapeutic potential of these whey derived bioactive peptides. A growing body of evidence from studies conducted have associated whey derived bioactive peptides with imparting a therapeutic benefits of antidiabetic, antihypertensive, antimicrobial activity and antiviral potential to name but a few (Sartorius, *et al*. 2019).

1.4.1 β-Lactoglobulin

The main protein constituent within whey derived from bovine milk is β-Lactoglobulin (β-Lg). It contributes to 50% of the total protein content that is present in the whey protein proportion of bovine milk. It is well documented that the β-Lactoglobulin protein is not present in human milk (Goulding, D.A., Fox, P.F. and O 'Mahony, J.A. 2020). β-Lg is described as having an excellent profile of essential amino acids. It is a globular in shape, (see **Figure 1.9**) and consists of 162 amino acid. The molecular weight (MW) of β-Lg is 18.4 kilodaltons (kDa) and it has an isoelectric point of 5.1 (Arona, *et al*. 2021).

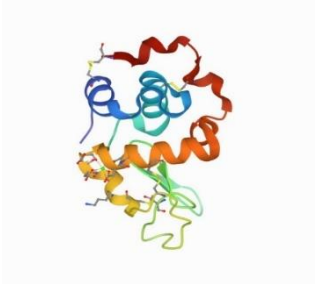


Figure 1.9 Schematic structure of β-Lactoglobulin (β-Lg

***Taken from:** (Kurpiewska & Biela, 2016).

1.4.2 α-Lactalbumin

The second most prevalent protein fragment that is present in bovine whey protein is that of α-Lactalbumin (α-La). It accounts for up to 20% of the protein content of whey (Arona, *et al.* 2021). Consisting of 123 amino acid residues, with a MW of 14.2 kDa and a pI of 4.2, it has been observed that α-La is more resistant to denaturation in comparison to that of β-Lg. **Figure 1.10** below provides a schematic diagram of α-Lactalbumins structure. As α-La is naturally present in human milk, it has evolved into a key ingredient used within infant formula, allowing for formulations to have a similar (but not identical) nutritional profile to that of human breast milk (Layman, *et al*. 2018).

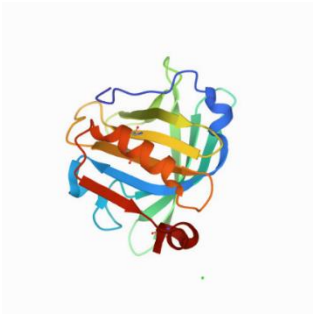


Figure 1.10 Schematic structure of α-Lactalbumin (α-La)

*** Taken from:** (Makabe, 2013).

1.4.3 Bovine Serum Albumin

A smaller protein constituent present in whey is that of Bovine Serum Albumin (BSA). It accounts for only a small portion (approximately 6%) of the proteins present in whey portion of liquid milk. Comprising of 582 amino acid, BSA has a MW of 69 kDa with an isoelectric point which varies between 4.7–4.9 (Arona, *et al*. 2021). Goulding *et al* previously described BSA as being both physically and immunologically identical to that of blood serum albumin found in human blood – making it an excellent ingredient to

include in infant nutrition formulations, thus helping to bridge the nutritional gaps between breast milk and infant milk formula (Goulding, D.A., Fox, P.F. and O 'Mahony, J.A. 2020).

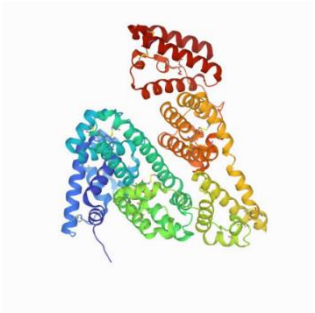


Figure 1.11 Schematic structure of Bovine Serum Albumin (BSA)

*** Taken from:** (Bujacz, A., Bujacz, G. 2003).

1.4.4 Immunoglobulins

A type of gamma globulin (γ-globulin) that is found in the blood and bodily fluids of all lactating mammals are referred to as Immunoglobulins (Igs) (Arona, *et al*. 2021). Immunoglobulins comprise of two light polypeptide chains which are held with two heavy polypeptide chains via disulphide bond linkages. The lighter chains have a MW of about 25 kDa, while the heavier peptide chains have MW between 50–70 kDa.

1.4.5 Lactoferrin

A minor protein that makes up about 3% of the total protein present in bovine whey is that of Lactoferrin (Arona, *et al.* 2021) (Tsermoula, *et al*. 2021). Lactoferrin is a single chain polypeptide molecule that comprises of 691 amino acids. It has a MW of 80kDa, with an pI of 8.6. Lactoferrin plays a vital role in the transportation of iron to the gut, therefore it is a key ingredient that can be utilised in infant nutrition formulations (Tsermoula, *et al.* 2021).

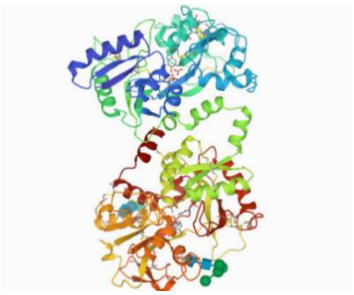


Figure 1.12 Schematic structure of Lactoferrin (LF)

*** Taken from:** (Moore *et al*. 2003).

1.5 Liberation Of Whey Bioactive Peptides

The liberation of peptides from the long protein chain of amino acids is something that naturally takes place during the digestion process when we eat sources of protein. The digestion of protein rich food through the gastrointestinal tract releases peptides, however the quantities of peptides released are in such small quantities that thus far it has proven to be very difficult to quantify in order to observe the exact beneficial effects that are imparted. At an industrial scale however, there are processes capable of both releasing specific peptides and the ability to concentrate them for use in nutritional applications and evaluate their effectiveness at imparting specific benefits to human health (Goulding, D.A., Fox, P.F. and O'Mahony, J.A. 2020). This is achieved by utilizing techniques such as enzymatic hydrolysis, microbial fermentation and ion exchange chromatography in order to extract from the readily available large volumes of liquid whey (Brandelli, *et al*, 2015), combined with membrane and drying technologies in order to purify and concentrate them for nutritional applications and evaluation (Brandelli, *et al*, 2015) (Goulding, D.A., Fox, P.F. and O'Mahony, J.A. 2020) (Pires, et al. 2021). The major obstacle currently associated with these processes is that these peptides are present in low concentrations within the large volumes of liquid whey, and there is a substantial whey waste stream that has been mined of is beneficial peptides, meaning that it is not nutritionally or functionally the same as unprocessed whey. The industry has not yet found suitable applications for this, other than animal feed, biofertilizer or a waste stream for biodigesters (Brandelli, *et al*, 2015) (Pires, et al. 2021).

1.6 Commercial Insights of Whey Protein Ingredients

To put into perspective the volumes of whey that are generated from the cheese production process, with every 1kg of cheese that is manufactured, a corresponding volume of about 10 litres of whey is generated (Pires, *et al*. 2021). If this is taken into account for sweet whey alone, a Eurostat report published by the European Commission Statistical Office in 2019 reported volumes in the region of 146 million tonnes of cow's milk being produced throughout Europe, of which up to 50% of this whole milk went

forward for the cheese manufacturing process. This works out at just under 55 million tonnes of liquid whey available for further processing and valorisation into nutritional ingredients (European Commission. Statistical Office of the European Union. 2020), (Tsermoula, *et al.* 2021) (Pires, *et al*. 2021). **Figure 1.13***Error! Reference source not found.* shows a breakdown of the % contribution regions around the global contributed to the overall 714 billion kg of cow milk production for the year 2019 (International Dairy Federation. 2019).

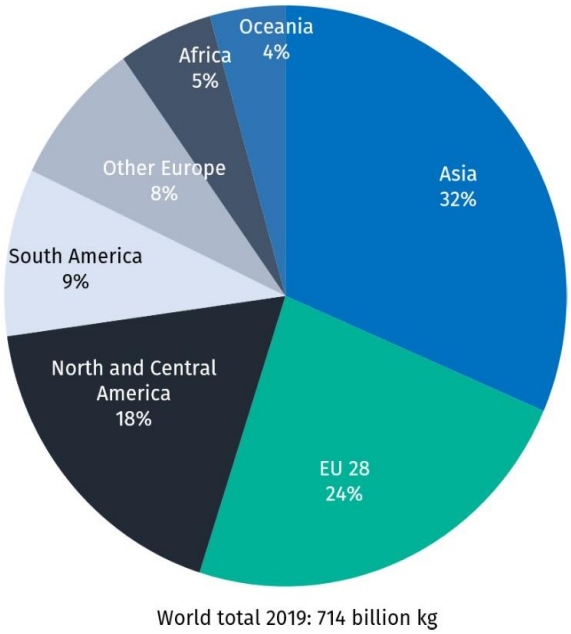


Figure 1.13 Cow's milk production % per world region

*** Taken from:** (International Dairy Federation. 2019).

The alpha-lactalbumin market is predicted to reach an overall value of 1.25 billion US dollars by 2023, as demand continues to grow for its inclusion in formulations for infants, sports athletes and health-conscious consumers. This is a CARG of 7.5% for the period 2021-2023 (Future Market Insights. 2022). Prices for bovine derived α-Lactalbumin at the time of this study are reaching €1,230 per gram from Sigma Aldrich (Sigma Aldrich. 2022).

Bovine β-Lactoglobulin powder with a purity of greater than or equal to 90% is currently retailing from Sigma Aldrich for the price of €235 for 1 gram (Sigma Aldrich. 2022).

The global market for bovine lactoferrin is projected to grow to in the region of 1461.3 billion US dollars by the year 2028 – which is a CAGR of 12.6% for the period 2021 – 2028. The pricing of lactoferrin had fluctuated in recent years, with more producers coming on stream, as well as tighter regulations surrounding the percentage purity that is required based on the applicational use. The price for lactoferrin has varied from its peak price of $3000/kg, to its current market value of about $1200/kg (LaFrenz. 2021)(Fortune Business Insights. 2021).

1.7 Viral Infections

A virus is best described as being a disease-causing agent, that is of small size and simple composition, and requires a living host cell of an animal, plant or bacteria in order to replicate themselves (Wagner, *et al*. 2022).

It is by this form of reproduction that we can describe all viruses as being obligate intracellular parasites. In other words, they thrive off of hijack the eukaryotic host cell and utilise its energy and intracellular organelles in order to replicate itself and spread throughout the cells of its host organism (Payne, 2017).

Figure 1.14 shows the basic elements of a typical eukaryotic cell. Some of which include: Nucleus – which contains the cells genetic material, Mitochondria – responsible for providing energy to the cell, Lysozymes - and Endoplasmic reticulum (Britannica. 2022).

In contrast, **Figure 1.15** shows a very basic depiction of a virus, which lacks the basic cellular components required replicate. The capsid contains surface proteins called hemagglutinin (HA) and neuraminidase (NA) which enable a virus to interact with and attach to host cell surfaces (Centers for Disease Control and Prevention. 2021). The relative size difference between a eukaryotic animal cell and that of a virus can be observed in **Figure 1.16** (Payne, 2017).

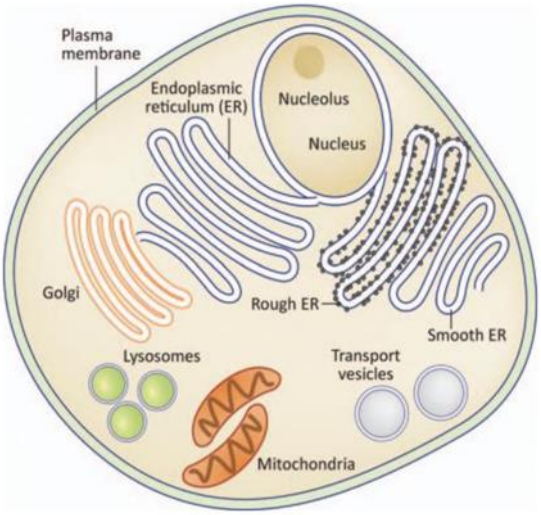


Figure 1.14 Schematic diagram of a eukaryotic cell, identifying major organelles

*** Taken from:** (Payne, 2017).

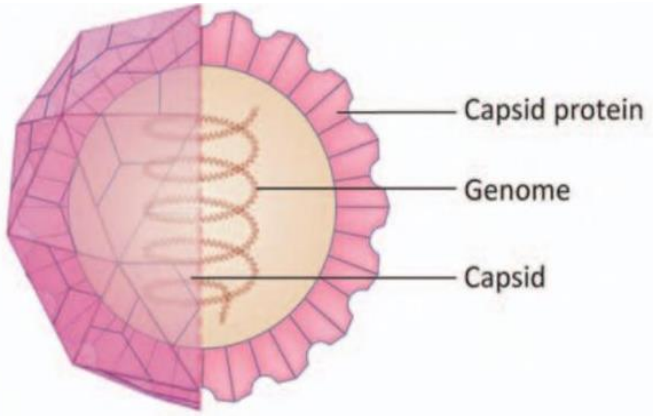


Figure 1.15 Schematic diagram of a virion

*** Taken from:** (Payne, 2017).

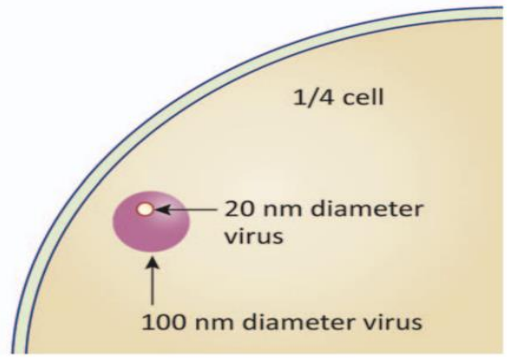


Figure 1.16 Comparison of the relative size of an animal cell versus virions

*** Taken from:** (Payne, 2017).

1.8 Viral Reproduction in human cells

A simplistic overview of the virus replication cycle in a human cell has been well outlined by (Payne, 2017). **Figure 1.17** below shows a simplistic schematic diagram of the basic steps that occur during viral reproduction within a host cell. These five steps are: Attachment, Penetration, Amplication, Assembly and Release, and have been summarized in the below sub-sections (Payne, 2017).

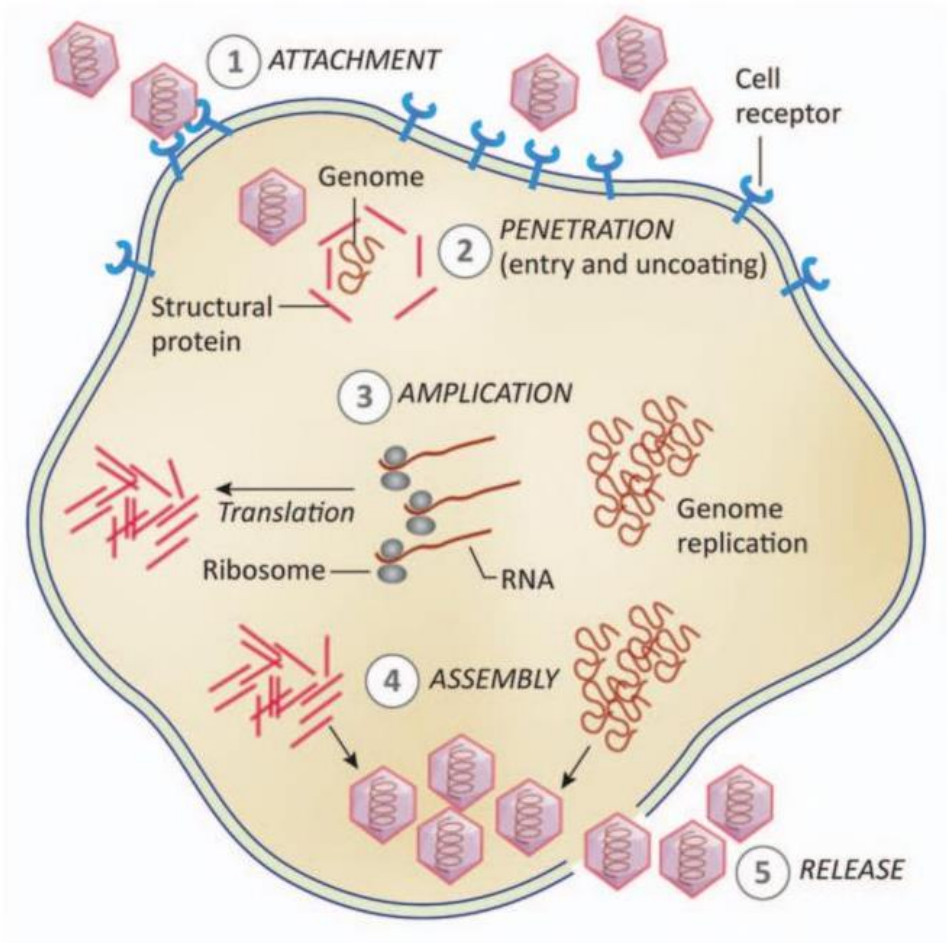


Figure 1.17 Schematic diagram of the 5 basic steps that occur during viral reproduction within a host cell.

***Taken from:** (Payne, 2017).

1.8.1 Attachment

Attachment is the initial step that takes place during viral replication. It requires specific interactions to occur between the viral surface capsid proteins and the molecules that are present on the surface of the host cell. The interactions are typically hydrophobic and ionic in nature, so environmental factors such as pH and salt concentration can impact

1.8.2 Penetration

Once a virus has successfully attached to its host cell, the next step involves the viral genome releasing from its capsid protein coating and penetrating the cytoplasm of the host cell. It must be noted that this process of uncoating and penetration is irreversible, and the virus cannot reassemble after this point.

1.8.3 Amplication

Following on from the viral genome penetrating the cytoplasm of the host cell, the synthesis of new viral proteins and genomes. This involves a number of complex intercellular processes within the host cell including: transcription – which is the synthesis of mRNA, translation – which is the synthesis of proteins and replication of the viral genome. This results in the generation of the necessary components that will

1.8.4 Assembly

The assembly step involves the newly synthesised genomes and proteins coming together to form new virus replication within the host cell.

1.8.5 Release

The final step of the viral replication process is the release of the newly formed virions from the host cell. This release occurs upon the rupturing of the infected host cell. The newly formed virions are then free to infect other cells within the host organism.

In order for each step of the viral reproduction process to take place, it is important to emphasise that very specific interactions between the viral proteins and the host cell membrane and intercellular proteins need to occur.

A certain cohort of viruses have the ability to infect many different types of organisms, due to their affinity for many types of protein interactions (Payne, 2017).

1.9 Human immune reaction to viruses

Upon a virus infecting a host, it quickly invades the host cells in order to survive and replicate throughout the cells of the host. When a virus has penetrated a host cell, the host's immune system cannot determine that cells have been infected because it cannot "see" the virus.

In order to have the ability to detect if a virus has penetrated a cell, host cells have employed a system whereby they use specialised molecules referred to as *Class I major histocompatibility complex proteins* (MHC class I). These work by making pieces of protein that are present within the cell to display on the surface of the cell. If a virus has infected a host cell, the visible pieces of protein fragments on the cell surface will contain those made by the virus (Payne, 2017).

A specific type of immune cell, referred to as a T cell, plays a role in looking for infections around the body. A particular type of T cell, called a *cytotoxic T cell*, have selective proteins on their surface, called T cell receptors (TCRs), that let them identify virally infected cells. They work by killing cells that have been infected through the use of toxic mediators. By destroying the virally infected cell, the host immune system prevents the invading virus from replicating.

Because of their relatively basic and simple structure, viruses are highly adaptable, and some have evolved to avoid detection by T cells.
One way by which they have managed to avoid detection is by preventing *MHC class I* molecules from getting to the surface of the host cell. This in turn does not signal to the T cells that the cell is infected, allowing the virus to continue replicating without being detected. There is however another immune strategy that the host can deploy - *natural killer cells* (NK cells). NK cells come into action and attack cells have a reduced number of *MHC class I* molecules on their surface (Laing. Unknown).

When the body's immune system is exposed to a new type of virus that it has not previously been exposed to, it can take a number of days for the immune system to adjust and determine the best course of action as to how to overcome this new infection. Following on from exposure to this new virus, the body's immune system is capable of adapting in order to protect itself should it come in contact with the same virus again. The T-lymphocytes are able to immediately recognise the virus, triggering the B-lymphocytes

to produce the appropriate antibodies that are capable of fighting off the infection (Centers for Disease Control and Prevention. 2018).

1.10 Vaccination & Medical Strategies against viral infections

As mentioned above, there are unfortunately instances when body's immune system cannot always fight the spread of harmful viral infections, particularly when it has not been exposed to a certain type of infection before. This can lead to the infected person becoming very ill, and in extreme cases can result in death – particularly if they already have an underlying health condition (HSE. 2019). An important strategy that has been established throughout the 18th and 19th centuries is the wides scale deployment of vaccinations (The British Society for Immunology. 2021).

1.10.1 Vaccines

The basic principle of how a vaccine works is by introducing selected antigen molecules from the virus of interest in order to trigger an immune response and train the immune system to recognise and attack the specific viral infection. By introducing these antigens in a safe and controlled manner, the body's immune system can have the appropriate time to develop the appropriate tools to identify the antigens and attack them well before the pathogen can spread and cause illness (PublicHealth. Unknown).

Edward Jenner is considered to be the founder father of vaccines. In 1796, he tested his hypothesis by inoculating a teenage boy with vaccinia virus (cowpox) and confirmed his theory that the cowpox vaccine was similar enough to smallpox (but not as deadly), that it could provide immunity to the smallpox virus. The first smallpox vaccine was developed in 1798. By 1979, smallpox was eradicated globally thanks to the roll out of mass immunisation against smallpox throughout the 18th and 19th centuries (The Immunisation Advisory Centre. 2017).

While the roll out of wide scale vaccination programmes have proven to be an effective measure in controlling the spread and lessening the severe symptoms of infectious viral diseases, viruses are constantly mutating and evolving in order to prolong their ability to spread infection amongst species. This process is referred to as antigenic drift – in which a virus will change its surface proteins (HA and NA), thus changing the was it interacts with and attaches to host cell membranes. These small changes in antigenic drift can

overtime, reduce the effectiveness and level of protection that vaccines can offer (Centers for Disease Control and Prevention. 2021).

1.10.2 Antiviral Medication

In order to counteract the impacts of viral infections, antiviral medications have been developed and work by interfering with viral replication by preventing the action of RNA replicase (Harper, *et al.* 2021) (Gambacorta, *et al.* 2021). Examples of anti-viral drugs currently utilised as influenza antiviral medications include Zanamivir and Oseltamivir. Antivirals are only prescribed under medical supervision, more often than not, serious side effects such as diarrhoea, nausea, sinusitis, nasal congestion, bronchitis, cough, headache, dizziness, as well as ear, nose, and throat infections can ensue (Centers for Disease Control and Prevention. 2016). Furthermore, antiviral medications can be quite costly, which can hinder their wide scale roll out at a global scale, which ultimately leads to the most vulnerable of cohorts remaining exposed to the devastating impacts of viral infections (Gambacorta, *et al.* 2021).

1.11 Viruses of Concern & Their Impact

As we begin to emerge for the depths of what can only be describes as a catastrophic pandemic, do we now realise the impact that viruses can have on our everyday lives. The impact of viruses are cause for serious concern, particularly amongst those who are immunocompromised and who have underlying health conditions (Ammendolia, *et al.* 2012). From preventing social interaction, suffering with symptoms, losing a loved one, on top of the economic burden that many faced when their form of employment was no longer feasible due to social distancing. While infectious diseases are not new to us, the speed by which they can be transmitted globally due to advances in global transportation and the ways in which they have been able to adopt, diversify and mutate to have the ability to jump from one species to another is a cause for concern when it comes to new emerging infectious diseases.

Infectious diseases are extremely common worldwide, but some are more common than others. Amongst some of the most common infectious diseases caused by viruses, as currently listed by the National Foundation for Infectious diseases include: Influenza, Human Papillomavirus (HPV), HIV/AIDS and Coronaviruses (National Foundation for Infectious Diseases. 2022).

1.11.1 Influenza

Influenza is a respiratory infection caused by a virus that targets the lungs and upper airways. Typically, influenza infections peak during the winter months. Symptoms of influenza can include weakness, aches and pains in muscles and a cough can develop. The severity of these symptoms can vary depending on the health status of the patient - with more vulnerable groups including the very young (aged 2 – 12 years), pregnant women, the elderly (over 65 years of age) and those who suffer from pre-existing medical conditions being at an increased risk of developing complications such as pneumonia (HSE. 2019).

Seasonal outbreaks of influenza contribute substantially to global mortality rates on an annual basis. Statistics published by the Centers for Disease Control and Prevention recorded mortality rates for influenza and subsequent cases of pneumonia reached 53.544 deaths for the year 2020 in the United States alone (National Center for Disease Control and Prevention. 2022).

1.11.2 Human Papillomavirus (HPV)

Human papillomavirus (HPV) is one of the most common sexually transmitted viral infections, for which there is no treatment available for. It is estimated that over 80% of both sexually active men and women will at some point contract HPV throughout their lifetime. In the US alone, it is estimated that up to 14 million new cases of HPV are diagnosed annually (National Foundation for Infectious Diseases. 2021).

Typically, patients do not display any symptoms, and the virus eventually clears from the body within a few years without treatment. The fact that typically no symptoms are displayed after infection means that transmission rates of HPV are quite high. Some patients may develop genital warts, which can be easily treated once diagnosed. More concerning however is that HPV is noted as the main contributing cause of cervical cancer – and with the absence of symptoms in the majority of cases, it can lead to delays in treatment during the early stages of cancer developing. Two strains of HPV in particular (types 16 and 18) are estimated to be responsible for over 50% of the development of pre-cancerous cells on the cervix (World Health Organisation. 2020).

When looking at the statistics for a country like Ireland alone, each year over 6,500 women require medical treatment for pre-cancerous cells found on the cervix. Over 300

women will develop cervical cancer annually and sadly almost 90 women die from cervical cancer caused by HPV every year on the Island Of Ireland (HSE. 2022). While The Centers for Disease Control and Prevention estimates that there were 43 million HPV infections in the United States in 2018, of which 13 million were new infections (Centers for Disease Control and Prevention. last updated April 2022).

Over the last decade or so, a number of global strategies such as vaccination programmes have been deployed in order to prevent the spread of HPV, but also to screen for instances where HPV may have infected individuals in order to detect the earliest signs of the development of pre-cancerous cells within the cervix (World Health Organisation. 2020).

1.11.3 HIV

Human Immunodeficiency Virus (HIV) is a virus that specifically targets a person's immune system, causing it to weaken and being unable to protect the body from minor infections that it was once able to defend it against (World Health Organisation. 2022). The transmission of HIV is through direct contact of bodily fluids such as: blood, semen, vaginal fluids and breast milk. The virus enters the blood stream when these fluids come into contact with mucosal membranes or open wounds (HIV.gov. 2022).

Since the beginning of the HIV epidemic in 1981 (HIV.gov. 2022), it is estimated that on average 84.2 million people globally have been infected with the virus, of which an average of 650,000 people have died from HIV and its associated complications. As of the year 2021, the World Health Organisation estimated that there are approximately 38.4 million people globally, living with HIV (World Health Organisation. 2022).

1.11.4 Coronaviruses

Coronaviruses are a large group of viruses that have the ability to cause diseases in both animals and humans. They are enveloped viruses containing positive stranded RNA. Coronaviruses were first identified as harmful infections in humans back in the 1960's. They typically circulate among animals such as camels, cats, and bats, however a number of coronaviruses have evolved and mutated with the ability to transmit and infect humans when they come into close contact with an infected species (National Foundation for Infectious Diseases. 2022).

At the time of this study, there are currently seven known coronaviruses that have the ability to transmit from animal to human and subsequently human to human. Of these, four are typically associated with causing mild to moderate symptoms in infected individuals. These include: HCoV-OC43, HCoV-HKU1 and HCoV-229E which can cause symptoms associated with common colds, while HCoV-NL63 is commonly associate with causing croup and bronchiolitis in young children. On the more extreme end of the scale of coronaviruses that have emerged in recent years are: SARS-CoV which leads to severe acute respiratory syndrome (SARS) and first emerged in; MERS-CoV, referred to as Middle East respiratory syndrome (MERS) which emerged in 2012; and SARS-CoV-2, known as coronavirus disease 2019 (COVID-19), which was first identified towards the end of 2019 (European Centre for Disease Prevention and Control. 2022).

1.11.4.1 Severe Acute Respiratory Syndrome (SARS)

Severe Acute Respiratory Syndrome (SARS) was first reported in China in 2002, with the widescale spread of the infection reaching over two dozen countries in North America, South America, Europe, and Asia. Although the exact species responsible for first transmitting the virus to humans was never identified, it was traced back to a number a small mammals including cats, ferrets and bats (European Centre for Disease Prevention and Control. Unknown).

Symptoms of SARS include muscle aches and pains, fever, and in extreme cases patients can develop pneumonia and suffer from respiratory distress. The global mortality rate of reached about 10% for all that were infected. This figure increased to a mortality rate that exceeded 50% in patients aged over 65 years (National Foundation for Infectious Diseases. 2022). According to statistics released by The World Health Organization (WHO), a total of 8,098 people worldwide were infected with SARS during the 2002/2003 outbreak, of which 774 individuals died (Centers for Disease Control and Prevention. 2017).

1.11.4.2 Middle East Respiratory Syndrome (MERS)

Middle East Respiratory Syndrome (MERS) was first reported in Saudi Arabia in 2012. It was determined that MERS originated from humans coming into contact with infected camels. As with the other coronaviruses mentioned, the typical symptoms included a fever, cough, and shortness of breath, which often progressed to pneumonia. It I estimated

that approximately 3 or 4 out of every 10 patients diagnosed with MERS died (National Foundation for Infectious Diseases. 2022).

1.11.4.3 SARS-CoV-2 (COVID-19)

The world is currently emerging from a global pandemic caused he novel coronavirus, SARS-CoV-2, which is commonly referred to as COVID-19 (National Foundation for Infectious Diseases. 2022). The origin of this virus was traced back to the city of Wuhan in China towards the end of 2019. By March 2020, SARS-CoV2 was declared a global pandemic (European Centre for Disease Prevention and Control. Unknown) (World Health Organisation. 2022). To date globally, there have been 585,950,085 confirmed cases of SARS-CoV2, resulting in 6,425422 deaths reported to the World Health Organisation. (World Health Organization. 2020 – Last updated 12th August 2022). **Figure 1.0.18** and **Figure 1.0.19** below, depict the timeline and scale to which infection rates and death have occurred since the beginning of the SARS-CoV2 global pandemic in 2020, up until today (2022).

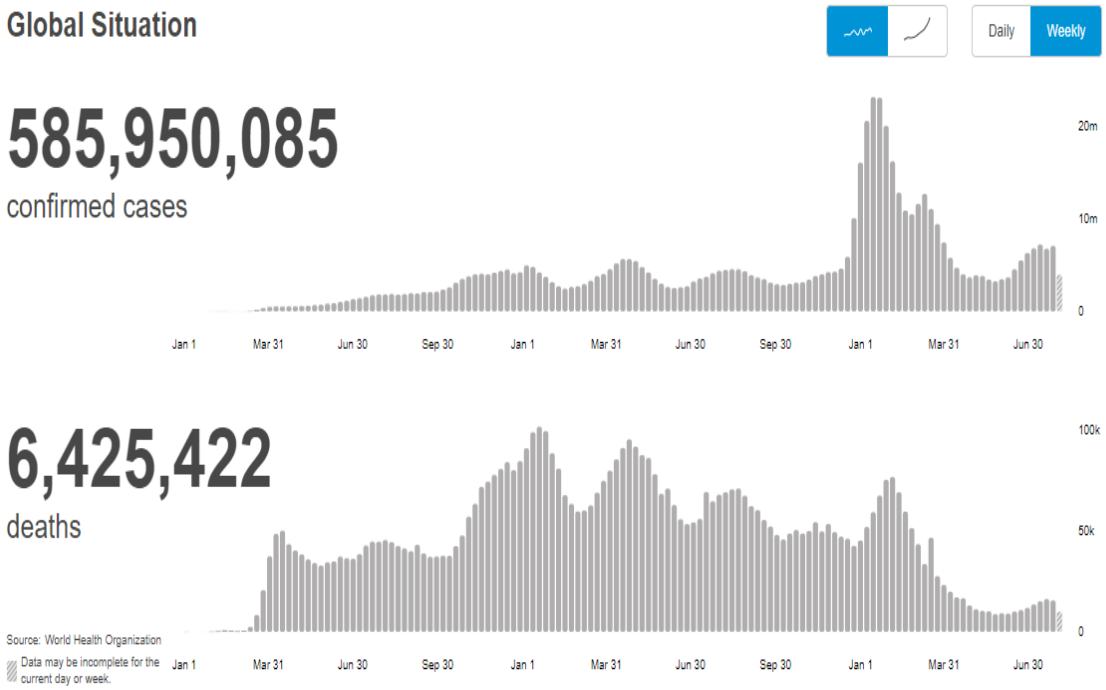


Figure 1.0.18 Overview of confirmed cases and deaths associated with SARS-CoV2

* Taken from: (World Health Organization. 2020 – Last updated 12th August 2022).

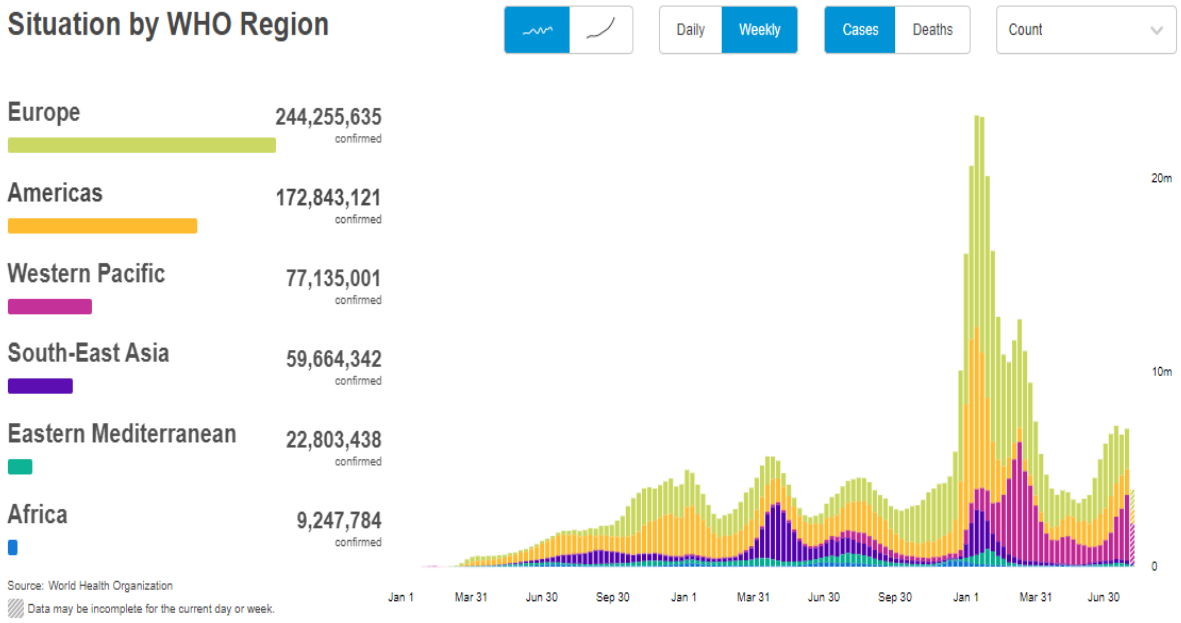


Figure 1.0.19 Overview of confirmed SARS-CoV2 cases reported to the WHO – broken down by region.

* Taken from: (World Health Organization. 2020 – Last updated 12th August 2022).

1.12 The Economic Impact of Viral infections

Despite the significant medical advances made in recent centuries, infectious diseases, particularly newly emerging ones pose a significant threat to our global society. While vaccination programmes and antiviral medication have proven to decrease overall mortality rates (Borràs *et al.* 2011), it must also be highlighted that there is an economic impact that needs to be taken into consideration. A briefing published by The European Parliament summarized the findings of various studies which have indicated that epidemic and pandemic outbreaks of infectious diseases can impact a country's economy through several channels, including the health, transportation, agricultural and tourism sectors (Delivorias, *et al*. 2020). In the case of the recent COVID-19 pandemic alone, it has been reported that the International Monetary Fund has forecasted that it will cost the global economy a forecasted $12.5 trillion – at the very minimum (Shalal. 2022).

Thesis Outline

This thesis will review the peptides present in whey proteins derived from bovine milk and assess their bioactive potential as antiviral agents in the prevention of viral infections. This will be achieved by providing insight into the bioactive components derived from the whey of bovine milk, whilst also detailing the mechanism of action by which viral infections take hold of cells within the human body, as well as a brief overview of how the human immune system responds to the detection of viral infections. Definition was given to milk and whey, as well as detail being provided on peptides constituents that can be derived.

A definition and understanding of viral infections and how they attack human host cells will be provided. Some of the most common viral infections globally will be discussed, their associated symptoms and mortality rates, as well as the current strategies that are available to control the spread of viral infections. The focus of this study will be on research study data that explores the potential of bovine whey derived bioactive peptides acting as anti-viral agents.

Chapter 2

Investigating The Therapeutic Potential Of Whey Derived Bioactive Peptides As Antiviral And Immune-Modulating Agents In The Prevention Of Viral Infections and Associated Methods

2.1 Methods – Study Design

This section provides an overview of the methods utilized in order to demonstrate the proposed mechanism of action by which whey bioactive peptides derived from bovine milk may act as antiviral agents in the prevention of viral infections (Haugh *et al*. 2007) (Smithers, 2008) (Guo, 2019). This will support the proposed use of food derived bioactive peptides to be used in combination with or as an alternative to traditional antiviral medications and drugs against the proliferation of, or prevention entirely of viral infections throughout host cells within the human body. The research method utilized have been a desk-based study of peer reviewed, published research papers, in conjunction with data from both government and non-governmental organisation sources.

2.1.1 Thesis Outline Of Research

In recent years a number of both in-vivo and in-vitro studies have demonstrated a positive effect of whey derived bioactive peptides on human health and wellbeing. The research question proposed in this thesis is, to what level is the therapeutic potential of whey derived bioactive peptides as antiviral agents in the prevention of viral infections. In order to address the research question, Chapter 1 reviewed the definition of milk and its derivatives, the technologies that can be implemented to isolate and concentrate whey protein ingredients, as well as defining what bioactive peptides are. An overview of the mechanism of action of how viral infections hijack host cells and the basic principle of how antivirals work was also outlined.

Chapter 2 will outline the materials and methods used in order to address the research question put forward. Chapter 3 will present the results and findings relative to the evidence supporting the role of bovine whey derived bioactive peptides potential as antiviral compounds. Chapter 4 will then critically review and discuss the findings, substantiating the conclusions drawn on the accuracy of the proposed research question. A synopsise of the key findings and suggested future work will be provided in Chapter 5.

2.1.2 Scope

The scope of the research question focuses on the beneficial role and mechanism of action that bioactive peptides derived from bovine whey can play in conjunction with, or as an alternative to traditional antiviral medication in the treatment of infectious viral diseases.

The scope will explore the processes by which whey is generated from a bovine milk, and the technologies that are utilised in order to purify and concentrate it and its subsequent components. Three key bioactive peptides have been focused on during this study, due to their percentage concentration within whey, as outlined in **Table 1.7**. These three peptides are β- Lactoglobulin, α-Lactalbumin and Lactoferrin.

2.1.3 Data Inclusions

Data included in the study was extrapolated from a selection of both *in-vitro* and *in-vivo* experimental studies.

Data included has been extrapolated from recent publication, typically those that have been published within the last 15 years or so. Data used, which was older than this, has only been used where the data is still current and relevant and where no new data had been generated.

2.1.4 Exclusions

Exclusions have been applied to publications in which data has not been peer-reviewed, as well as data that was deemed to be unsubstantiated.

2.1.5 Statistics Size Cohort

For this study, only data from experimental and observational studies, which contained sizeable data sets and sizable number of study subjects, have been included ensure only statistically sound data was utilised.

Statistical analysis has not been carried out as part of this study, as to do so would have been inappropriate, due to the lack of available raw data. Within the research material used for the study, researchers applied statistical analysis, to generated data within all of the cohort studies outlined and within all research cited as *in-vitro, in-vivo* or meta-analysis in nature. Statistical analysis was also used by the primary researchers of the cited studies.

2.2 Materials

2.2.1 Population

The review focused on the global population, encompassing both people from developed and developing countries.

2.2.2 Male and Female

For the purpose of the review, data relating to both male and female persons has been included, due to the cross-sex prevalence and impact of viral infections across all age categories

Morbidities:

As outlined in **section 1.11** and its associated subsections, some of the most common infectious diseases caused by viruses, as currently listed by the National Foundation for Infectious diseases include: Influenza, Human Papillomavirus (HPV), HIV/AIDS and Coronaviruses (National Foundation for Infectious Diseases. 2022). These specific infectious diseases have been highlighted due the current and projected high rates of incidence in the global population and where instances of bovine whey derived bioactive peptides have been evaluated in order to determine their specific impact.

Inclusion:

Only bovine dairy and its whey derivatives have been included within the data set used for this study. This is due to the global prevalence of bovine dairy over that of other dairy sources in the human diet due to availability and accessibility on a global scale – as outlined in the introductory chapter (see **Figure 1.2** and **Figure 1.13**). Due to their availability at a commercial scale, as a result of their higher concentration percentage to the overall protein content of whey, this study focuses on the antiviral potential of the following protein peptides found within bovine whey: α-Lactalbumin, β-Lactoglobulin and Lactoferrin.

Exclusion:

Dairy derived from non-bovine sources has been excluded due to the relatively low levels of non-bovine dairy produced and consumed globally, as demonstrated by **Figure 1.2 Summary of global milk production by species, for the period 2010 – 2019Figure 1.2** in the introductory chapter. As a result, the likely scalability required in order to isolate and concentrate these relevant peptides of interest and their potential to have a positive

impact on human health would not be in a global scale. Due to the lack of their commercial availability – primarily due to their low concentration levels in raw whey and difficulty to extract and concentrate to commercial volumes, studies based on the antiviral potential of the following protein peptides found within bovine whey have been excluded from this study: Serum Albumin, Immunoglobulins, Lactoperoxidase and Enzymes.

Chapter 3

Results

The impact of infectious diseases are now, more than ever a growing concern for the global population as we begin to emerge from the devastating impacts, at both a mortality and economical level, from the SARS-CoV-2 pandemic. As we gain greater insights into the threat that emerging viruses have to humans, their mechanism of adaptation, combined with external factors that facilitate their proliferation around the globe; including but not limited to climate change, the increased mobility of people and goods around the world and rapid changes to demographics do we appreciate and understand the need for a wide range of strategies and therapies in order to counteract the spread of infection and subsequent symptoms that can lead to increased mortality rates globally (Harper, *et al.* (2021).

In this chapter, an outline of the therapeutic and potential of bioactive peptides derived from bovine whey as antiviral agents will be provided. A number of studies and reviews have been conducted, investigating the potential antiviral benefit that peptides derived from bovine whey protein can impart. Particular focus will be paid to the peptides β-Lactoglobulin, α-Lactalbumin and Lactoferrin due to their percentage concentration in liquid whey, as well as their availability at a commercial scale due to the technologies currently available to isolate these peptides of interest.

A review recently conducted by Gallo. *et al.* (2022) provided an insightful overview of the key studies which summarized the antiviral activity of the three main peptides of interest for this study; β-Lactoglobulin, α-Lactalbumin and Lactoferrin.

Table 3.1 below provides a collative summary of key studies which looked at the whey derived bioactive peptides of interest whereby antiviral activity was displayed.

Whey Protein	Virus	References
β-lactoglobulin	Human immunodeficiency Virus (HIV)	Neurath. *et al.* (1996)
	Influenza virus A (H1N1)	Sitohy. *et al.* (2010)
α-lactalbumin	Herpes simplex virus type 1 (HSV-1	Oevermann. *et al.* (2003)
	Human immunodeficiency Virus	Marshall. (2004).
Lactoferrin	Herpes simplex virus type 1	Marchetti. *et al.* (1998)
	Hepatitis C virus	Ikeda. *et al.* (2000)
	Human Immunodeficiency Virus (HIV)	Berkhout. *et al.* (2004)
	Human papillomavirus (HPV)	Mistry *et al.* (2007)

Table 3.1 Summary of studies where antiviral activity of B-Lg, a-Lac and Lactoferrin derived from bovine whey displayed antiviral activity against viral infections

*** Adapted from:** Gallo. *et al.* (2022)

3.1 Antiviral Effects of Whey Derived Beta-Lactoglobulin

A number of studies and reviews have been conducted in order to assess the potential of whey derived β -Lactoglobulin as both an immune modulating and anti-viral agent. β -lactoglobulin has the potential to be extracted from the whey stream in large volumes, as it is the most predominant protein present in whey, as shown in **Error! Reference source not found.** within the introductory chapter (Pires, *et al*. 2021).

In 2003, Oevermann. *et al.* conducted an in-vitro study which set out to investigate the antiviral activity of naturally occurring proteins and their peptide fragments after they had been modified by the action of the proteolytic enzyme's trypsin, chymotrypsin and pepsin respectively. β -Lactoglobulin was one of the three protein fragments that Oevermann. *et al.* focused on for this study. By applying an enzymatic digestion as a pre-treatment to these proteins, the potential bioactive fragments could be exposed in order to evaluate their effectiveness, in comparison to un-modified proteins. In order to first identify and extract the protein fragments of interest from bovine whey, reverse phase chromatography was applied before exposing the proteins to enzymes.

The antiviral activity of the enzymatically modified β -Lactoglobulin was evaluated against Herpes Simplex virus type 1 (HSV-1) and was determined via the method of Neutral red uptake assay. The percentage of antiviral protection that was achieved from enzymatically modified β -Lactoglobulin was calculated by the absorbance measurement that was measured at 550 nm using a microplate reader. The data presented was derived from experiments conducted in triplicate.

In this study, the native protein peptides were used as a control to compare to the enzymatically modified peptides before, during and after infection with the HSV-1 viral strain. From the results obtained, the native protein fragment of β -Lactoglobulin did not display antiviral activity against HSV-1. The enzymatically modified β -Lactoglobulin, referred to as 3-HP- β -Lactoglobulin did in fact show antiviral activity against the virus HSV-1, as seen in **Figure 3.1**, where 3-HP-β-lactoglobulin **(■)**, ability to inhibit HSV-1 was measured when it was added before, during and after infection.

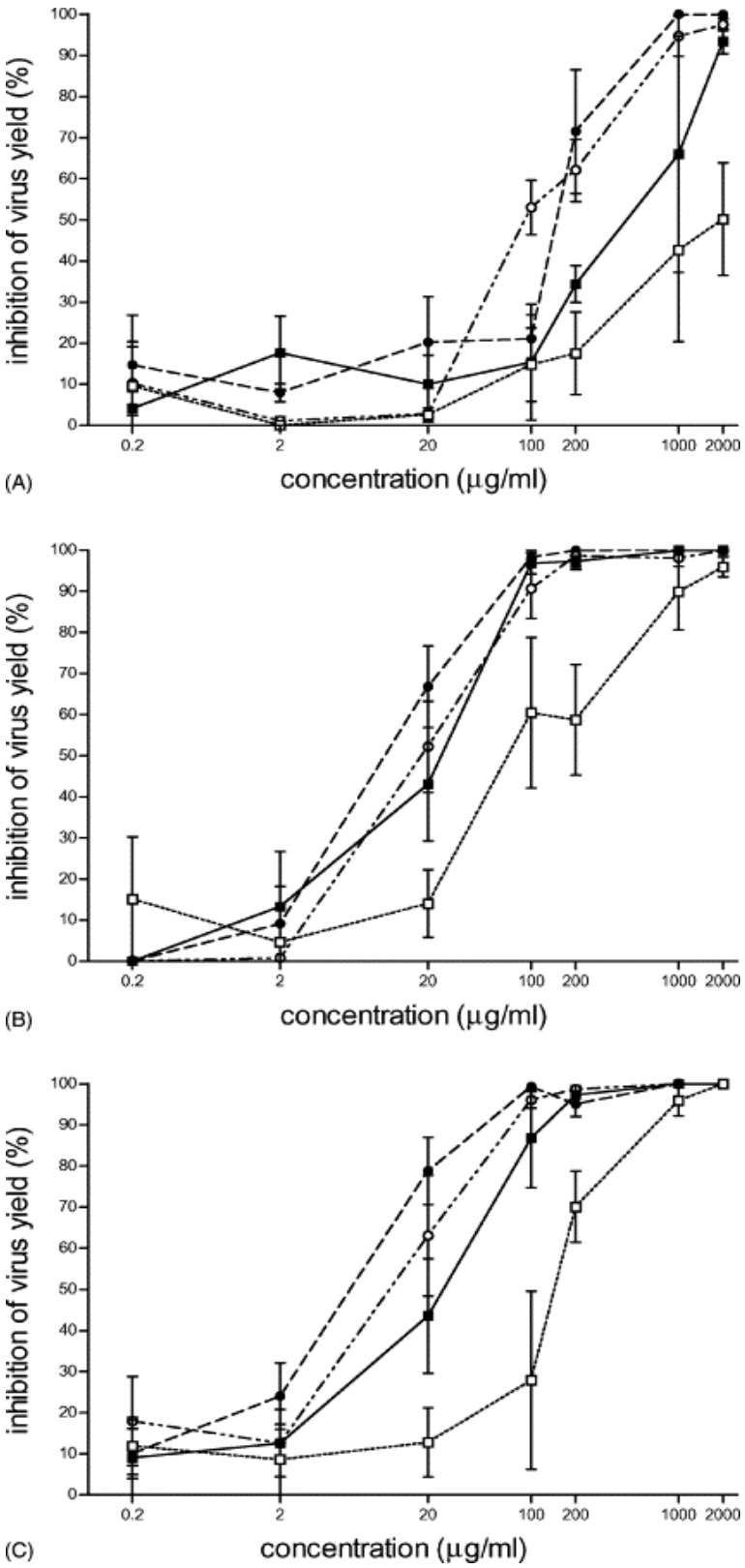


Figure 3.1 Dose–response of the inhibitory effect on the virus yield of 3-HP-α-lactalbumin (□), 3-HP-β-lactoglobulin (■), 3-HP-albumin (○) and 3-HP-lysozyme (•).

A 2020 in vitro study conducted by Afrin *et al*, investigated the antiviral potential of *β*-lactoglobulin against Avian Influenza virus. Strains of avian flu viruses are a persistent threat to public health (Li, *et al*. 2019). Afrin *et al's* study displayed that the anti-viral impact of *β* -lactoglobulin was concentration dependent over a 24-hour period.

A glycoprotein called hemagglutinin is present in the external coating of the virus. This plays a role viral replication as it facilitates the virus having the ability to associate to cell receptors on the surface of a host cell. It is currently understood that salt bridges inside the hemagglutinin (HA) monomers on a virus's surface, play a critical role in its ability to fuse with receptors on the surface of human host cells. It is from this, that it is thought a methylated *β* -Lactoglobulin that is saturated with a positive charge could play a role in inhibiting the action of hemagglutinin (HA).

To test this theory, Afrin *et al.* firstly esterified *β* -Lactoglobulin with a range of alcohols including: methanol, ethanol, n-propanol, and isopropanol, over a range of acidities in order to generate methylated *β* -Lactoglobulin (Met-BLG), which has an increased in positive charges on its surface.

The impact of Met-BLG when applied to viral cells was observed under electron microscope, as per **Figure 4.2**.

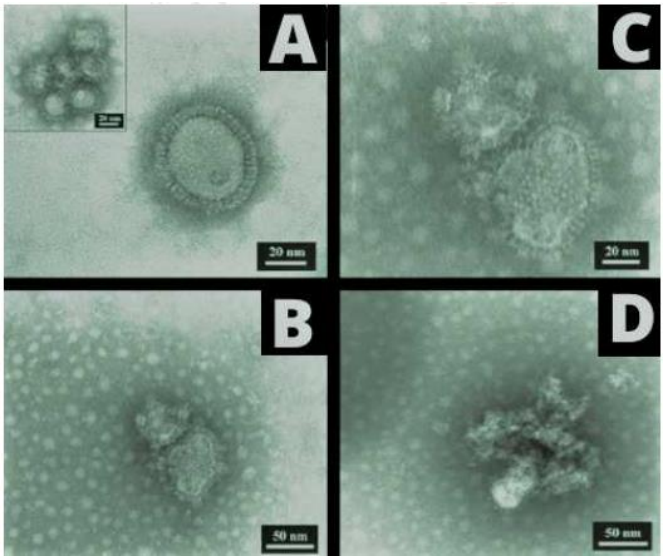


Figure 4.2 Electron microscope images of flu virus H1N1, before and after being exposed to Met-BLG.

***** A= local structure, B - D = in contact with 50 mg/ml Met-BLG At room temperature for 1 hr

From **Figure 4.2** above, it can be observed that the introduction of 50 mg/ml of Met-BLG, which is positively charged, caused the viral coat looks twisted and distorted (B – D). It is postulated that this distortion to the (HA) monomers on the viral surface inhibits its ability to fuse to receptors present on the surface of host cells by disrupting the electrostatic charge of hemagglutinin action (HA), thus impacting its ability to fuse to and spread infection to the host cell.

The impact of Met-BLG on the infection development curve was monitored in this study, whereby the advancement of H1N1 infection contaminated MDCK cells was compared against infected cells treated with Met-BLG by monitoring levels of HA and replicated RNA via RT-PCR over a 48-hour window at 37.8°C. Madin-Darby canine kidney (MDCK) cells are commonly used as a model mammalian cell for studying viruses (Takada, *et al.* 2019). This evaluation was carried out in triplicate, with the mean and standard deviation figures obtained being presented as the results in **Figure 4.0.3**.

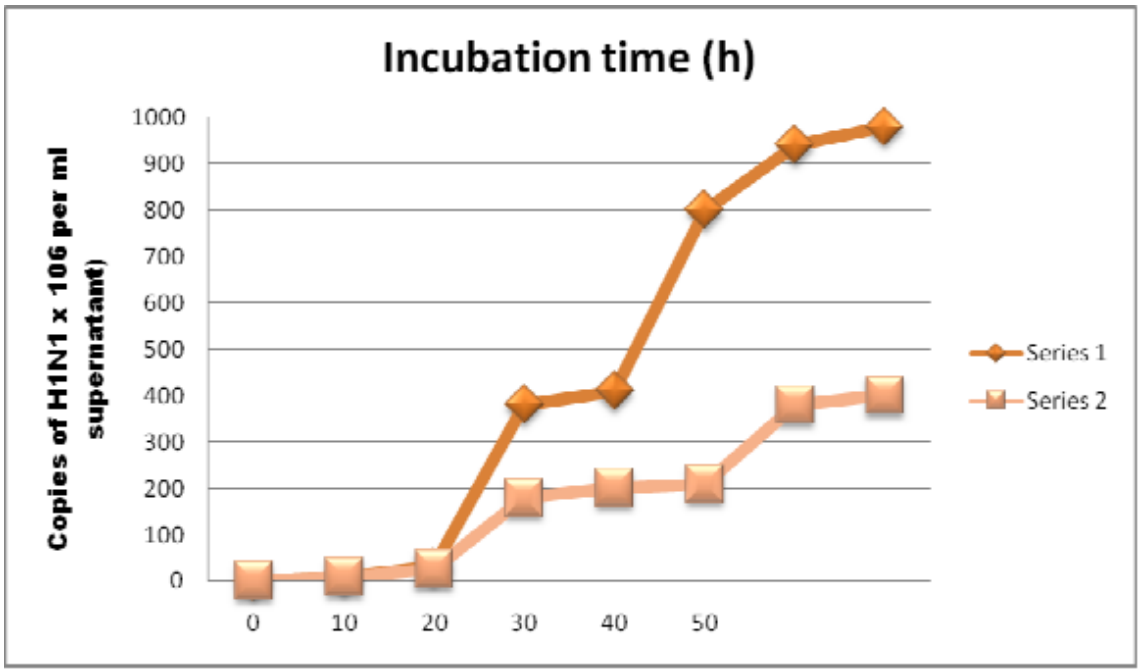


Figure 4.0.3 Advancement of hem agglutination movement and of the measure of imitated RNA (tested by RT-PCR) of H1N1 infection filled in MDCK cells in the absence of (Series 1) and presence of (Series 2) 50 mg/ml Met-BLG throughout a 48-hour window of observation.

It was observed that the hemagglutinin action (HA) of the control reached exponential infection of cells after 24 hours. In contrast, series 2, which shows the cells treated with

50 mg/ml Met-BLG displayed retarded proliferation – demonstrating the inhibitory action of Met-BLG on the hemagglutinin action of H1N1 viral cells.

3.3 Antiviral Effects of Lactoferrin

Lactoferrin is perhaps the most studied of the whey derived bioactive peptides, due its presence in both human and bovine milk. It plays a vital role in the transportation of iron to the gut, making it a key ingredient that can be utilised in infant nutrition formulations (Tsermoula, *et al.* 2021).

A number of both in-vitro and in-vivo studies have been conducted in order to investigate the proposed anti-viral activity that Lactoferrin may be able to impart.

In a double-blind, in-vivo trial, Iwasa, *et al.* (2002) assessed the effect of lactoferrin in 27 patients (16 men and 11 women), over a wide age range, who suffered with chronic hepatitis C, exhibiting high viral loads and HCV genome subtype 1b.

They were randomized into two groups, a low dose and a high dose group; who over the course of a six-month period received doses of 0.4g and 3.6g of lactoferrin per day respectively. The serum concentration of HCV RNA in the high dose group reduced after the six-month period obtaining a p value of < 0.01 (Iwasa, *et al.* 2002).

Ammendolia, et al. 2012, conducted an in-vitro assessment of bovine Lactoferrin against a range of influenza viruses, including: H1N1, H3N2, H5N1 and H7N1 subtypes, via the method of Hemagglutination inhibition assay (HI). This study also went a step further to investigate which portion of bLf was actively involved and responsible in the inhibition of influenza viruses by splitting bLf into its C and N-lobes and investigating the antiviral potential of each lobe individually. From the results obtained, it was concluded that all viruses HI activity was exclusively expressed by bLf C-lobe. It was found that bLf, ranging in concentrations from 0·05 pM to 6 nM had the ability to inhibit Hemagglutination activity (HA), of all tested viruses, including the above mentioned H1N1, H3N2, H5N1 and H7N1 subtypes (Ammendolia, *et al.* 2012).

	Viral strain	Subtype	CC50*	EC50°	SÎ
Lactoferrin	A/Roma-ISS/2/08	H1N1	>25 µM	25±0·95 pM	>106
	A/Parma/24/09	H1N1	>25 µM	25±1·01 pM	>106
	A/Parma/05/06	H3N2	>25 µM	0·25Â±0·01 nM	>105
C-lobe	A/Roma-ISS/2/08	H1N1	>25 µM	10±0·62 pM	>2·5 x 10^6
	A/Parma/24/09	H1N1	>25 µM	10±0·81 pM	>2.5 x 10^6
	A/Parma/05/06	H3N2	>25 µM	50±1·96 pM	>5·0 x 10^5
N- lobe	No data available				

Table 3.1 In vitro antiviral activity of bLf and C-lobe towards influenza virus infection

*** Taken from:** (Ammendolia, *et al.* 2012).

Given the growing body of evidence supporting lactoferrin as a useful antiviral tool against infectious diseases, as well as the outbreak of a global pandemic in late 2019 more recent research has focused in on investigating the impact of lactoferrin against the SARS-CoV-2 virus.

In February 2021, Hu, *et al.* published an in-vitro study which investigated the antiviral activity of lactoferrin against SARS-CoV-2. For the initial screening, the author evaluated both human lactoferrin (HLF) and bovine lactoferrin (BLF). From the results of this initial test, as observed in **Table 3.2**, BLF had significantly more of an impact on SARS-CoV-2 than that of HLF. For this reason, the remainder of the study focused on BLF.

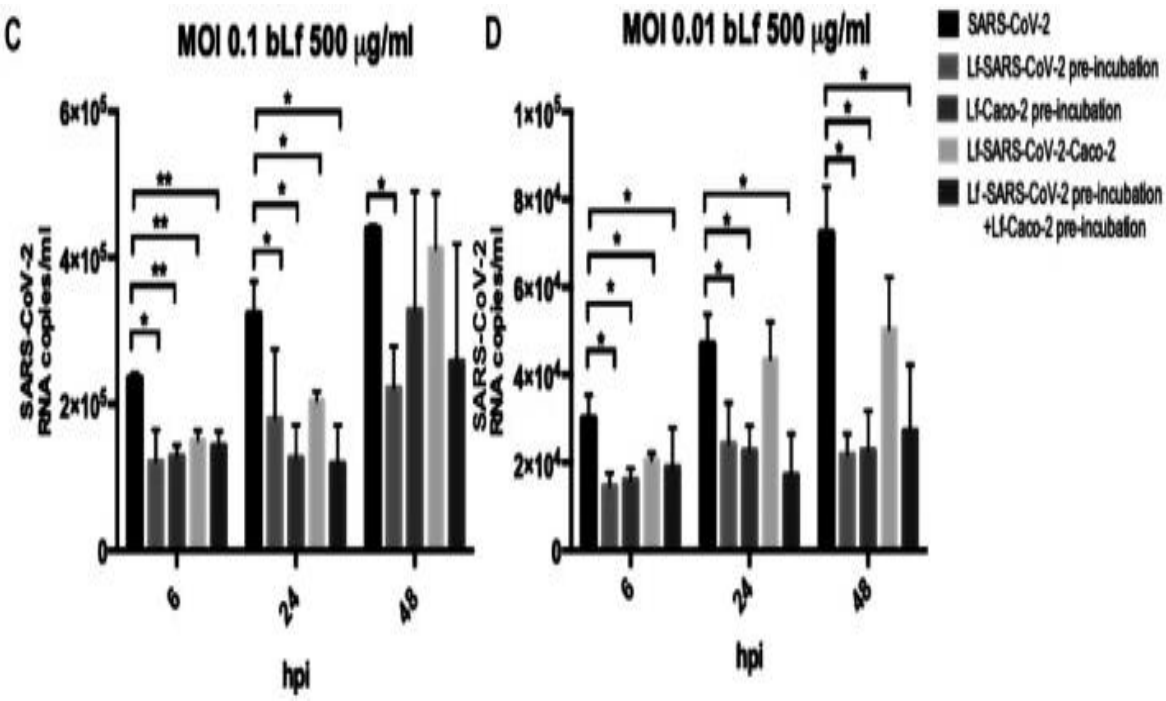
HCoVs	EC50 and CC50	BLF	HLF
SARS-CoV-2 (Vero E6 cell)	EC50 (µg/ml)	571.5 ± 72.8	N.T.
	CC50 (µg/ml)	>10,000	>10,000

Table 3.2 EC50 and CC50 values of BLF and HLF against SARS-CoV-2 in cell culture.

Through extensive study of its viral structure, it has been concluded by many that SARS-CoV-2 attaches to either its animal or human host cells via interactions between the viral spike protein and the host cell receptor ACE2 (Clausen, *et al*. 2020). A follow-on outcome from this in-vitro study was to determine the mechanism by which LF interferes with viral attachment, either by through interacting with the host cell or the virus. The results suggested that BLF inhibits viral attachment through binding to host cells instead of the virus (Hu, *et al*. 2021).

Similar conclusions were also drawn later in 2021 by Campione, *et al.* through a combination of both in vitro and in silico experimentation. The mechanism of lactoferrins antiviral action was also observed to be through binding to the host cell and not through interacting directly with the virus (Campione, *et al.* 2021).

Cells were firstly pre-incubated with bLf before being exposed to and infected with SARS-CoV-2. Supernatant of the viral samples were collected at the set time points of: 6, 24, and 48 hours post infection (hpi). Viral loads were determined using the quantitative method of rT-PCR.



From this study, a decrease of viral load up to 24 hpi was observed for MOI 0.1 compared to untreated SARS-CoV-2 infection ($p < 0.001$ after 6 hpi and $p < 0.05$ after 24 hpi for MOI 0.1) (Figure 2C). Although at MOI 0.01 the decrease of viral load remained statistically significant up to 48 hpi compared to untreated SARS-CoV-2 infection ($p < 0.05$) (Figure 2D). Data represent the mean values of three independent experiments. Error bars: standard error of the mean. Statistical significance is indicated as follows: *: $p < 0.05$, **: $p < 0.001$ (Unpaired student's t test).

3.3.1 Lactoferrin Intervention Study

Campione, *et al.* (2021) continued to publish research which developed on the knowledge obtained from the in-vitro study discussed above, by conducting a preliminary clinical trial which investigated the efficacy of an oral and intranasal bovine lactoferrin formulation when treating asymptomatic COVID-19 patients, as well as those suffering from mild-to-moderate symptoms. This was a short-term study was conducted between the months of April and June 2020.

Campione recruited a total of 92 patients with COVID-19, confirmed through rRT)-PCR nasal swab testing. Patients were divided into three groups. The first group consisted of 32 patients who had tested positive for SARS-CoV2. The second group included 14 SARS-CoV2 patients that required hospitalization and the final group consisted of 18 individual who were in home-based isolation. 32 healthy volunteers who were confirmed to test negative for COVID-19 infection via rRT)-PCR nasal swab testing, were used as a control group for the study.

Patients received oral and intranasal liposomal bLf; 32 hospitalized patients were treated only with a SOC regimen (hydroxychloroquine and lopinavir/darunavir); while 28 patients in home-based isolation did not receive any anti-COVID-19 treatment.

Demographic Data		Liposomal bLf Treated COVID 19 Group		SOC – Treated COVID-19 Group		Untreated COVID-19 Group		Control Group (COVID-19 Negative	
		Mean ± SD	N %	Mean ± SD	N %	Mean ± SD	N %	Mean ± SD	N %
Age		54.56± 16.86		49.9 ± 13.20		41.32 ± 11.77		52.80 ±15.54	
Sex	**Male**		14 (44%)		17 (53%)		10 (36%)		13 (41%)
	Female		18 (56%)		15 (47%)		18 (64%)		19 (59%)
Asymptomatic Patience			10 (31%)		3 (10%)		12 (43%)		
Mild-to-moderate patience			22 (68.7%)		29 (90%)		16 (57%)		

Table 3.3 Demographic of the patients enrolled in the preliminary clinical trial

* bLf = bovine lactoferrin, SOC = Standard of Care SD = Standard Deviation

*** Taken from:** Campione, *et al.* (2021)

The liposomal bLf-treated COVID-19 patients required a mean time of 14.25 ± 6.0 days in order to test negative for SARS-CoV2, which was a shorter time period than that observed in the SOC-treated and the untreated with bLf COVID-19 groups (p-value < 0.0001). The SOC-treated COVID-19 patients showed a mean time of 27.13 ± 14.4 days to test negative for SARS-CoV2, whereas the untreated COVID-19 patients revealed a mean time to test negative of 32.61 ± 12.2 days, as showcased below in **Table 3.3.**

	Number of Enrolled Patients	Mean Time ± SD to rRT-PCR SARS-C0V-2 RNA Negative Conversion of Naso-Oropharyngeal Swab	*p*-Value
Liposomal bLf Treated COVID-19 Group	32	14.25 ± 6.0	
SOC – Treated COVID-19 Group	32	27.13 ± 14.4	<0.0001
Untreated COVID-19 Group	28	32.61 ± 12.2	<0.0001

Table 3.3 Mean time and standard deviation (SD) to reverse transcriptase real time (rRt)-PCR SARS CoV-2 negative conversion in liposomal bovine lactoferrin (bLf0-treated, standard of care (SOC), or untreated COVID-19 patients.

3.4 Synopsis of Major Findings

The overall findings of the study found linkage between the selected bioactive peptides (β-Lactoglobulin, α-Lactalbumin and Lactoferrin) and the suppression of viral attachment to host cells, thus preventing their ability to proliferate an infect a larger number of cells within the body, leading to infection and subsequent symptoms.

Chapter 4

Discussion

The purpose and objective of this study was to assess the therapeutic potential of bioactive peptides derived from bovine whey as antiviral agents against viral infections. Globally, the most prevalent viral infections that have high rates of infection and associated mortality rates particularly amongst those who find themselves to be immunocompromised, as listed by the National Foundation for Infectious Diseases include: Influenza, Human Papillomavirus (HPV), HIV/AIDS and Coronaviruses, to name but a few (National Foundation for Infectious Diseases. 2022). In recent centuries, a number of medical advances and global strategies such as vaccination programmes and use of antiviral medication have been deployed in order to suppress the spread of infectious viral diseases. There however a number of drawbacks to these strategies.

While the roll out of wide scale vaccination programmes have proven to be an effective measure in controlling the spread and lessening the severe symptoms of infectious viral diseases, viruses are constantly mutating and evolving in order to prolong their ability to spread infection amongst species. This process is referred to as antigenic drift – in which a virus will change its surface proteins (HA and NA), thus changing the way it interacts with and attaches to host cell membranes. These small changes in antigenic drift can overtime, reduce the effectiveness and level of protection that vaccines can offer (Centers for Disease Control and Prevention. 2021).

Antivirals are only prescribed under medical supervision, more often than not, serious side effects such as diarrhoea, nausea, sinusitis, nasal congestion, bronchitis, cough, headache, dizziness, as well as ear, nose, and throat infections can ensue (Centers for Disease Control and Prevention. 2016). Furthermore, antiviral medications can be quite costly, which can hinder their wide scale roll out at a global scale, which ultimately leads to the most vulnerable of cohorts remaining exposed to the devastating impacts of viral infections (Gambacorta, et al. 2021).

It is by realising the drawbacks of these traditional approaches used to combat the spread of, as well as limiting the economic and mortality impact of viral infections that the research question put forward for this study chose to investigate the potential therapeutic role that bovine whey derived bioactive peptides could play.

This study aimed to investigate the proposed mechanisms of action by which three selected whey derived bioactive peptides (β-Lactoglobulin, α-Lactalbumin and Lactoferrin) and their ability to inhibit viral attachment to host cell membranes. This

investigation provided a detailed overview of bovine milk and the various industrial processes that can be applied in order to valorise it, and its subsequent by-products. Definition was given to the terms milk, whey, peptide and bioactive peptide, as well as understanding the volumes of bovine milk is produced globally.

Figure 1.2 looked at the Global milk volumes produced by different species year on year for the period between 2010 – 2019. It is noted that cow milk volumes reached over 700 million tonnes in 2019, while other sources of milk available globally are small in comparison (International Dairy Federation. 2019). These figures supported that the focus of this study should be maintained of peptides derived from bovine milk only, due to it being produced at a global scale across a number of regions. The volumes of bovine milk derived whey that are generated from the cheese production process were put into perspective, with it being noted that for every 1kg of cheese that is manufactured, a corresponding volume of about 10 litres of whey is generated (Pires, *et al*. 2021). If this is taken into account for sweet whey alone, a Eurostat report published by the European Commission Statistical Office in 2019 reported volumes in the region of 146 million tonnes of cow's milk being produced throughout Europe, of which up to 50% of this whole milk went forward for the cheese manufacturing process. This works out at just under 55 million tonnes of liquid whey available for further processing and valorisation into nutritional ingredients (European Commission. Statistical Office of the European Union. 2020), (Tsermoula, *et al.* 2021).

The peptides present and their percentage concentration in the whey fraction of bovine milk were outlined in **Table 1.7**. Due to their availability at a commercial scale, as a result of their higher concentration percentage to the overall protein content of whey, this study focuses on the antiviral potential of the protein peptides: β-Lactoglobulin, α-Lactalbumin and Lactoferrin.

Definition was given to a virus and a detailed overview of the viral replication cycle in within a human host cell was provided in **Section 1.8**. The five steps of viral replication; Attachment, Penetration, Amplication, Assembly and Release, were summarized in basic terms. The conditions, associated cost (in terms of mortality) and prevalence of the current most common infectious diseases globally were described, as well as the mechanisms by which the human immune system deals with infectious diseases and how vaccines and antiviral medication work to counteract viral infections, as well as their associated risks

and side-effects were discussed. The estimated economic cost of viral infections at a global scale was also briefly touched on in the introductory chapter.

In response to the need for a wide range of strategies and therapies in order to counteract the spread of infection and their associated symptoms, bioactive peptides derived from bovine whey offer a widely available and cost-effective option

From the research, it has been established that the main mechanisms of action by which the three whey derived bioactive peptides work against infectious viral infections mainly involved these peptide interfering with the virus's surface proteins, thus limiting and even preventing attachment to the host cell membrane proteins from occurring. This restricted the rate at which viruses could proliferate amongst host cells, allowing adequate time for the body's own natural immune system to register that an infection is occurring when *MHC class I* molecules reach the surface of the host cell. This in turn sends a signal to the T cells that the cell is infected. The majority of the studies within this research topic are in-vitro based,

The findings from investigating the antiviral effects of whey derived beta-lactoglobulin, studies conducted by Neurath. et al. (1996), Sitohy. et al. (2010), Oevermann. et al (2003) and by Afrin et al. (2020) all derived a similar consensus that β-Lactoglobulin required some form of chemical or enzymatic modification. This was required in order to expose the specific bioactive components that have the ability to interact with viral surface proteins, limiting their ability to interact with host cell membranes. An excellent depiction of this was observed in Afrin et al. (2020) study, in Figure 4.2, where by electron microscope images of flu virus H1N1, before and after being exposed to Met-blg. After a certain exposer time, the capsid layer of the H1N1 virus appears to become distorted.

Lactoferrin is perhaps the most studied out of all three of the whey derived peptides discussed in this study. At present there is a major focus on the impact of Lactoferrin against the SARS-CoV2 virus. Through extensive study of its viral structure, it has been concluded by many that SARS-CoV-2 attaches to either its animal or human host cells via interactions between the viral spike protein and the host cell receptor ACE2 (Clausen, *et al*. 2020). A follow-on outcome from this in-vitro study was to determine the mechanism by which LF interferes with viral attachment, either by through interacting

with the host cell or the virus. The results suggested that BLF inhibits viral attachment through binding to host cells instead of the virus (Hu, *et al*. 2021).

Looking at the findings from this study in combination, a therapeutic benefit of bioactive peptides derived from the whey of bovine milk can be demonstrated by their ability to inhibit viral infections from proliferating and spreading in both in-vitro and in-vivo experimental settings.

Chapter 5

Conclusion & Future Work

5.1 Conclusion

In conclusion, whey protein derived from bovine milk is undoubtably a rich source of biologically active peptides that have the potential to play a role in new therapeutic strategies for the prevention of viral infections. There is a need for a wide range of alternative therapies and more affordable approaches in order to better counteract the spread and devastating impacts of harmful viral infections; especially for those who find themselves to be immunocompromised or prone to suffering side-effects from traditional anti-viral medications. Peptides derived from the whey of bovine milk offer this alternative to traditional treatments.

From the reviewed research, the selected whey derived peptides β-Lactoglobulin, α-Lactalbumin and Lactoferrin were shown to exert antiviral activity when evaluated against selected viruses of concern due to their prevalence at a global scale. Their mechanisms of action against infectious viral infections mainly involved these peptide interfering with the virus's surface proteins, thus limiting and even preventing attachment to the host cell membrane proteins from occurring. This restricted the rate at which viruses could proliferate amongst host cells, allowing adequate time for the body's own natural immune system to register that an infection is occurring when *MHC class I* molecules reach the surface of the host cell. This in turn sends a signal to the T cells that the cell is infected.

In conclusion, the antiviral potential of whey derived bioactive peptides is one such benefit that this study's findings have displayed a growing body of evidence is supporting – showcasing them as a viable, widely available and more cost-effective option to work in conjunction with or as an alternative to traditional vaccines and anti-viral medication.

5.2 Future Work

The selected whey derived peptides β-Lactoglobulin, α-Lactalbumin and Lactoferrin were shown to exert antiviral activity when evaluated against selected viruses of concern due to their global prevalence.

From the findings of this study, it has been noted that the majority of research has been conducted in-vitro, or amongst relatively small, short term human intervention studies. This area of research would greatly benefit from more long term in-vivo studies that are conducted globally, across a range of gender, age and diseased states in order to move a step closer in proving the efficacy of whey derived peptides as a suitable therapeutic application that is cost-effective for the treatment of; either in conjunction with or instead of traditional medical approaches.

A benefit could also be derived by increased research and development within the dairy industry to fund suitable applications for the waste streams that are generated from the isolation and extraction of these peptides. This would make the large-scale production of these peptides commercially viable.

Chapter 6

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