TÉCNICO LISBOA



A comparative study between the correlation of the *in silico* and *in vitro* hydrolysis of whey proteins

Discovery of potential new antimicrobial peptides

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“Life is not easy for any of us. But what of that?
We must have perseverance and above all confidence in ourselves.
We must believe that we are gifted for something and that this thing must be attained.”
- Marie Curie

Acknowledgments

"We are what we want to be', and in this way, we know that you will be what you want to be. Why?! Because you can't resist a challenge. Because you always try to go beyond. Because within the struggles you find what it takes to go on. Because you put yourself in everything that you do. This is what your parents see in you: character and determination". My parents wrote this for my High School graduation ceremony.

Taking my parents words, I really can't say no to a challenge, and I clearly don't give up that easily. See, my master thesis has truly been a challenge for me, and I'm not talking about my supervisor running of to Brazil during the most critical months of the work. I'm talking about all of the times I had no idea what I was doing, the times that my colleagues seemed to be so advanced in their work and I had not even started, the times they were all finishing and I was still in the lab. But I kept going. I was not going to be defeated.

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Alexandra Salvado

Abstract

Whey proteins are present in the yellowish liquid remaining after the precipitation of caseins, a procedure used in the cheese manufacture. Throughout the years, whey has become a high value product, not just because of its high biological value and content in essential amino acids, but also because it has been proven to be a precursor of a wide range of bioactive peptides. These bioactive peptides are inactive within the parent molecule but can be released by diverse methods, being the enzymatic hydrolysis the most common method.

This work is thus divided into two main goals. The first one was the release, *in silico,* of peptides from whey proteins upon hydrolysis with α-chymotrypsin and the characterization of the antimicrobial peptides. The second goal was the production, *in vitro*, of whey protein hydrolysates that resulted from the hydrolysis of whey also with α-chymotrypsin and their characterization in terms of molecular weight, isoelectric point, and antimicrobial potential against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Burkholderia contaminans.*

The *in silico* hydrolysis of whey proteins revealed 13 potential new peptides: α-LA f(80-99), β-LG f(78-98), LF f(19-27), f(85-91), f(102-111), f(113-123), f(129-138), f(291-297), f(443-446), f(594-608), f(701-705), and k-CN f(1-5) and f(83-95). The *in vitro* hydrolysis of whey proteins revealed that at least one of the peptides identified with antimicrobial activity mainly against *S. aureus* appears to correspond to the fragment LF f(443-446) with a molecular weight of 830 Da and a pI of 6.12 identified by Tricine SDS-PAGE and IEF.

Keywords: Whey proteins, hydrolysis, *in silico*, *in vitro*, α-chymotrypsin, antimicrobial peptides

Resumo

As proteínas do soro de leite estão presentes no líquido amarelado que resulta da precipitação das caseínas, um procedimento muito utilizado na indústria do queijo. Ao longo dos anos, o soro de leite tem-se tornado um produto de valor adicional, não só devido ao seu elevado valor biológico e presença de aminoácidos essenciais, mas também por ter sido comprovado como precursor de uma gama alargada de péptidos com atividade biológica. Estes péptidos estão inativos e normalmente encriptados dentro da sequência da proteína precursora e podem ser libertados através de vários métodos, sendo a hidrólise enzimática o mais utilizado.

Este trabalho está assim dividido em dois objetivos principais. O primeiro consistiu na hidrólise, *in silico*, de proteínas do soro do leite com α-quimotripsina e a caracterização dos péptidos antimicrobianos. O segundo objetivo consistiu na produção, *in vitro,* de hidrolisados de soro de leite, obtidos também pela ação da α-quimotripsina e a sua caracterização em termos de massa molecular, ponto isoeléctrico e potencial antimicrobiano em *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Burkholderia contaminans.*

Da hidrólise *in silico* resultaram treze novos péptidos antimicrobianos: α-LA f(80-99), β-LG f(78-98), LF f(19-27), f(85-91), f(102-111), f(113-123), f(129-138), f(291-297), f(443-446), f(594-608), f(701-705), and k-CN f(1-5) and f(83-95). Da caracterização dos hidrolisados obtidos *in vitro* foi identificado pelo menos um péptido, com atividade maioritariamente contra *S. aureus,* correspondente ao fragmento LF f(443-446), com um peso molecular de 830 Da e ponto isoelétrico de 6.12, identificado através de SDS-PAGE Tris-tricina e focagem isoelétrica.

Palavras-chave: Proteínas do soro de leite, hidrólise, *in silico*, *in vitro*, α-quimotripsina, péptidos antimicrobianos

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List of Abbreviations

Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	Angiotensin-converting enzyme
AMPs	Antimicrobial peptides
ANN	Artificial neural networks
APD	Antimicrobial Peptide Database
APTS	(3-Aminopropyl)triethoxysilane
B. cepacia	*Burkholderia cepacia*
BCA	Bicinchoninic acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BLF	Bovine lactoferrin
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
BV	Biological value
CAMP	Collection of Anti-Microbial Peptides
CEP	Cell envelope proteinases
CMP	Caseinemacropeptide
CPPs	Caseinophosphopeptides
DA	Discriminant analysis
DPP IV	Dipeptidyl peptidase IV
DPPH	2,2'-diphenyl-1-picrylhydrazyl
E. coli	*Escherichia coli*
ESI	Electrospray ionization
FAB	Fast atom bombardment
FGF	Fibroblast growth factor
FRAP	Ferric ion reducing antioxidant power
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucakon-like peptide 1
GRAS	Generally recognized as safe
HPLC	High-performance(pressure) liquid chromatography
HTST	High temperature short time
IEF	Isoelectric focusing
IEP	Isoelectric point
IGF	Insulin-like growth factor
IGs	Immunoglobulins
LAB	Lactic acid bacteria

LC	Liquid chromatography
Lc. lactis	*Lactococcus lactis*
LP	Lactoperoxidase
MALDI	Matrix assisted laser desorption ionization
MDR	Multi-drug resistant
MS	Mass spectrometry
ORAC	Oxygen radical absorbance capacity
P. aeruginosa	*Pseudomonas aeruginosa*
PDGF	Platelet-derived growth factor
PG	Propyl gallate
p-κ-CN	Para-κ-casein
RF	Random forest
S. aureus	*Staphylococcus aureus*
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SVM	Support vector machines
SW	Sweet whey
SWH	Sweet whey hydrolysate
TBHO	Tert-butylthydroquinone
TCA	Trichloroacetic acid
TEAC	Trolox equivalent antioxidant capacity
TGF	Transforming growth gactor
UHT	Ultra-high temperature
WPC	Whey protein concentrate
WPI	Whey protein isolate
α-LA	α-Lactalbumin
α_{s1}-CN	α_{s1}-Casein
α_{s2}-CN	α_{s2}-Casein
β-CN	β-Casein
β-LG	β-Lactoglobulin
κ-CN	κ-Casein

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Chapter I – Introduction

I.1. Study Motivation

Whey, one major part of milk, has traditionally not been paid as much attention as happened with source milk, probably because it is a by-product of the cheese industry, viewed for many years as of little value, besides additive to animal feed. However, recent decades have witnessed an increased interest in whey protein products, owing to their nutritional role, and more and more to their active role upon human health[1].

Over the past approximately 50 years, the transformation of whey from a waste to a high value product has resulted in increasingly sophisticated products, which consequently enhanced the wealth of dairy manufacturers and the communities that rely on them. Now that whey and whey proteins are prized by the dairy industry, what does the future hold for these once maligned co-products of cheese and casein manufacture? While the future is yet to be written, science and technology advances that have taken place over the recent past, allowed whey proteins and peptides to be seen as preferential ingredients for functional foods and nutraceuticals, and also as active medicinal agents[2].

Considering the great potential of whey as a source of bioactive peptides, an effective knowledge on the production and characteristics of these peptides would be very relevant. In particular, the focus has been on the generation of new potential antimicrobial peptides that could be an alternative to antibiotics presently available at the market and whose activity has been discredited by multidrug resistance bacteria. Additionally, taking into account that whey is an ingredient in several food formulations such as whey protein powders for bodybuilders, a whey-derived product rich in bioactive peptides would be an interesting added-value product. Indeed, commercial proteases have been successfully tested for the production of bioactive hydrolysates from whey proteins, with special attention given to peptides showing antioxidant, antihypertensive and antimicrobial activities[3].

I.2. Objectives and Outline

This work focused on the release of peptides upon non-specific hydrolysis of whey proteins with α-chymotrypsin and their assessment as antimicrobial peptides against models of gram-negative and gram-positive bacteria. It also aims to correlate the *in silico* and the *in vitro* production of antimicrobial peptides derived from whey proteins.

Initially, a revision of milk proteins, caseins and whey proteins, and the evolution of the valorization of whey as a source of bioactive peptides is presented. Also, a definition for bioactive peptides and the methodologies behind their production, since their release from the parent molecule to their synthesis, is also outlined. Moreover, the different bioactive peptides derived from whey are also explored accordingly to their activity.

Chapter II – Milk and Milk Proteins

This chapter focuses mainly on the role of milk proteins to young and adult mammals, including humans, discussing their nutritional and physiological value. Furthermore, the two major families of milk proteins are reviewed, providing the reader with a brief description of each of their main constituents.

The major role of milk proteins is to supply amino acids to young mammals and constitute an important part of dietary proteins for the adult. Intact milk proteins also have specific functions such as micelle formation[4]. Furthermore, milk proteins have physiological importance, they facilitate uptake of several important nutrients such as trace elements and vitamins and contain a group of proteins which perform a protective function, therefore indicating their importance as highly functional substances[5].

From the nutritional point of view, the protein fraction of milk contains many valuable components and biologically active substances capable of influencing the development and growth of the gastrointestinal tract, other specific organs immunoregulation, and modulation of the gut microflora population[6]. Also, from an evolutionary perspective, it also provides a mechanism to confer health potential benefits for the support of infant development and growth beyond basic nutrition, in the form of bioactive peptides released in the intestine via digestion.

Milk is often described as a complex biological fluid and as a colloidal suspension, containing water, emulsified globules of fat, a heterogeneous family of major and minor proteins, the carbohydrate lactose, minerals, vitamins and enzymes[7]. An overview of the composition of bovine milk is given in Table 1.

Table 1. Proximal analysis of bovine milk. Adapted from Illanes, A. (2011)[8].

	Milk	
Component	**% wet basis**	**% dry basis**
Caseins	2.8	21.9
Whey Proteins	0.7	5.5
Lactose	4.9	38.3
Fat	3.7	28.9
Ash	0.7	5.4
Total Solids	12.8	100

The insoluble caseins and the soluble whey proteins constitute the major families of proteins in the protein system of milk. While caseins account for 80% (w/w) of the whole protein inventory, whey proteins account for the remaining 20% (w/w) of the whole protein inventory[9]. The distribution of these proteins in bovine milk is depicted in Figure 1.

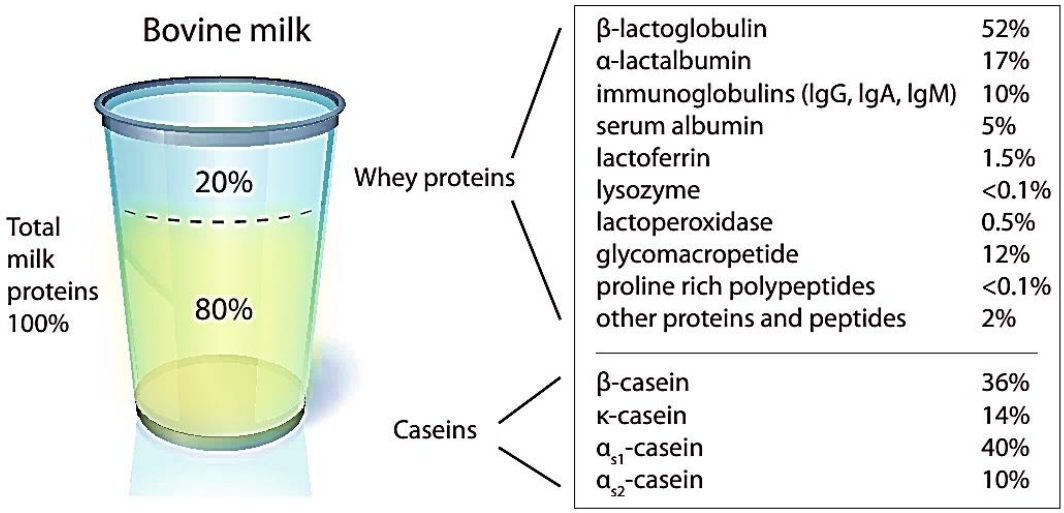

Figure 1. Typical content of milk proteins in mature bovine milk. Shown are the major milk proteins, as well as additional proteins with relevance in health. The contents of proteins are expressed as percentage (%) of whey proteins and caseins in the total milk proteins and also whey proteins as a percentage of total whey proteins and caseins as a percentage of total caseins. Adapted from Artym, J. *et al.* (2013)[9].

II.1. Caseins

Caseins are phosphoproteins that can easily be recovered from skimmed milk via isoelectric precipitation at pH 4.6 by addition of, or *in situ* production of acid or rennet-driven coagulation[10]. In the cheesemaking industry, the most common procedure for the precipitation of caseins is conducted by the enzyme chymosin, that specifically cleaves the Phe_{105}-Met_{106} bond of κ-casein, to give two macropeptides – a hydrophobic N-terminal extreme named para-κ-casein (p-κ-CN), and a hydrophilic C-terminal moiety called caseinemacropeptide (CMP)[11].

Caseins interact with calcium and calcium phosphate, and self-aggregate to organize into a supramolecular structure known as the casein micelle. The central physiological function of the casein micelle is to supply proteins, phosphate and calcium to neonates[12]. In milk, almost all caseins are organized in casein micelles with an average diameter of about 120 nm containing 93% proteins and 7% inorganic salts, mainly calcium and phosphate[13]. The casein micelles contain the four caseins: α_{S1}, α_{S2}, β and κ in an approximate ratio of 4:1:3.5:1.2[13].

II.1.2. α_{S1}-Casein

The α_{S1}-casein (α_{S1}-CN) family represents about 40% and shows a diverse secondary structure. The percentage of α-helix in α_{s1}-CN has been estimated in a range between 5 and 20%. For β-sheet, values of 17–20% were reported whereas for β-turn structures, the value is 29-35%. Moreover, α_{s1}-CN can self-associate in dimers, tetramers, hexamers and others, and this is dependent on pH and ionic strength. Also, α_{s1}-CN is one of the calcium-sensitive caseins and its precipitation occurs in the range of 3-8 mM $CaCl_2$. In addition, the concentration of calcium required to induce precipitation of α_{s1}-CN also increases with increasing ionic strength, but not proportionally to calcium binding. The molecular weight of α_{S1}-CN is between 22.07 and 23.7 kDa[14].

II.1.3. α_{S2}-Casein

The α_{S2}-casein (α_{S2}-CN) family constitutes up to 10% of the total casein fraction in bovine milk and exhibits varying levels of phosphorylation and intermolecular disulfide bonding. In α_{S2}-CN isolated from bovine milk (25.23 kDa), more than 85% of the protein is in monomeric form containing the intramolecular disulphide bond, with the remaining fraction consisting of dimers. Various suggestions have been made for the composition of the secondary structure of α_{S2}-CN, but mainly it consists in α-helix, β-sheets, and turns[14]. Also, its structure is amphipathic and highly charged, so its self-association properties strongly depend on ionic strength. α_{S2}-CN exhibit consecutive self-associations, the extent of which reaches a maximum at an ionic strength of 0.2-0.3 at 20°C, but decreases at higher ionic strength[14]. Moreover, since α_{S2}-CN has the highest number of phosphorylated residues, it is also the most sensitive casein to calcium-induced precipitation. Calcium-induced precipitation of α_{S2}-CN occurs at calcium concentrations less than 2 mM[14].

II.1.4. β-Casein

The β-casein (β-CN) family constitutes up to 35% of the casein of bovine milk and is highly amphipathic. This 209 amino acid protein has a molecular weight which is increased from 23.6 kDa to 24.0 kDa following phosphorylation of its five Ser residues. The pI of the non-phosphorylated amino acid is estimated at 5.1, which decreases to approximately 4.7 as a result of phosphorylation[14]. In relation to the secondary structure of β-CN, this is mainly composed of α-helix. Moreover, the presence of distinct polar and hydrophobic domains makes its self-association behavior extremely dependent on temperature. At 0–4°C, primarily monomers of β-CN are observed and as the temperature is increased above 4–5°C, it undergoes a highly cooperative, reversible, rapidly equilibrating discrete self-association, yielding large polymers[14]. Considering the interaction of β-CN with calcium, this casein is less sensitive to calcium-induced precipitation than α_{S1}-CN and α_{S2}-CN. Nonetheless, at 37ºC, β-CN precipitates in the range of 8-15 mM Ca^{2+}. However, at 1°C, β-CN remains in solution at concentrations up to 400 mM $CaCl_2$. In this way, binding of calcium by β-CN increases with increasing temperature, whereas an increase in ionic strength reduces the binding capability. In addition, the binding of calcium by β-CN decreases with decreasing pH[14].

II.1.5. κ-Casein

Within the caseins, κ-casein (κ-CN) displays some rather unique features. It is the smallest of the caseins, it has a low level of phosphorylation, a low sensitivity to calcium and is the only one of the caseins to occur in the glycosylated form. This highly glycosylated casein, preferentially localizes at the periphery of the micelle and forms a layer at the protein-water interface, stabilizing the structure and preventing it from aggregating[10].

κ-CN displays an unevenly distributed hydrophobicity and charge throughout the protein's169 amino acid residues. This protein as a reported molecular weight of around 19 kDa and a pI of approximately 5.9 if the post-translational modifications are not accountable[14]. Regarding the secondary structure of this protein, estimates suggest that κ-CN may contain 10-20% α-helix, 20-30% β-structure and 15-25% turns. However, the degree of estimated α-helical structure has been shown to increase with increasing temperature (10–70°C), while the proportion of β-structure and turns decreases with temperature[15]. Several structural motifs have also been suggested, including possible antiparallel and parallel β-sheets or βαβ structure in the hydrophobic domain and a β-turn-β-strand-β-turn motif centered on the chymosin-sensitive Phe_{105}-Met_{106} region[14]. Also, this latter motif appears to be conserved in κ-CN from various species, as would be expected for specific sensitivity to aspartyl proteinases[14]. Finally, in terms of interaction with calcium ions, unlike the above mentioned caseins, κ-CN is calcium insensitive, meaning that it does not precipitate in the presence of excess calcium.

II.2. Whey Proteins

Cheese whey is a green-yellowish liquid resulting from the precipitation and removal of milk casein in cheesemaking processes[16]. After the casein curd separates from the milk, following coagulation of the casein proteins through the action of chymosin (rennet) or mineral/organic acid, the remaining watery and thin liquid is called whey. Depending on the type of precipitation the whey can be called sweet or acid. Acid whey – the pH of which is equal to or smaller than 5.1 is obtained by direct acidification of milk, whereas sweet whey – the pH of which is equal to or higher than 5.6, is obtained after rennet-coagulation, as happens in most cheesemaking processes worldwide[17]. Whey can be made from any type of milk, with cow's milk being the most popular in western countries, while in some regions of the world, goats', sheeps', and even camels' milk can be used in the manufacture of dairy products that result in the generation of whey[17]. Whey proteins are globular molecules with a substantial content of α-helix motifs, in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a fairly balanced way along their polypeptide chains[18]. The proteins that constitute the family of whey proteins are β-Lactoglobulin (β-LG), α-Lactalbumin (α-LA), immunoglobulins (IGs), bovine serum albumin (BSA), bovine lactoferrin (BLF) and lactoperoxidase (LP), together with other minor components[19].

II.2.1. β-Lactoglobulin

β-LG is quantitatively the dominant whey protein (58% w/w in cow's milk) and was first discovered in 1934[20]. When in its isolated form, it exhibits a low solubility (despite its globular nature). Synthesized in the mammary gland of ruminants (and other species) and designed to be included in milk, this protein has several genetic variants – of which β-LG A is the most common. It is composed mainly of β-sheet motifs, and consists of 162 amino acid residues, which lead to a molecular weight of approximately 18 kDa[21].

II.2.2. α-Lactalbumin

α-LA is quantitatively the second most important protein in whey, representing approximately 20% (w/w) of the total whey protein inventory, and is fully synthesized in the mammary gland. Here, α-LA acts as coenzyme for biosynthesis of lactose – an important source of energy for the newborn[22]. It contains 123 amino acid residues, which lead to a molecular weight of approximately 14 kDa[23].

II.2.3. Bovine Serum Albumin

BSA is not synthesized in the mammary gland, but appears instead in milk following passive leakage from the blood stream. It contains 582 amino acid residues, which lead to a molecular weight of nearly 66 kDa[24]. Because of its size and higher levels of structure, BSA can bind to free fatty acids and other lipids, as well as flavor compounds – a feature that is severely hampered upon denaturation[25].

II.2.4. Immunoglobulins

IGs constitute a complex group, the elements of which are produced by B-lymphocytes and they make a significant contribution to the whey protein content – besides exerting an important immunological function. These proteins are present in the serum and physiological fluids of all mammals; some of them attach to surfaces, where they behave as receptors, whereas others function as antibodies, which are released in the blood and lymph. IGs are subject to postnatal transfer via colostrum – as the placenta does not permit passage of macromolecules[26]. In terms of quaternary structure, IGs are either monomers or polymers of a four-chain molecule, consisting of two light polypeptide chains (with a molecular weight in the range of 25 kDa) and two heavy chains (with molecular weight between 50-70 kDa)[27]. There are three basic classes of IGs: IG_G, IG_A and, IG_M. Up to 80% (w/w) of all IGs in milk or whey is accounted for IG_G[28].

II.2.5. Lactoferrin

Bovine LF is an iron-chelating, monomeric glycoprotein, characterized by a molecular weight of 80 kDa to which two carbohydrate groups are attached[29]. It is present in the human body as a secretory protein synthesized by glandular epithelial cells and mature neutrophils, and

can be found in milk, saliva, tears, nasal and intestinal secretions, pancreatic juice and seminal fluid, as well as in secondary granules of neutrophils[29].

II.2.6. Lactoperoxidase

LP is present in a variety of animal secretions like tears, saliva and milk. A member of the family of mammalian peroxidases, it is one of the most abundant enzymes in plain milk – it represents approximately 1% (w/w) of the total protein pool in whey[30]. The complete LP system (enzyme plus substrate) was originally characterized in milk by Reiter *et al.* (1963)[31] and its activity depends on many factors such as animal species, breed and lactation cycle[32].

Chapter III – Valorization of Whey

This chapter outlines the importance that whey proteins have gained throughout the years. The reader is guided through key events that have transformed whey from a waste product of the cheesemaking industry to a valuable source of proteins and peptides that have been shown to have important roles in the human health and nutrition.

As mentioned before, whey corresponds to the liquid fraction remaining after milk clotting and casein removal during cheese manufacturing, and represents an abundant by-product of the dairy industry, which is characterized by about 85-90% of milk volume and retains approximately 55% of milk nutrients[33].

Historically, whey has been considered a waste stream and nuisance by cheesemakers and casein manufacturers, and dairy companies have chosen the most economical management of whey – disposal methods[2]. Such methods have included: i) spraying the whey onto fields, ii) discharging the whey into rivers, lakes, or the ocean, iii) discharging the whey into municipal sewage system, and iv) selling the whey for a low return as animal feed. All these approaches have been based on the premise that this dairy stream is a waste product with little value, and therefore needed to be disposed of in the most economical manner. However, this perspective has changed, and the transformation of a so-called nuisance into a valuable and prized raw material has happened (Figure 2). This transformation has been facilitated by key events over the recent past, some imposed on the industry, and some championed and exploited by the industry, which are going to be mentioned through this chapter.

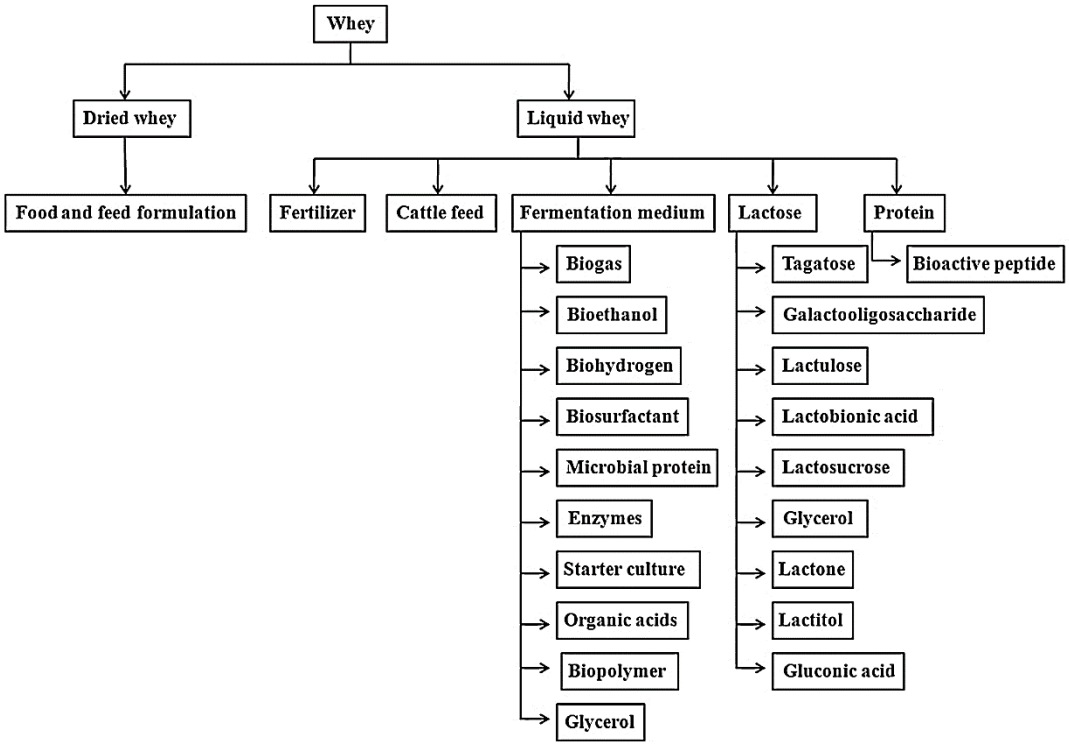

Figure 2. Utilization of whey in different aspects. Adapted from Nath, A. *et al* (2015)[19].

III.1. Environmental considerations

Due to its elevated contents in lactose, non-casein proteins and organic matter, whey is associated with a high biochemical oxygen demand (BOD) and potential for decomposition[34]. The elevated BOD values encouraged governments and other regulatory authorities to restrict and/or ban the disposal of untreated whey. Notably, community pressure in regional areas, has also forced dairy factories to reconsider how they manage the large and growing volumes of whey generated from cheese and casein manufacture. The United States, Canada, Australia, New Zealand, and the European Union countries have all introduced strict environmental protection legislation, and such regulation has forced a re-think within the dairy industry in regard to whey disposal[2].

III.2. Scientific and technical advances

Legislative restrictions on whey disposal encouraged a deeper exploration of the widely recognized but less well understood physical, chemical, nutritional, and biological properties of whey components, notably the proteins and peptides. In parallel with advances in establishing a sound science foundation for whey components and their behavior, similar advances have occurred in whey-processing technologies. Several process technologies, namely anaerobic and aerobic digestion, ethanol fermentation, lactic acid production, electrochemical precipitation and coagulation of biomolecules have been meticulously used for valorization of whey and dairy waste management[35]. These techniques, together with the introduction of modern biochemical techniques, some from outside the dairy and food industries, have been applied for the manufacture of new whey-based ingredients with an expanded applications base for increasingly receptive markets[36].

III.3. Nutritional value of whey proteins

III.3.1. Biological Value

Whey protein has an exceptional biological value (BV) that exceeds that of egg protein by about 15%, the former benchmark, and a range of other common edible proteins (Figure 3A). In essence, BV refers to how well and how quickly the body can utilize the protein consumed[37]. In this regard, whey protein excels and is the protein of choice for body builders, elite athletes, and those whose health is compromised[38].

III.3.2. Essential amino acids

Whey protein is a rich source of essential amino acids when compared with other typical food proteins (Figure 3B), and is also rich in the branched chain amino acids Leu, Ile, and Val (>20% w/w). These latter amino acids are thought to play a role as metabolic regulators in protein and glucose homeostasis, and in lipid metabolism, and as such may play a role in weight control[39].

III.3.3. Sulphur amino acids

Whey protein is a rich and balanced source of the sulphur amino acids Met and Cys (Figure 3C). These amino acids serve as critical role as antioxidants, as precursors to the potent intracellular antioxidant glutathione, and in one-carbon metabolism[40].

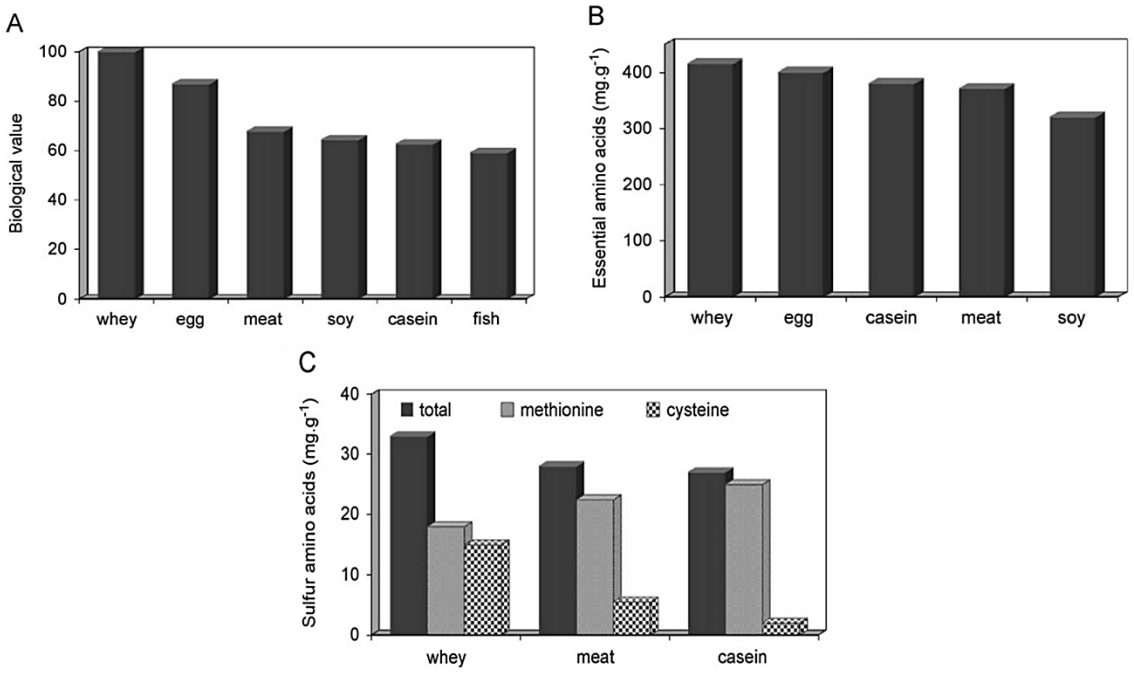

Figure 3. Nutritional value of whey protein when compared with other common edible proteins, as measured by biological value (A), essential (B), and sulphur (C) amino acid content. Adapted from Smithers, G (2008)[2].

III.4. Expanded and new applications

The growing understanding of the characteristics of whey proteins and their interactions with other components in complex food systems, together with the advances in technologies for processing, concentrating, fractionating, and dehydrating whey has formed a foundation for an expanding application base for whey solids and whey-based ingredients[41]. For instance, the nutritional value of whey solids has encouraged their use in a variety of beverages and whey-based cheeses[42]. Beyond nutrition and food, whey proteins are used as surfactants in different cosmetic applications, and as plastic films for coating foods, drugs and special papers[43]. Likewise, it has been reported that whey protein hydrolysates are more easily absorbed and have been used as protein supplements for infants, senescent, athletes and adults[44]. Moreover, the hydrolysis of whey proteins has shown that they release bioactive peptides that are capable to exert specific biological activities, such as antihypertensive, antimicrobial, opioid, antioxidant, immunomodulatory, mineral binding, among others[45].

Moreover, while the polluting power of whey is well known, this dairy stream also represents an excellent source of functional proteins and peptides, lipids, vitamins, minerals, and lactose, that until relatively recently have been less well recognized. It is these latter component

of whey, notably the proteins and peptides, and their properties that have helped transform whey from a waste material that has often been shunned, to a valuable dairy stream containing a multitude of components available for exploitation in the agro-food, biotechnology, medical, and related markets[2].

III.4.1. Functional foods "revolution"

The food industry at large, and the dairy industry in particular have indeed been searching for innovative modes of conversion of their effluents into added-value products, following economically feasible routes[46]. One booming market is that of health-promoting foods, generically known as functional foods[47]. These foods are those that provide a specific health benefit to the consumer over and above their nutritional value, promote health and reduce the risk of diseases[48]. The global market size has already reached $73.5 billion from a base just almost 20 years ago. The United States market dominates (>30% of the total global market) and is showing a sustained growth of ~14% per year. Other significant markets include the European Union and Japan. Growth in the functional foods market across the world is currently ~8% per year, and at 2012 the market was valued at >$100 billion[49]. In this large and burgeoning marketplace, the food industry is demanding economical, high-quality, novel, and substantiated ingredients. In such a setting, whey (protein)-based ingredients provide the industry with an excellent choice.

III.4.2. Bioactivity of whey proteins and peptides

Whey contains a multitude of biologically active proteins and peptides, and these and other components have at very least been implicated in a variety of nutritional and physiological effects, including (i) physical performance, recovery after exercise, and prevention of muscular atrophy; (ii) satiety and weight management; (iii) cardiovascular health; (iv) anti-cancer effects; (v) wound care and repair; (vi) management of infections; (vii) infant nutrition, and (viii) healthy aging[2]. While some of these effects remain putative, a number have received considerable scientific scrutiny and have been substantiated in several laboratories around the world. The addition of whey components, and their derivatives bearing physiological roles has responded to demand by specific consumer groups – and the possibility of releasing bioactive peptides encrypted in their proteins has motivated several studies in recent decades. In this way, the following review is related to the definition of bioactive peptides, their modes of release from whey proteins and also their activities.

Chapter IV – Bioactive Peptides

This chapter continues the subject of bioactive peptides and offers the reader with a brief definition of said peptides. Also, the reader is guided through the several stages involved in the production of bioactive peptides, from their release from the parent molecule to their synthesis.

IV.1. Definition of Bioactive Peptides

Bioactive substances of food origin can be defined as genuine or generated components of consumption ready food which may exert regulative activities in the human organism beyond basic nutrition[50]. A great diversity of food-derived bioactive substances having a non-nutrient character can be considered as an aid in maintaining good health. Although there are other animal as well as plant proteins that contain potential bioactive sequences, milk proteins are the principal source of bioactive peptides encrypted within primary amino acid sequence. These bioactive peptides are generally specific and short peptides (3–20 amino acids) and are often functionally inactive within the native proteins, but they can be released by digestive enzymes (both *in vivo*, during gastrointestinal transit, or *in vitro*), fermentation or ripening during food processing to achieve their specific bioactive roles[51]. Their bioactive roles and potential health benefits are diverse and they include, among others, immunomodulatory, antihypertensive, anticarcinogenic, antimicrobial and anti-inflammatory effects[52].

A growing number of such peptides are being identified in dairy protein hydrolysates and fermented dairy products and for this reason the interest in developing products with those potential benefits in human health has increased. Consequently, there are already a number of such products available in different countries around the world, such as Japan, USA and Europe[52] (Table 2).

Table 2. Commercial dairy products and ingredients with health or function claims based on bioactive peptides. Adapted from Korhonen, H. (2009)[53].

Brand name	Type of Product	Claimed functional bioactive peptides	Health/function claims	Manufacturer
Calpis	Sour milk	VPP and IPP derived from β-casein and k-casein	Reduction of blood pressure	Calpis Co., Japan
Evolus	Calcium enriched fermented milk drink	VPP and IPP derived from β-casein and k-casein	Reduction of blood pressure	Valio Oy, Finland
BioZate	Hydrolysed whey protein isolate	β-lactoglobulin fragments	Reduction of blood pressure	Davisco, USA
Lactium	Flavoured milk drink, confectionery, capsules	a_{s1}-casein f(91–100)	Reduction of stress effects	Ingredia, France
Capolac	Ingredient	Caseinophosphopeptide	Helps mineral absorption	Arla Foods Ingredients, Sweden

IV.2. Production of Bioactive Peptides

In a general way, the production of bioactive peptides follow a number of strategies, being the first obvious step the choice of a protein source. Secondly, there is a step of hydrolysis, where the bioactive peptides can be released from their parent molecule. The following steps are regarding the purification, separation, and enrichment of the bioactive peptides, as well as their biological activity assessment. The bioactive peptides of choice may be further identified and then synthesized. All these approaches involve a number of specific techniques that may be different at laboratory and industrial scale. The different stages involved in the production of bioactive peptides are summarized in Table 3 and will be explained next.

Table 3. Different stages involved in the production of bioactive peptides at laboratory and industrial scale. Adapted from Dziuba, B. et al. (2014)[10].

Stage	Laboratory Scale	Industrial Scale
Protein source	Food proteins	Waste products rich in proteins
Release of bioactive peptides	Hydrolysis by digestive enzymes Hydrolysis during food processing (e.g. fermentation) Hydrolysis with other proteolytic enzymes	Hydrolysis with microbial hydrolases Hydrolysis during fermentation Immobilized enzymes
Separation and Purification	Ultrafiltration membranes Mass exclusion chromatography Ion Exchange chromatography Affinity chromatography High-Performance Liquid Chomatography	Ultrafiltration membranes Ion Exchange chromatography Ion Exchange membranes Nanofiltration
Biological Activity Assessment	*in vitro*, *ex vivo*, and *in vivo* research	*in vitro* methods
Determination of peptide sequence	Edman's degradation Mass spectrometry	
Peptide Synthesis	Chemical synthesis Recombinant DNA technology Enzymatic synthesis	Chemical synthesis (liquid phase) Recombinant DNA technology

IV.2.1. Release of bioactive peptides

The release of bioactive peptides from their immediate precursor sequence is a prerequisite for any functional role in the living system and they can be released in three different ways: (a) hydrolysis by digestive enzymes; (b) proteolytic microorganisms and/or (c) the action of plant or microbial proteases[53].

a) Enzymatic hydrolysis by digestive enzymes

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules[54]. A large number of studies have demonstrated that hydrolysis of milk proteins by digestive enzymes can produce biologically active peptides[55]. The most prominent enzymes are pepsin, trypsin and chymotrypsin that have been shown to release a number of

antihypertensive, antibacterial, immunomodulatory and opioid-like peptides both from different caseins (α-, β- and k-casein) and whey proteins (α-LA and β-LG)[56]. Also, other proteolytic enzymes, such as alcalase, thermolysin, subtilisin, papain and pancreatin and successive treatment with pepsin and trypsin in order to stimulate gastrointestinal digestion have been employed to release various bioactive peptides[57]. These included caseinophosphopeptides (CCPs), angiotensin-converting enzyme (ACE) inhibitory, antibacterial, antioxidative, immunomodulatory and opioid-like peptides[58].

An important aspect of the enzymatic hydrolysis method is that the production of bioactive peptides must be performed at the optimal conditions of the enzyme(s), and thus the type of peptides generated is dependent on the hydrolysis specificity of the enzyme(s)[59]. Also, digestion of proteins can be conducted either in homogenous phases, where both the protein(s) and enzyme are in solution, or in heterogeneous phases, where the protein(s) are in solution and the enzyme is immobilized onto a solid support[60]. The use of immobilized enzymes in industrial-scale conditions over the conventional soluble enzymes may offer several advantages such as: i) enzymatic hydrolysis under milder and more controlled conditions, ii) immobilization makes the enzyme autolysis almost negligible and therefore iii) the presence of peptides belonging to the enzyme itself may be avoided[60].

b) Microbial fermentation

Industrially used dairy starter cultures useful for the generation of bioactive peptides through microbial fermentation are proteolytic in nature. The enzymes involved in milk protein degradation are thought to be cell envelope proteinases (CEP) and/or intracellular peptidases[61]. In a study performed by Algaron *et al.* (2004) milk was fermented with mutant *Lactococcus lactis* (*Lc. Lactis*) strains lacking either aminopeptidases N, pepX or tripeptidase to test these mutants for the ability to produce peptides with antihypertensive or immunomodulatory activities, and to relate the resultant bioactivity to the resulting mutation. They reported that, in some cases, the modified proteolytic systems of *Lc. Lactis* gave rise to a significant difference in the mixture of peptides produced, indicating that the specific peptidase activity of lactic acid bacteria (LAB) affects the bioactive nature of the peptides produced[62].

Moreover, several studies have demonstrated that *Lactobacillus helveticus* strains, are capable of releasing antihypertensive peptides, the best known of which are ACE-inhibitory tripeptides VPP and IPP[63]. Also, yoghurt bacteria, cheese starter bacteria and commercial probiotic bacteria have been demonstrated to produce different bioactive peptides in milk during fermentation[64]. For example, Virtanen *et al.* (2006) demonstrated that fermentation of milk with single industrial dairy cultures generated antioxidant activity in the whey fraction[65]. In another study, Chen *et al.* (2007) observed that fermentation of milk with a commercial starter culture mixture of five LAB strains followed by hydrolysis with a microbial protease increased ACE inhibitory activity of the hydrolysate[66].

c) Microbial and Plant proteases

Proteolytic enzymes derived from several sources have been employed in the hydrolysis of milk proteins[67]. For example, in addition to live microorganisms, proteolytic enzymes isolated from LAB have been successfully employed to release bioactive peptides from milk proteins. For instance, in a study by Mizuno *et al.* (2005), they measured the ACE-inhibitory activity of casein hydrolysates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease isolated from *Aspergillus oryzae* showed the highest ACE-inhibitory activity *in vitro*. Besides other peptides, VPP and IPP were identified[68].

Moreover, less conventional sources of proteolytic enzymes have been reported that can cleave the whey protein backbone at specific and usual sites. This is the case of aspartic proteinases present in the flowers of *Cynara cardunculus,* usually named cyprosins. These proteinases were found to be able to cleave the whey protein backbone next to hydrophobic amino acid residues, especially Phe, Leu, Thr and Tyr, and act mainly on α-LA, while β-LG appears not to be hydrolysed to a significant extent[69]. Other plant enzymes usually used for the production of whey protein hydrolysates are mainly papain and bromalin[70].

IV.2.2. Separation and Purification

After hydrolysis, the peptides in hydrolysates need to be fractioned and enriched by means of various methods, in order to facilitate their further identification[71]. Until now, membrane separation techniques have provided the best technology available for the enrichment of peptides with a specific molecular weight range. Among the membrane separation techniques, ultrafiltration membrane reactors have been shown to improve the efficiency of enzyme-catalyzed bioconversion and to increase product yields[55]. Membranes with different nominal molecular weight cut-off have been used. The retentate, or reaction mixture, is discarded whereas the permeate, so called total hydrolysate, may undergo further fractionation before use[72]. Besides, enzymatic hydrolysis can be performed through conventional batch hydrolysis or continuous hydrolysis and can be easily scaled up[72].

Moreover, several chromatographic methods, such as High-Performance Liquid Chromatography (HPLC) have been developed for the enrichment of peptides derived from milk hydrolysates, but the costs for scale-up make them prohibitive for large-scale applications[55].

IV.2.3. Determination of Peptide Sequence

In the last decade many research efforts have been done to develop techniques and methods for the separation, purification and characterization of food peptides and proteins[73]. The complexity of protein hydrolysates, which can contain hundreds of peptide sequences, of which the bioactive peptide may represent only a minor constituent, combined with the complex nature

of food matrices has meant that the task of identifying, characterizing and quantifying food-derived bioactive peptides has been a difficult and often labor-intensive work[52]. Once the bioactive sub-fractions have been prepared, mass spectrometry (MS) is the method of choice to determine the peptide sequence and thus bioactive identity[74].

The advent of soft ionization MS techniques such as fast atom bombardment (FAB), electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) has introduced a new era for protein characterisation[13]. MS techniques are highly selective and sensitive, with the result that they have been successfully used to not only identify and characterize, but also when combined with other techniques such as HPLC (i.e. LC/MS or LC/MS/MS), to quantify food-derived bioactive peptides in both foods and biological fluids[75].

IV.2.4. Peptide synthesis

The selection of the most suitable method for peptide synthesis mainly depends on the length and quantity of the desired peptide. Peptides short in length are usually produced via enzymatic synthesis, whereas recombinant DNA technology is the preferred choice for relatively large peptides, consisting of up to several hundred amino acids[48].

At laboratory scale, the most widely used approach to the synthesis of peptides is still the chemical one. Two variants of this methodology exist, namely the liquid-phase and solid-phase synthesis, but the latter is still the most powerful approach to synthesize peptides composed of about 10 to 100 residues on a small-scale. Moreover, solid-phase synthesis also allows the rapid production of peptide libraries for screening purposes[48]. On the contrary, at the industrial scale the liquid-phase chemical synthesis is preferred.

Nonetheless, recombinant DNA technology is the most widely used technique at industrial scale for the purpose of synthesis of peptides. Although it usually requires a long and expensive research and development phase, once the system is established the product can be obtained in large quantities[48]. The production of larger amounts of bioactive peptides needed for food supplementation may be possible by using food-grade fermentation bacteria as peptide production systems. In a study performed by Renye and Somkuti (2008) they described the cloning and expression of synthetic genes in *Streptococcus thermofilus* that encode BL-11 antimicrobial and C-12 antihypertensive bioactive peptides which are normally derived from bovine milk proteins[76].

IV.2.4. Bioinformatics

The major drawbacks of the classical approach include limited sample scope, time consumption especially during the purification steps, low yields of isolated peptides, and the likelihood that individually potent peptides may not be discovered after extensive processing

typically when bioactivity is associated with additive or synergistic effects of various components of the enzymatic protein hydrolysates[77]. Nevertheless, most of the times the bioactive peptides are newly derived sequences and can be deposited in web-based open access databases of bioactive peptides such as BIOPEP (http://www.uwm.edu.pl/biochemia) and PepBank (http://pepbank.mgh.harvard.edu) for reference by the community of researchers. BIOPEP contais information on bioactive peptides, major allergens and their epitopes as well as sensory peptides including amino acids[78]. PepBank is a database of peptides based on sequence text mining and public peptide data sources and only peptides that are 20 amino acids or shorter are stored within the database[79]. The structures of the bioactive peptides can be used as templates for designing more active peptides and peptidomimetics, and for structure-function relationship studies.

In order to circumvent some challenges of the classical approach, computer-based (often referred to as "*in silico*") simulation has been recently applied towards the discovery of bioactive peptides encrypted in food proteins[80]. The *in silico* approach involves the use of information accrued in databases, to determine the occurrence frequency of cryptic bioactive peptides in the primary structure of food proteins. The protein sequences can be obtained from databases, such as the universal protein knowledgebase UniProtKB and occurrence frequency calculated as a/N, where "a" is the number of peptides in the sequence that exhibit a particular bioactivity, and "N" is the total number of amino acid residues in the protein[81]. Since occurrence does not necessarily indicate liberation of the cryptic peptides, bioinformatics software can also be used to simulate proteolytic specificities of enzymes in order to generate profiles of peptides *in silico*. Popular *in silico* proteolysis tools include BIOPEP "enzyme action" tool and ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter). The peptides resulting from *in silico* proteolysis can then be matched with bioactive peptides in databases for predetermined bioactivities.

Additionaly, there are databases specific for certain bioactivities, as is the case of APD (http://aps.unmc.edu/AP/main.php), a database of natural antimicrobial peptides with less than 100 amino acid residues[82], and CAMP (http://www.camp.bicnirrh.res.in/), which provides a useful resource for the study of antimicrobial peptides as it holds experimentally validated and predicted antimicrobial peptide sequences[83]. Other tools, such as Innovagen's Peptide property calculator (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp), ProtParam (http://web.expasy.org/protparam/), and APD2: Antimicrobial Peptide Calculator and Predictor (http://aps.unmc.edu/AP/prediction/prediction_main.php), provide information regarding some of the physicochemical characteristics of a given polypeptide sequence. The development of such databases and tools has made possible the search for peptides with a given activity or sequence. Also, they allow to study the possibility to generate new bioactive peptides from parent proteins.

Chapter V – Whey-derived Bioactive Peptides

In this chapter, the reader is guided through an extensive review on the bioactivities associated to peptides obtained from the hydrolysis of whey proteins. These activities aim to elucidate the reader to the importance of whey proteins and peptides and reinforce the idea of the importance that whey proteins and peptides have gained along the years.

Regarding whey proteins in particular, apart from the inherent bioactivity of its proteins, their primary amino acid sequences contain peptides with bioactivity, additional and often varied from that of the parent molecule[84]. Although less information is available about bioactive peptides derived from whey proteins, some important biological activities have been associated with protein hydrolysates derived from whey (Figure 4) and different bioactive compounds have already been synthesized from whey proteins (Table 4).

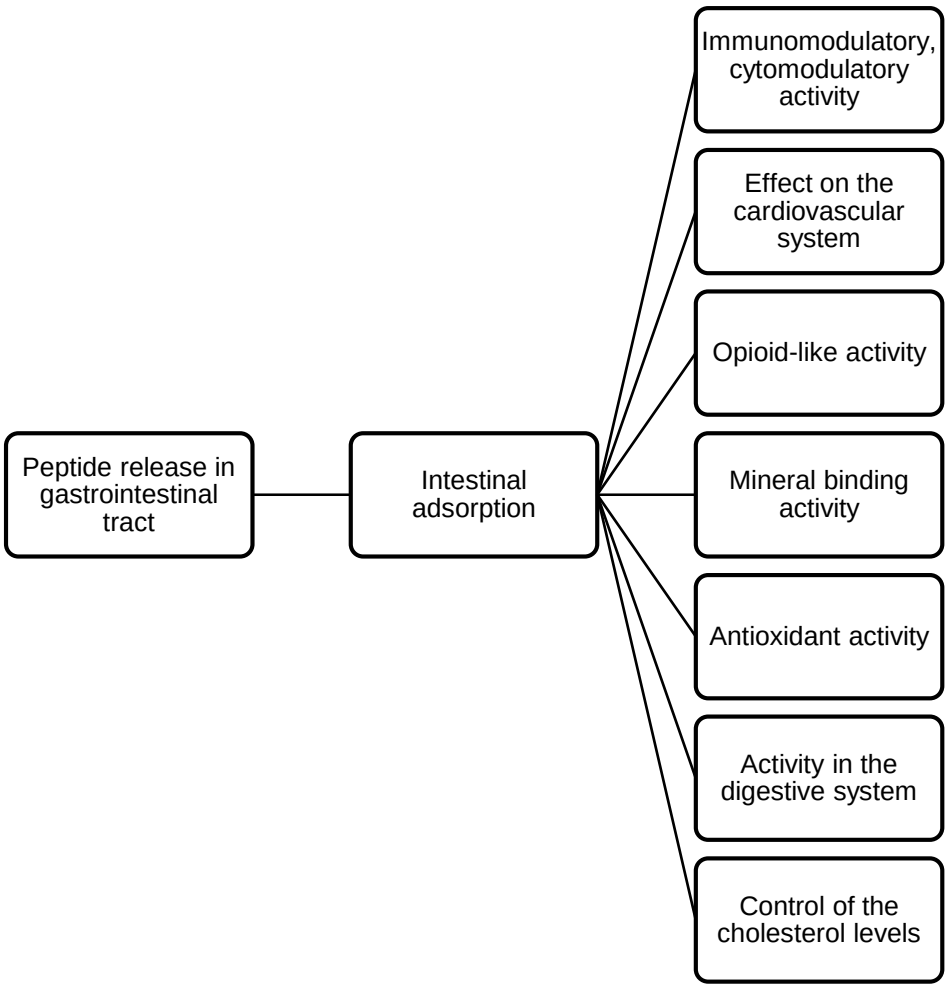

Figure 4. Different physiological and biological activities of whey protein hydrolysates. Adapted from Nath, A. et al. (2015)[19].

Table 4. Different bioactive compounds synthesized from whey proteins through enzymatic hydrolysis. Adapted from Nath, A. et al. (2015)[19].

Whey Proteins	Enzyme treatment	Amino acid sequence
α-LA	Pepsin	YGLF
	Trypsin	EQLTK
		GYGGVSLPEWVCTTF|ALCSEK
	Chymotrypsin	CKDDQNPH:α-chain| ISCDKF :β-chain
	Cheese microflora	WLAHK
β-LG	Trypsin	VAGTWY
		AASDISLLDAQSAPLR
		IPAVFK
	Pepsin; trypsin	YLLF
	Porcine trypsin	IIAEK
	Chymotrypsin	HIRL
	Thermolysin	LQKW
		LLF
BSA	Trypsin	IIVTQTMK
		YGFGNA
LF	Pepsin or chymosin	FKCRRWQWRMKKLGAPSICVRRAF/A

V.1. Anticancer peptides

Cancers represent about one-eighth of all deaths worldwide and have become a leading cause of mortality. For instance, in 2008, the lung (12.7%), breast (10.9%), and colorectal (9.7%) cancers were the most commonly diagnosed cancers worldwide[85]. New cases of cancers are reported every day and mortality rates continue to rise[86]. Cancer is a term for a class of diseases identified by uncontrolled growth and spread of abnormal cells, which may be induced by external environmental factors (radiation, chemicals, and infectious organisms) or internal factors (mutations, hormones, and altered immunity)[87]. Fortunately, rapid progress in treatment has switched some fatal cancers to survivable chronic diseases because of development of anticancer drugs such as hormone agonists/antagonists, antimetabolites, and DNA-alkylating agents[88].

Peptide-based drug therapies have shown to possess various key benefits, including low cytotoxicity, strong specificity, tumor-penetrating ability, small size, and easy modification[89]. Recently, researchers have shown an increased interest in the generation of biologically active peptides encrypted within various proteins, with a perspective of using such peptides as functional components, since they have been reported to display activities against cancer cells mainly through cytoplasmic membrane disruption and apoptosis induction[90].

Some whey proteins have shown to be remarkable in cancer therapy. For instance, the multimeric form of α-LA was found to accelerate apoptotic processes in transformed and juvenile cells, but to protect mature epithelial cells. β-LG has been found to provide protection against cancer development in an animal model when administered orally. Moreover, both BSA and

bovine LF prevented the growth of human breast cancer cell line in a dose-dependent way. Also, antitumor activities of bovine lactoferrin are also mediated through the retardation of angiogenesis and diminishing of endothelial cell proliferation. Also, some fragments released after hydrolysis of whey proteins may have a key role in cancer therapy. On example are the bovine lactoferrin fragments, namely lactoferricin, which have shown to exhibit cytotoxic activities on various human and rat malignant cell lines, including fibrosarcoma, leukemia, different carcinoma, and neuroblastoma cells[91].

The discovery of various anticancer peptides is very likely to create a “new era” for more efficient and effective antitumor medications. Nevertheless, molecular research is still needed to elucidate the mechanisms of the anticancer activities. Moreover, the innovation behind the discovery of new anticancer peptides derived from fermented dairy products and milk-protein hydrolysates will also contribute towards the advancement of various functional foods[92].

V.2. Antidiabetic peptides

Type 2 diabetes is a metabolic disorder characterized by impaired insulin secretion by β-cells and insulin resistance in tissues, a condition that is associated with the development of several complications, including hypertension and cardiovascular disease. Considering the prevalence of type 2 diabetes (estimated to affect 370 million people) and its increasing trend, different strategies are developed to properly treat hyperglycemia[93].

It is reported that oral administration of whey proteins and their hydrolysates positively affect blood glucose control and insulinotropic responses in humans[94]. Similar effect are described in animal models of diabetes and BRIN-BD11 pancreatic β-cells[95]. Nongonierma *et al.* (2013) observed that whey protein hydrolysates enriched in free amino acids and hydrophilic peptides could have been responsible for the increased insulinotropic response of BRIN-BD11 cells[96].

Although not completely understood, the effects of whey proteins and their hydrolysates on glycemia appear to be mediated, *in vivo*, by the release or the presence of bioactive peptides and amino acids from whey proteins that could stimulate the secretion of gut hormones, and also act as dipeptidyl peptidase IV (DPP IV) inhibitors[94].

V.2.1. Inhibition of DPP IV

Upon food ingestion, the hormones glucacon-like peptide 1 (GLP-1) and the glucose-dependent insulinotropic polypeptide (GIP) are released from the gastrointestinal tract, increasing insulin secretion by β-cells. This characterizes the incretin response, which contributes for up to 70% of insulin release in healthy subjects. In type 2 diabetes, although the incretin effect is greatly depressed, the action of GLP-1 seems to be preserved. However, both hormones are

substrates of DPP IV, a ubiquitous enzyme that due to its activity decreases rapidly the plasma levels of GLP-1 and GIP. In this sense, DPP IV inhibitors could increase the half-life of active GLP-1, potentiating the insulinotropic effect and glycemic control[97].

Diverse proteins are considered as precursors of DPP IV inhibitory peptides[98]. In fact, *in silico* approaches revealed several peptides encrypted within the amino acid sequences of dietary proteins that could act as DPP IV inhibitors[99], including bovine, ovine and caprine whey proteins[100]. Thus, treatment of whey proteins by proteases is investigated to generate hydrolysates able to inhibit DPP IV activity. Such approach represents a valuable strategy, expanding the knowledge on DPP IV inhibitory peptides (Table 5), also suggesting that hydrolysates and peptides might be useful in the management of type 2 diabetes.

Table 5. Bovine whey protein-derived peptides with dipeptidyl peptidase IV (DPP IV)-inhibitory activity. Adapted from Brandelli, A. *et al.* (2015)[3].

Whey proteins	Enzyme treatment	Amino acid sequence	Protein sequence
α-LA	Pepsin	WLAHKAL	f(104–110)
		WLAHKALCSEKLDQ	f(104–117)
		LAHKALCSEKL	f(105–115)
		LCSEKLDQ	f(110–117)
		TKCEVFRE	f(4–11)
		IVQNNDSTEYGLF	f(41 –53)
		ILDKVGINY	f(95–103)
β-LG	*In silico*	IPA	f(78–80)
	Trypsin	VAGTWY	f(15–20)
		IPAVF	f(78–82)
		IPAVFK	f(78–83)
		TPEVDDEALEK	f(125–135)
		VLVLDTDYK	f(92–100)
	Pepsin	LKPTPEGDL	f(46–54)
		LKPTPEGDLEIL	f(46–57)
		IPAVFKIDA	f(78–86)

From the studies summarized in Table 5, DPP IV inhibitory peptides identified from hydrolysates usually have molecular weights below 2kDa, and most contain proline and/or hydrophobic amino acid residues within their sequence[101]. Length, net charge, and polarity of whey-derived peptides do not appear to have, *per se*, a predictable impact on inhibitory action or potency. Nevertheless, amino acid sequence seems to play more important roles for DPP IV inhibition[102].

Moreover, peptide inhibitory capabilities seem to result from competitive, un-competitive or non-competitive modes of inhibition. Thus, peptides displaying the same mode of inhibition could share some features, as well as distinct peptide properties might be more relevant for each mode of action.

Diprotin A (IPI) and diprotin B (VPL) are potent DPP IV inhibitors showing an apparent competitive behavior by acting as enzyme substrates. *In vitro* evaluation of the synthesized peptide IPA, derived from β-LG digestion, was demonstrated to inhibit DPP IV in a competitive manner. Similarly, DPP IV competitive inhibition was reported for the peptide IPAVFKIDA, which might result from its substrate-like structure[102]. Peptides LAHKALCSEKL and ILDKVGINY, from α-LA were also demonstrated to inhibit DPP IV by direct interaction with the enzyme active sites.

On the contrary, the β-LG peptides LKPTPEGDL and LKPTPEGDLEIL, that are among the most potent DPP IV inhibitors identified from whey protein hydrolysates, seem to act upon DPP IV in an un-competitive manner, that is, by potentially binding to enzyme-substrate complexes outside the active site and decreasing reaction velocity[102]. Also, the α-LA peptide WLAHKALCSEKLDQ displayed an un-competitive inhibitor behavior, whereas WLAHKAL and LCSEKLDQ were reported to act as non-competitive inhibitors that is, potentially binding outside the DPP IV catalytic center on either the enzyme or the enzyme-substrate complex and LAHKALCSEKL acted as a competitive inhibitor. Therefore, structure-activity relationships and the exact means by which whey-derived peptides exert DPP IV inhibition are not completely elucidated[97].

In this sense, considering the diversity of peptides and their modes of action, whey protein hydrolysates or mixtures of peptides therein could act synergistically, leading to a greater inhibition of DPP IV when compared to individual peptides[102]. However, conventional drugs employed as DPP IV inhibitors, such as sitagliptin, are much more potent than whey protein hydrolysates and peptides. Thus, they might not be intended to replace available drugs but they could be used as natural complementary approaches that could be implemented through dietary intervention and food-drug therapies for the management of type 2 diabetes[103]. For instance, an additive effect on DPP IV inhibition was observed when whey-derived peptides and diprotin A were tested in combination with sitagliptin[104].

V.2.2. Inhibition of α-glucosidase

Another strategy to manage type 2 diabetes is the inhibition of carbohydrate-hydrolyzing enzymes, such as the membrane-bound α-glucosidase from the epithelial mucosa of the small intestine, which releases monosaccharides from complex carbohydrates, delaying the degradation of carbohydrate in the gastrointestinal tract, and thus decreasing the levels of blood glucose[93].

The inhibition of *Saccharomyces cerevisiae* α-glucosidase by fractions of bovine whey protein concentrate (WPC) and β-LG hydrolysates, produced with a protease from *Cucurbita ficifolia*, was also investigated[105]. Moreover, peptic hydrolysates of bovine whey protein isolates (WPI), β-LG and α-LA were observed to inhibit rat intestinal α-glucosidase by 36, 33, and 24%, respectively, whereas the non-hydrolysed counterparts have not displayed enzyme inhibition[106]. However, as few non-saccharide inhibitors of α-glucosidase are reported, investigations should

focus on the identification of the peptides responsible for enzyme inhibition, also aiming to gain insight on the inhibitory mechanism and *in vivo* significance[107].

V.3. Antihypertensive peptides

Hypertension, also known as high blood pressure, is one of the main risk factors for cardiovascular diseases[108]. Cardiovascular diseases are a group of disorders of the heart and blood vessels and include: coronary, cerebrovascular, peripheral arterial, rheumatic and congenital heart-disease[109]. The prevalence of hypertension varies with gender, age and nationality[110]. A large worldwide data analysis estimated that 26.4% of the adult population had hypertension in the year 2000, and it has been projected that it will increase to 29.2% by 2025[111]. Therefore, prevention and early identification of hypertension are the best ways to reduce the overall risk of cardiovascular mortality. Indeed, one of the most studied bioactivity of peptides is the capacity of reducing blood pressure[112].

Many antihypertensive peptides are characterized by their ability to inhibit ACE (peptidyl-dipeptide hydrolase, EC 3.4.15.1), an enzyme that plays a key physiological role in the renin-angiotensin, kalliknein-kinin, and immune systems[113]. In the renin-angiotensin system in particular, an increase in blood pressure is observed when ACE catalyzes the hydrolysis of angiotensin-I to angiotensin-II, a potent vasoconstrictor agent, and the degradation of bradykinin, which has vasodilative action, to a greater extent than needed[114].

Synthetic ACE inhibitors such as enapril, lisinopril, captopril and ramipril have been widely used as antihypertensive drugs[115]. Nonetheless, these synthetic drugs have demonstrated diverse side effects such as increased potassium levels, reduced renal function, allergic reactions, skin rashes, and taste disturbances[116]. Therefore, the search for nontoxic, safer, economical, and innovative ACE-inhibitors is required for the control and treatment of high blood pressure. In this way, various food protein-derived bioactive peptides have been isolated and evaluated for their antihypertensive activity aiming to avoid undesirable side effects of synthetic drugs and to also avoid increasing cost of drug therapy.

It has been demonstrated that the major whey proteins contain encrypted peptides that inhibit ACE. The primary sequences of some ACE-inhibitory peptides derived from α-LA and β-LG are summarized in Table 6.

Table 6. ACE-inhibitory peptides derived from bovine whey proteins.
Adapted from Brandelli, A. *et al.* (2015)[3].

Whey proteins	Enzyme treatment	Amino acid sequence	Protein sequence
α-LA	Pepsin and trypsin	LAHKAL	f(105–110)
	Trypsin	WLAHK	f(104–108)
		VGINYWLAHK	f(99–108)
	Protease preparation from *Cynara cardunculus*	RELKDL	f(10–15)
		DKVGINY	f(97–103)
		KGYGGVSL	f(16–23)
		DKVGINYW	f(97–104)
		KGYGGVSLPEW	f(16–26)
β-LG	Pepsin and trypsin	GLDIQK	f(9–14)
		VAGTWY	f(15–20)
		IIAEK	f(71 –75)
		IPAVFK	f(78–83)
	Proteinase K	IPA	f(78–80)
	Trypsin	ALPMHIR	f(142–148)
		VFK	f(81 –83)
		LAMA	f(22–25)
		LDAQSAPLR	f(32–40)

The structure-activity relationship of ACE inhibitory peptides from food proteins is not yet well studied. However, some general features have been found. The binding to ACE is strongly influenced by the C-terminal sequence, whereby hydrophobic amino acids are more active if present at each of the three terminal positions. In addition, the presence of the positive charge of lysine (Lys) and arginine (Arg) as the C-terminal residue may contribute to the inhibitory potency[117].

V.4. Antimicrobial peptides

Antimicrobial peptides (AMPs) are a special subset of evolutionary conserved peptides that play an important role in the defense mechanisms of numerous organisms. Found in bacteria, plants, insects, and vertebrates, AMPs protect against an extensive array of infectious agents[118].

Due to the emergence of new multidrug resistant (MDR) bacteria, there is a need to develop new antimicrobial agents, and, in contrast to most antibiotics, which target specific proteins, AMPs act on bacterial membranes and other generalized targets, such as DNA. Such characteristic mechanisms of action enables AMPs to avoid the common resistance mechanism observed for classic antibiotics, and therefore, the development of resistance is unlikely[119].

Moreover, the research for new AMPs is somehow influenced by the changing mindset of consumers, who demand more products without chemical additives, pushes the industry to develop alternatives, including use of those antimicrobial peptides, as food-grade biopreservatives or as health-promoting food supplements[120]. Over the past few decades, the impact of dairy AMPs on human health and the manufacture of novel functional food ingredients therefrom have been subject to intensive investigations and researchers have proposed the

possibility of incorporating milk-derived AMPs directly into pharmaceutical products and food systems[120].

Among whey proteins, lactoferrin, lysozyme, and their proteolytic fragments, are the most well studied regarding antimicrobial activity; on the contrary the antimicrobial potential of peptides encrypted within the β-LG and α-LA sequences seems to be less exploited[121]. Examples of antimicrobial peptides derived from whey include bovine and human lactoferricin, corresponding to the fragments f(17-41) and f(1-47), released following enzymatic digestion with trypsin and pepsin, respectively[122]. Both bovine and human lactoferricin display antimicrobial activity against a broad spectrum of gram-positive and gram-negative bacteria, including *Listeria monocytogenes*[123]. Moreover, several peptides with bactericidal activity have also been obtained by enzymatic digestion of α-LA and β-LG[124]. For instance, proteolytic digestion of β-LG by trypsin yielded four bactericidal peptides against gram-positive bacteria corresponding to fragments f(15-20), f(25-40), f(78-83), and f(92-100)[125]. Several others antimicrobial peptides have been obtained by enzymatic hydrolysis of α-LA and β-LG, from which examples are presented in Table 7.

Table 7. Antimicrobial peptides derived from α-Lactalbumin and β-lactoglobulin. Adapted from Brandelli, A. *et al.* (2015)[3].

Whey protein	Enzyme treatment	Amino acid sequence	Protein fragment	Antimicrobial activity
α-LA	Porcine trypsin	EQLTK GYGGVSLPEWVCTT F/ALCSEK	f(1-5) f(17-31)S-S(109-114)	Mainly Gram-positive bacteria
	Bovine chymotrypsin	CKDDQNPH/ISCDKF	f(61-68)S-S(75-80)	
β-LG	Commercial preparation from bovine pancreas	IDALNENK	f(84-91)	Mainly Gram-positive bacteria (*S. aureus* and *L. monocytogenes*)
	Trypsin and chymotrypsin	TPEVDDEALEK	f(125-135)	Mainly Gram-positive bacteria (*L.ivanovii*)
	Porcine pepsin	KVAGT VRT IRL	f(14-18) f(123-125) f(147-149)	
	Bovine trypsin	VAGTWY AASDISLLDAQSAPLR IPAVFK VLVLDTDYK	f(15-20) f(25-40) f(78-83) f(1-8)	Only Gram-positive bacteria

Generally, AMPs exhibit a net positive charge (cationic) and a high ratio of hydrophobic residues, which allows them to selective bind to negatively charged microbial membranes through electrostatic interactions between the cationic peptide and anionic LPS in the outer membrane, leading to perturbations in the integrity of the bacterial cytoplasmic barrier, which becomes compromised following initial AMPs adsorption (Figure 5)[126]. A specific interaction of the AMPs with the bacterial membrane culminates in the leakage of the cell contents. Although this

interaction is most likely essential for the antimicrobial activity of AMPs, many different mechanisms have been suggested. These include the formation of pores by the “barrel stave pore” or the “toroidal-pore” mechanisms[126], as well as the “carpet” mechanisms, based on detergent-like binding properties, highlighted in.

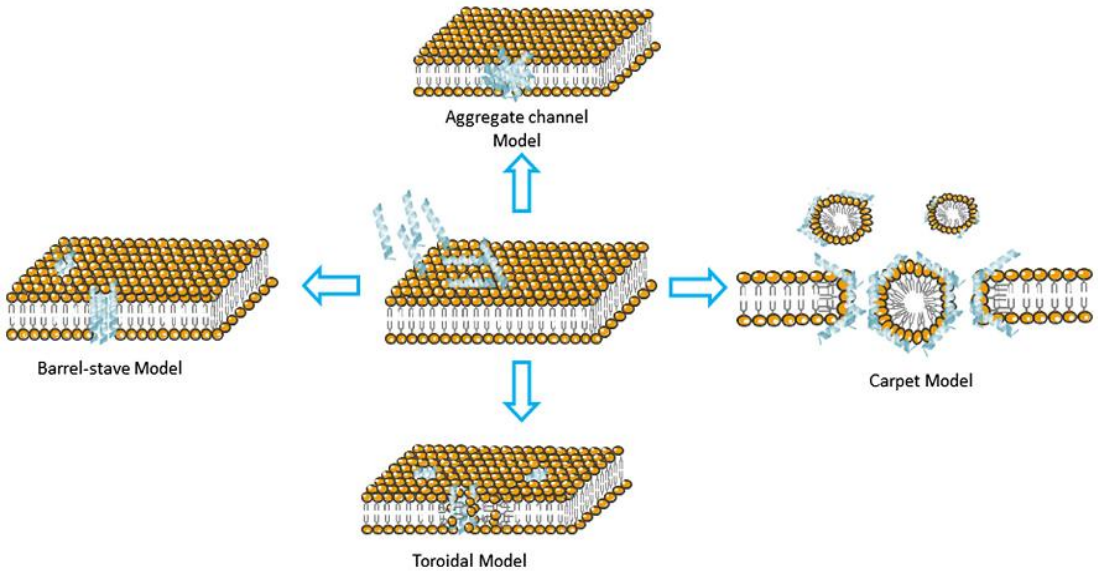

Figure 5. Bacterial membrane disruptions mechanisms following initial adsorption of AMPs. Note that these are not mutually exclusive. Adapted from da Costa et al.[118].

According to the barrel-stave pore model, the amphipathic peptides reorient perpendicular to the membrane via electrostatic interactions and align in a manner in which the hydrophobic sidechains face outwards into the lipid environment while the polar sidechains align inward to form transmembrane pores. These interaction results in the formation of a structure that behaves like a protein ion channel that is proposed to allow leakage of cytoplasmic components and also disrupt the membrane potential[127]. Peptides may also affect the local curvature of the membrane cooperatively, as happens in the toroidal pore model, but in contrast with the previous model is that lipids are intercalated with peptides in the transmembrane channels[128]. Another proposed mechanism is the aggregate model that suggests that only short-lived transmembrane clusters are shaped, which tolerate the crossing of the peptides without causing significant membrane disruption. Once inside the cell, peptides aim at intracellular targets[129]. In the alternative carpet model, the peptides do not insert into the membrane but accumulate parallel to the bilayer, remaining in contact with the lipid head groups and effectively coating the surrounding area. When a critical local concentration is reached, changes in the membrane fluidity and/or reductions in the membrane barrier properties lead to permeabilization[130].

Nonetheless, peptides that do not appear to act on membranes are thought to act on cytoplasmic targets leading to cell death broadly by membrane dysfunction, inhibition of intracellular functions, or inhibition of extracellular biopolymer synthesis events[128]. Figure 6 highlights these mechanisms of action.

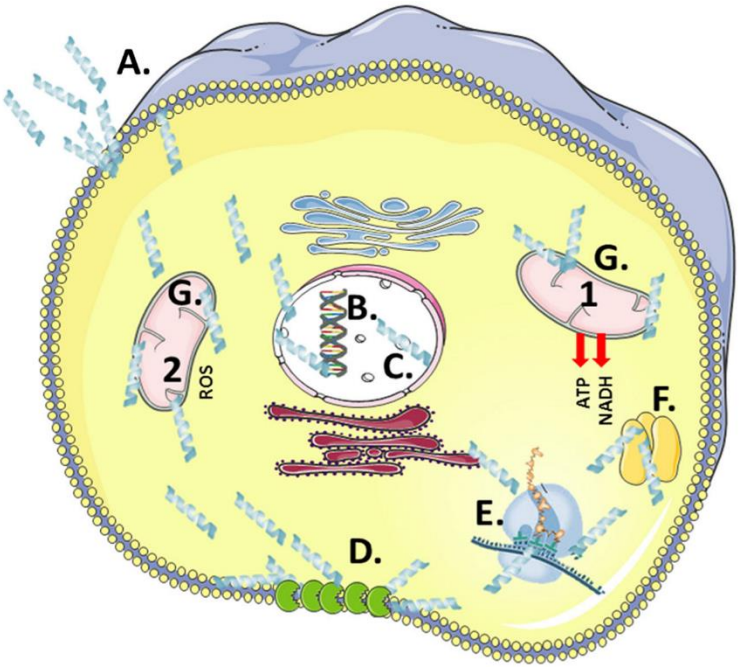

Figure 6. Mechanisms of cell death by AMPs. A) disruption of cell membrane integrity, B) blocking of RNA synthesis, C) inhibition of enzymes necessary for linking of cell wall structural proteins, D) inhibition of ribosomal function and protein synthesis, E) blocking of chaperone proteins necessary for proper folding, and F) targeting of mitochondria: inhibition of cellular respiration and induction of ROS formation (1) and disruption of mitochondrial cell membrane integrity and efflux of ATP and NADH (2). Adapted from da Costa *et al.* (2015)[118].

Membrane dysfunction is based on the fact that the cytoplasmic barrier is key in the mediation of many essential cell functions, such as selective permeability, and motility[131]. Conceivably, damages in the integrity of the lipid bilayer may interfere with one or more of these processes, leading to cell death, either directly or indirectly[128]. Inhibition of biopolymer synthesis may also result in cell death. For example, the biosynthesis of peptidoglycan is essential for maintaining cell integrity and function[132]. Hence, any perturbation in this synthesis may culminate in the death of the targeted cell. Lastly, it has been demonstrated that the disruption of key intracellular processes contribute or may be required for cell death and cell permeabilization alone allows for cell survival[133]. Ultimately, these observations suggest that AMP-induced cell death may occur due to the contribution of multiple independent or cooperative mechanisms, and, even when acting over the same microbial species, different mechanisms may prevail over others, depending on factors such as environmental settings, growth phase, and presence of immune responses[134]. While these models largely contribute for a better understanding of the action mechanisms of antimicrobial peptides, the molecular organization of the cellular membrane undergoing disruption is still poorly understood. Therefore, the prediction of structure-sequence-activity correlations remains a challenge, and, subsequently, so does the design of novel AMPs[135].

V.5. Antioxidant peptides

In the last years special attention has been dedicated for searching whey-derived peptides with radical scavenging and lipid peroxidation inhibitory activities. It is well known that lipid peroxidation of food products can cause deterioration in food quality, shorten the shelf life and decrease the acceptability of processed foods. Lipid peroxidation can generate free radicals that can lead to fatty acid decomposition, which may reduce the nutritional value and safety of food by producing undesirable flavors and toxic substances[136]. Furthermore, free radical-mediated reactions have a significant role in many biological phenomena such as cellular damage and aging by stimulating oxidation of lipids and formation of secondary lipid peroxidation products[137]. Free radicals can also modify DNA, proteins, and small cellular molecules and are believed to play a significant role in the occurrence of some diseases such as cardiovascular diseases, diabetes and neurological disorders, such as Alzheimer's disease[138].

The use of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylthydroquinone (TBHO) has been restricted because of their potential toxic effect on humans. On the other hand, bioactive peptides are considered natural antioxidants and have attracted a great deal of interest because of their safety and wide distribution properties[139].

Regarding milk-derived peptides in particular, the antioxidant activities are mostly related with bovine casein[140]. However, hydrolysis of whey proteins may also result in the production of antioxidant peptides[141]. Conway *et al.* (2013) suggested that peptides originating from the major whey proteins (α-LA and β-LG) were likely responsible for the antioxidant activity of enzyme-hydrolysed whey concentrate and skim milk, because the casein content of skim milk did not improve its scavenging activity to any significant degree[142]. The hydrolysis of WPI with different proteases has originated several peptides with antioxidant activity that are summarized in Table 8.

Table 8. Antioxidant peptides derived from bovine whey proteins. Adapted from Brandelli, A. et al. (2015)[3].

Whey protein	Enzyme treatment	Amino acid sequence	Protein fragment
α-LA	Thermolysin	INYW	f(101 –104)
		LDQW	f(115–118)
β-LG	Corolase PP	MHIRL	f(145–149)
		YVEEL	f(42–46)
		WYSLAMAASDI	f(19–29)
	Thermolysin	FNPTQ	f(151 –155)
		LQKW	f(58–61)
		LDTDYKK	f(95–101)
	Trypsin	VAGTWY	f(15–20)
	Alcalase	WYSL	f(19–22)

Peptides from protein hydrolysates are reported to act as antioxidant through mechanisms of inactivation of reactive oxygen species, free radical-scavenging, inhibition of lipid peroxidation, chelation of metal ions, or a combination of these mechanisms[143]. In addition, the major mode of action is related to the inherent amino acid composition and sequence of peptide. For instance, the fragments LQKW, LDTDYKK, and FNPTQ contain the amino acids tyrosine (Y) and tryptophan (W), which have been described by different authors as mainly responsible for the antioxidant activity of peptides[144]. Other hydrophobic amino acids such as proline (P) and histidine (H) are described as important for the antioxidant activity, in a way that this activity is related with a greater number of ionizable groups and the exposure of hydrophobic groups[145].

Therefore, considering a protein hydrolysate as a complex mixture of peptides and amino acids, distinct antioxidant mechanisms are possible, which highlights the importance of evaluating the antioxidant potential of proteins and peptides by different methods[146]. Usually, these assays measure the ability of a compound (the potential antioxidant) to transfer hydrogen atoms or electrons to an oxidant. Among the antioxidant capacity assays are the Trolox equivalent antioxidant capacity (TEAC) assay utilizing the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical, and the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay, which detects both electron transfer and hydrogen atom transfer, and the ferric ion reducing antioxidant power (FRAP) assay, which evaluates electron transfer. These simple and inexpensive methods are widely used, although commonly performed under conditions that do not resemble physiological situations. Other assays, such as the oxygen radical absorbance capacity (ORAC), are considered more suitable for detecting the antioxidant relevant radicals[146]. Also, the utilization of food and/or cell model systems of antioxidant capacity is a required approach to better characterize antioxidant potentials[147].

Although the antioxidant properties of whey-derived peptides have been extensively investigated, further research about the structure-activity relationship of peptides and synergistic and antagonistic effects among amino acids and other antioxidant compounds should be carried out[148]. Whey hydrolysis could also be promising from a food technology perspective, since transition metal ions promote lipid oxidation and their chelation helps to retard the peroxidation and subsequently prevent food rancidity. This is particularly relevant when considering the potential health risks associated with synthetic antioxidants. In this perspective, the antioxidant activities of peptides present in whey-derived products could meet the increasing demand for more natural antioxidants aiming human health and food quality[149].

V.6. Growth-promoting peptides

Growth factors are generally considered to be peptides that act via specific receptors triggering intracellular secondary messengers, ultimately resulting in cell proliferation[150]. Despite their considerably lower concentrations in milk and whey, growth factors may be the next category of dairy ingredients for new health promoting product concepts, for instance for the prevention or

treatment of certain gastrointestinal diseases. Whey-derived growth factors could also have an impact on biotechnological and pharmaceutical applications since they may serve as a reliable replacement or as a supplement for fetal bovine serum[151].

Mammalian cell growth factors, including insulin-like growth factor (IGF)-1, IGF-2, transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), betacellulin, and others, have been isolated as a concentrated mixture from cheese whey, and characterized and commercialized[152]. This growth factor preparation shows (i) potent mammalian cell growth stimulatory activity, particularly for fibroblast cells; (ii) impressive wound healing effects; (iii) the ability to protect against gut damage and address gastrointestinal disorders; and (iv) efficacy in delaying the onset of mouth ulcers in an animal model of oral mucositis, a debilitating condition associated with chemo and/or radiation therapy of cancer patients[153]. Milk-derived growth factors are nowadays increasingly used in health products, such as in the treatment of skin and bone disorders, and gastrointestinal diseases (Table 9). These growth factors can be extracted from milk whey, and a number of technological approaches have been developed[154]. Figure7 shows a simplified experimental procedure for obtaining growth factor extracts from milk.

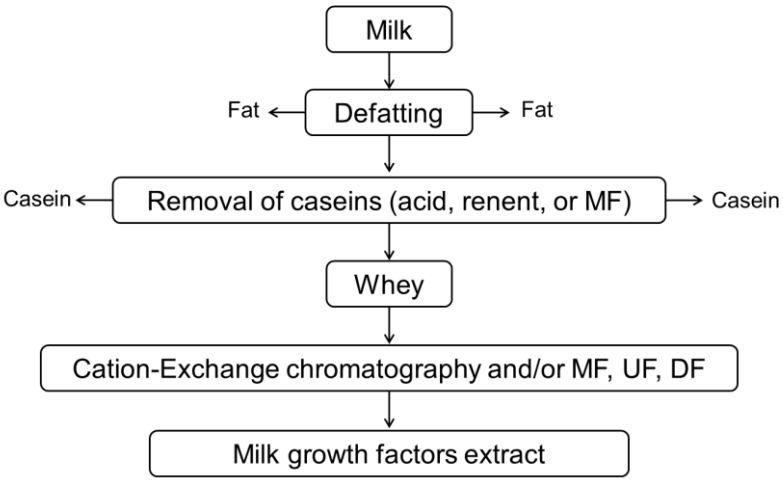

Figure 7. Technological approaches to extract growth factors from milk. MF stands for microfiltration, DF for diafiltration, and UF for ultrafiltration. Adapted from Pouliot, Y. and Gauthier, S.F. (2006)[154].

Table 9. Health-promoting effects associated with growth factor extracts. Adapted from Pouliot, Y. and Gauthier, S.F. (2006)[154].

Health target	Application	Models
Skin disorders	Wound repair	*In vitro, r*ats[155]
	Leg ulcers	Humans[156,157]
	Psoriasis	
Gut health	Crohn's disease	Humans[158]
	Intestinal mucositis	Rats, Hamsters[153,159]
Bone health	Bone resorption (osteoporosis)	Humans[160,161]

V.7. Immunomodulatory peptides

Milk provides essential nutrients during neonatal development and plays an important protective role by educating the immune system of newborns. While milk contains components that can modulate the immune system, an increasing number of studies suggest that milk proteins, including whey proteins, may contain biologically active peptides with immunomodulating properties in their primary sequences[162].

For example, peptides derived from WPI hydrolysis with trypsin/chymotrypsin seem to modulate immune parameters *in vivo* using non-infected and *E. coli* infected mice model. In particular, the basic F3 peptide fraction showed promising results, stimulating serum TGF-β1 secretion, which coincided with a significant increase in IgA levels[163]. Also, Eriksen *et al.* (2008) investigated if whey-derived products with different immunological responses may be generated by using different enzymes[164]. Samples of cow and goat whey were hydrolysed with either commercial enzymes, pepsin and Corolase PP, or by using human gastric and human duodenal juices. Whey protein samples from both goat and cow showed dose-dependent inhibition of peripheral blood mononuclear cell proliferation *in vitro*.

In other studies, eleven synthetic peptides derived from theoretical release from β-LG and α-LA by hydrolysis with trypsin or chymotrypsin were evaluated for their immunomodulatory properties. The peptides β-LG f(15–20), f(55–60), f(84–91), f(92–105), f(139–148), f(142–148) and α-LA f(10–16) stimulated proliferation to different extents, whereas β-LG f(15–20), f(55–60) and f(139-148) also induced various inhibiting and/or stimulating effects on cytokine secretion[165]. These results confirm that hydrolysis of α-LA and β-LG by digestive enzymes may result in peptides that have the potential to influence the specific immune response through the modulation of splenocyte proliferation and cytokine secretion. Moreover, recent reports indicate that the addition of whey peptides has a positive effect in development of immune-modulating diets in both murine and rat models[166].

V.8. Mineral-binding peptides

A number of peptides from milk proteins with mineral binding abilities have been reported. Among them, peptides from whey proteins, β-LG, α-LA and LF, have been derived[167]. Some minerals cause a change in conformation, and thereby alter (inhibit/accelerate) the enzymatic attack on the protein and peptide[168].

Among the whey proteins, LF is regarded as the most important iron-binding protein[169]. Conformational changes of lactoferrin have been shown to have a role in iron binding and release[170]. The role of peptides obtained from the other milk proteins, for example BSA, immunoglobulins, lysozyme and lactoperoxidase are currently unknown. A summary of the whey proteins as precursors of peptides with mineral-binding abilities is shown in Table 10.

Table 10. Mineral binding capacity of whey-derived peptides. Adapted from Vegarud, G.E.et al. (2000)[167].

Whey protein	Enzyme treatment	Mineral-binding
α-LA	Pepsin Trypsin/chymotrypsin	Cu, Ca, Zn, Fe
β-LG	Thermolysin	Cu, Ca, Zn, Fe
	Pepsin Trypsin/chymotrypsin	Fe
LF	Pepsin/trypsin Trypsin	Fe

Measurements of the mineral content of the various peptides are complex and dependent on several factors. Among these, the equilibrium status, red/ox form, solubility and binding affinity are regarded as some of the most important ones. Also sensitivity of the selective method is of importance. Analysis of the total calcium, iron, zinc, magnesium and some trace elements are most commonly detected by atomic absorption spectroscopy[171]. Moreover, the mineral binding and release is affected by differences in mineral affinity, competition at binding sites, pH and ionic strength of the solvent[172].

V.9. Opioid peptides

Opioid peptides are short amino acid sequences present in dairy products which play an active role in the nervous system. These peptides have pharmacological similarity to opium (morphine) and exert their activity by binding to specific receptors on the target cell[173]. Opioid receptors are integral membrane proteins of the central nervous system thought to be responsible for mediating effects such as the analgesic effect, feelings of euphoria, and changes in the endocrine immune system produced by ingestion of opioid drugs in man. At least three types of opioid receptors are known to date; these are termed μ-(morphine), δ-(enkephalin) and κ-(dynorphin) receptors[174]. The individual receptors are responsible for specific physiological effects: the μ-receptor is responsible for emotional behavior and suppression of intestinal mobility, the δ-receptor for emotional behavior and the κ-receptor for sedation and food intake[71]. These are called the typical opioid peptides because they all originate from the precursor proteins endorphins, encephalin and dynorphins, and they have the same N-terminal sequence, Tyr-Gly-Gly-Phe[71].

Opioid peptides derived from other precursor proteins, such as whey proteins, are called “atypical”, that is, they carry various amino acid sequences at their N-terminal regions where only the N-terminal Tyr is conserved. Thus, the N-terminal sequence of “atypical” opioid peptides is Tyr-X-Phe or Tyr-X-X-Phe. It seems that the presence of Tyr and another aromatic amino acid form a structural motif important in ligand-receptor binding[175].

Whey proteins contain opioid-like sequences in their primary structure, as has been described earlier[176]. These opioid-like sequences are encrypted in the primary structure of major

whey proteins: α-LA f(50–53) and β-LG f(102–15), which have been termed α- and β-lactorphins, respectively[71] (Table 11). The whey protein-derived peptide, α-Lactorphin, with proved opioid activity can also induce mucin secretion and mucin gene expression in human colonic goblet-like cells[177]. Similar effect was observed for a trypsin β-LG hydrolysate and β-lactorphin, probably operating through an opioid pathway[178]. These hydrolysates with the ability to modulate mucin production could be promising for improving gastrointestinal protection.

Table 11. Opioid peptides derived from bovine whey proteins. Adapted from Pihlanto-Leppälä, A. (2001)[71].

Whey protein	Enzyme treatment	Amino acid sequence	Protein fragment
α-LA	Pepsin	YGLF	f(50-53)
β-LG	Pepsin then trypsin	YLLF	f(102-105)
	Chymotrypsin	HIRL	f(146-149)

Moreover, opioid peptides can be found encrypted within the primary sequence of whey proteins such as lactoferrin, β-LG and BSA. For example, β-lactorphin (YLLF), corresponding to fragment f(102-105), is the result of the digestion of bovine β-LG with pepsin and trypsin, or trypsin with chymotrypsin[117].

Chapter VI – Experimental Design

This chapter summarizes the materials and methods utilized during this work. Initially it presents the reader with a bioinformatic analysis on the generation, *in silico*, of new antimicrobial peptides derived from whey proteins. Following this analysis, the reader is guided through the experimental design employed to study the *in vitro* release of potential antimicrobial peptides from whey proteins and compare the results obtained.

VI.1. Bioinformatic analysis

In the first part of this experimental work, in the same framework as the work performed by Dziuba, B. and Dziuba, M. (2014)[179], new peptides with potential antimicrobial activity, encrypted in whey protein sequences, were searched with the use of bioinformatic tools. The whey proteins, β-LG, α-LA, bovine LF and κ-CN (soluble portion), were hydrolysed *in silico* by α-chymotrypsin, the enzyme used in this study. The obtained peptides were characterized by the following parameters: molecular weight, isoelectric point, composition and number of amino acid residues, net charge at pH 7.0, aliphatic index, instability index, Boman index, and GRAVY index, and compared with those calculated for known 40 antimicrobial peptides from bovine milk proteins listed in the BIOPEP database. Moreover, new whey-derived peptides with antimicrobial potential were searched for, applying computer simulated proteolysis of whey proteins and prediction algorithms such as SVM (support vector machines), RF (random forest), ANN (artificial neural networks) and DA (discriminant analysis) available at the interface of the CAMP database.

VI.1.1. Physicochemical properties of bovine milk antimicrobial peptides

The physicochemical properties of antimicrobial peptides derived from bovine milk proteins and listed in the BIOPEP database were calculated with the use of software and algorithms freely available on the internet. Molecular weight, isoelectric point, the number and composition of amino acid residues, values of instability index, aliphatic index and GRAVY index (grand average of hydropathicity) of AMPs were calculated in the ProtParam application (accessed from 10 to 17 December 2014). The instability index provides an estimate of the stability of a given protein; when the instability index is smaller than 40, the protein is stable, whereas a value above 40 predicts that the protein may be unstable. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). The GRAVY value for a peptide or protein is calculated as the sum of hydropathy value of all the amino acids, divided by the number of residues in the sequence.

The Boman index was calculated with APD2 algorithm: Antimicrobial Peptide Calculator and Predictor (accessed from 10 to 17 December 2014), and net charge was determined using Innovagen's Peptide Property Calculator (accessed from 10 to 17 December 2014).

VI.1.2. *In silico* proteolysis of whey proteins

The amino acid sequences of reference whey proteins were subjected to *in silico* proteolysis in the BIOPEP database. The “Enzyme(s) action” application was used to determine the release of peptides from the precursor proteins. The option involving a single enzyme was applied for protein hydrolysis with chymotrypsin (E.C. 3.4.21.1). Of the peptides released, the ones composed of 5 to 30 amino acid residues were submitted to statistical prediction of antimicrobial activity.

VI.1.3. Prediction of antimicrobial activity of peptides released during *in silico* proteolysis of whey proteins

The antimicrobial activity of the released peptides was determined with the use of the Prediction Antimicrobial Peptides tool in the CAMP database[180]. Four multivariate statistical methods were used for prediction: RF, SVM, ANN and DA. The process of model development and evaluation has been described in detail by Waghu *et al.* (2014)[83]. The prediction results are presented with the relevant scores, excluding ANN, and peptides are classified as AMPs or non-AMPs. In this study, peptides were classified as AMPs if the resulting score was higher than 0.45 and if a positive recognition was obtained for at least two statistical methods. Then, the selected peptides were processed in the APD database to describe their amino acid content and structure and to determine the presence of residues on the same hydrophobic surface of the molecules[181].

VI.2. Casein precipitation

VI.2.1. Isoelectric precipitation

In its natural state milk is negatively charged, which allows the dispersion of caseins. However, when an acid is added to milk, the protons neutralize the anionic charges of the casein micelles (specifically κ-casein). If milk is acidified to a pH near 4.6, the isoelectric point of casein, a pellet is formed. In this work, precipitation of caseins was done by adding a solution of HCl 5M drop-by-drop to a pre-heated (~42ºC) volume of 500 mL of Ultra-High Temperature (UHT) and High Temperature Short Time (HTST) skimmed milk purchased in a local super market (UHT milk was from Pingo Doce brand, whereas HTST milk was Vigor). Samples were retrieved for pH’s 4.8, 4.6, and 4.3 because it is in this interval that the precipitation occurs. The protein content of the samples was measured at 280 nm and compared with the initial sample. The remaining liquid, acid whey, was centrifuged at 7000 rpm and 4ºC for 15 minutes and the quantification of total protein was done by the BCA assay. Whey was stored in the freezer (-20 ºC) for future assays.

VI.2.1. Enzymatic precipitation

The precipitation of κ-caseins can also be performed via enzymatic hydrolysis with chymosin. Chymosin is the predominant milk-clotting component of rennet, a crude mixture of enzymes traditionally extracted from the stomachs of calves. The enzyme specifically cleaves the Phe_{105}–Met_{106} bond of κ-casein to give two macropeptides, a hydrophobic N-terminal extreme named p-κ-CN and a hydrophilic C-terminal moiety called CMP[182]. This C-terminal region protrudes to the media, and provides electrostatic and steric stabilization for the protein suspension. When the chymosin causes the enzymatic destabilization of κ-CN, the second stage of spontaneous aggregation of p-κ-CN occurs, followed by the formation of a 3D network of gel[11]. For the enzymatic precipitation of caseins, approximately 10 drops of liquid chymosin, purchased in a local pharmacy, were added to 500 mL of pre-heated (37 ºC) HTST skimmed milk. The milk was left to clot for approximately 30 minutes and the remaining liquid (sweet whey) was centrifuged at 7000 rpm and 4ºC for 15 minutes. The quantification of total protein was done by the BCA assay and the liquid was stored in the freezer (-20 ºC) for future assays.

VI.3. Enzymatic Activity Assay

For enzyme activity determination, casein (P>96%), trichloroacetic acid (P>99.5%), sodium carbonate (P>95%) and Folin-Ciocalteu's phenol reagent (2N) and L-tyrosine (P> 99%) were purchased from Merck. Calcium acetate (P>98%) and α-chymotrypsin type II from bovine pancreas (40 U/mg solid) were purchased from Sigma-Aldrich. Sodium carbonate (P>99.5%) and potassium phosphate dibasic trihydrate (P>99%) were purchased from Panreac. Quantification of the enzyme activity was done using Hitachi's U-2000 double beam UV/Vis spectrophotometer (Hitachi High Technologies America, Inc.), with a cell size of 1 cm, and a SIGMA 1-15P microcentrifuge (Sartorius AG, Germany). During the times of incubation, a thermostatic orbital shaker Agitorb 160E (Aralab Equipamentos de Laboratório e Electromecânica Geral LDA, Lisbon, Portugal) was used. Protein digestion was performed in capped microtubes of 2 mL from Eppendorf (Hamburg, Germany).

To test the enzyme activity, 15±1 and 7±1 mg of enzyme was diluted in 1 mL of 10 mM sodium acetate buffer (pH 7.5) with 5 mM of calcium acetate. From this standard solution, several others were prepared to evaluate the correlation between enzyme activity and degree of dilution. The dilutions of 1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:400 were prepared. The estimation of the enzymatic activity was performed according to a Sigma protocol[183] as follows: 0.9 mL of 0.65% (w/w) casein as the substrate and 0.18 mL of the appropriate diluted enzyme solution were incubated at 37ºC for 10 minutes. The reaction was stopped by adding 0.9 mL of 110 mM trichloroacetic acid (TCA). The mixture was then kept at 37ºC for 30 minutes and finally centrifuged at 13,000 rpm for 5 minutes. Additionally, 0.5 mL of the supernatant fluid was collected, and the volumes of 1.25 mL of sodium carbonate and 0.25 mL of Folin-Ciocalteu's phenol reagent were added to this fraction and the solution mixed by vortexing. The solution was

then again centrifuged at 13,000 rpm for 5 minutes. The colour developed by the solution was then measured at 660 nm. A standard graph was generated using standard tyrosine solutions of 0-0.111 µmol. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of amino acid equivalent to tyrosine used as standard in 1 min at pH 7.5 and 37ºC. Equations 1 and 2 were used to determine the enzyme units per mg of solid preparation. The remaining 1.5 mL of the supernatant was used to assay the soluble total protein concentration by the BCA method.

$$U/mL = \frac{\mu mol\ T \times V_{total}}{V_{enzyme} \times Incubation\ time \times V_{assay}}$$

Equation 1

$$U/mg = \frac{U/mL}{mg\ enzyme/mL\ diluent}$$

Equation 2

Where T is tyrosine, V_{total} is the total volume (2 mL), V_{enzyme} is the volume of diluted enzyme used (0.18 mL), the incubation time is 10 minutes and V_{assay} is the volume of the supernatant used in the colorimetric assay determination (0.5 mL). It is important to note that this assay was also performed on the hydrolysis of skimmed milk, acid whey and sweet whey with α-chymotrypsin.

VI.4. Protein Quantification

The colorimetric determination of protein concentration in solution was done by the Bicinchoninic acid (BCA) assay[184]. A calibration curve was obtained using a set of standards with the following concentrations of BSA (ChemCruz): 2 mg/mL, 1.5 mg/mL, 1 mg/mL, 0.75 mg/mL, 0.50 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 25 mg/mL. Absorbance was measured at 562 nm on the SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany). Each well contained 25 µL of sample or standard and 200 µL of BCA working solution (50 parts solvent A to one part solvent B). The microplates were incubated 30 minutes at 37ºC before reading.

VI.5. Tricine SDS-PAGE

For this experiment, tricine (P≥99%), acrylamide (P≥99%), bis-acrylamide (P=99%), Tris(hydroxymethyl)aminoethane (P≥99.9%), N, N, N', N'–tetramethyl-ethylenediamine (TEMED), and Ammonium persulfate (APTS, P>98%), were purchased from Sigma-Aldrich. Coomassie Brilliant Blue R-250 (P) was purchased from Fluka. β-mercaptoethanol and dodecyl sulfate sodium salt (SDS) were purchased from Merck. Glycerol (86-88% w/w) was purchased from Scharlam. Analysis of the whey fractions obtained after hydrolysis with α-chymotrypsin and purification in small-scale SP-Sepharose XL™ columns was performed by Tricine sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to Shägger & von Jagow[185]. Briefly, Tricine SDS-PAGE was carried out in 16.5%T, 6%C polyacrylamide gels (~4.5 cm), with a "spacer" gel of 10%T, 3%C (~1.2 cm), and a stacking gel of 4%T, 3%C (~0.6cm) on a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories). Electrophoresis was performed at room temperature, cooled only by the ambient air, using a voltage stepped procedure: voltage was kept constant (30 V) until the samples completely left the stacking gel and then the voltage was increased 15 V per min for 4 times. Voltage was maintained constant at 90V until the tracking dye reached the bottom of the gel. The polypeptide SDS-PAGE standards molecular weight marker (MW ~1,400 ~26,600 Da; Bio-Rad Laboratories) was used and it is composed of Trisephosphate isomerase (26.625 kDa), Myoglobin (16.950 kDa), α-Lactalbumin (14.437 kDa), Aprotinin (6.512 kDa), Insulin b chain (oxidized, 3.496 kDa), and Bacitracin (1.423 kDa). Gels were firstly stained with Coomassie Brilliant Blue and, whenever the intensity of the bands was too low, the gels were subsequently stained with silver nitrate. Gels were scanned with the GS-800™ Calibrated Densitometer (Bio-Rad Laboratories) and saved as .jpeg files.

VI.6. Isoelectric Focusing

Isoelectric focusing (IEF) was performed in order to assess the isoelectric points of the peptides present in the hydrolysates. The IEF was performed in a Pharmacia PhastSystem separation module using PhastGel® IEF 3-9 with 50×46×0.45 mm (GE Heatlthcare). Gels were run at 2000 V for 410 Vh (~30 minutes), after a 75 Vh prefocusing step at 200 V for 15 Vh (~10 minutes). The pI markers used were a Broad pI Kit, pH 3-10 (GE Healthcare), composed by, amylglucosidase (pI=3.50); methyl red (pI=3.75); trypsin inhibitor (pI=4.55); β-lactoglobulin A (pI=5.20); bovine carbonic anhydrase B (pI=5.85); human carbonic anhydrase B (pI=6.55); myoglobin, acidic band (pI=6.85); myoglobin, basic band (pI=7.35); lentil lectin, acidic (pI=8.15); lentil lectin, middle (pI=8.45); lentil lectin, basic (pI=8.65) and trypsinogen (pI=9.30). In addition a sample of myoglobin (from horse skeletal muscle, Sigma Aldrich) was also run in the gel to help with the identification of the pI protein samples. The myoglobin sample was prepared by adding 1 mL of ultra-pure water (Merck Millipore), 300 µL NaCl 1M and 50 µL of Tris-HCl 1.5 M pH 8.5 to 2.5 mg myoglobin. Gels were stained with silver nitrate.

VI.7. Small-scale Ion-Exchange chromatography

The small scale purification was performed using 1 mL adsorbent (SP-Sepharose XLTM, GE Healthcare). Each column was washed previously with approximately 10 column volumes (CV) of ultra-pure water. The preparation of the adsorbent for purification consisted in the equilibration of the adsorbent with the working buffer (citrate 20 mM pH 4.5) until the working pH is reached. The soluble sweet whey hydrolysate (SWH) was also in the same pH conditions as the column. Two conditions were tested: in the first one SWH (10 mL) was corrected to pH 4.5

with the working buffer (240 mL) yielding a solution diluted 24x ("diluted" SWH); in the second one SWH was corrected to pH 4.5 with NaOH 1M ("concentrated" SWH).

One mL of soluble SWH was loaded into the column, then a washing step of the adsorbent was performed with working buffer (~ 4 CV). The elution step was carried out with the working buffer containing NaCl 150, 250 or 350 mM, collecting 10 fractions of approximately 1 mL each, for diluted SWH. When concentrated SWH was used, 500 µL were loaded into the column and 5 fractions of 2 mL were collected. In this case the elution step was done with working buffer with 250 mM NaCl and citrate buffer 20 mM pH 6. The adsorbent regeneration was performed using 2 CV of NaOH 1M and then washed with ultra-pure water (~5 CV). The adsorbent was stored in distilled water in the fridge, at approximately 4ºC.

VI.8. Antibacterial Testing

VI.8.1. Model organisms and growth conditions

The bacterial isolates tested were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PA14, *Burkholderia contaminans* IST408 and *Staphylococcus aureus* Newman. The *S. aureus*, *P. aeruginosa* and *B. contaminans* strains used were specifically chosen for being isolated from human infections[186–188], while the *E. coli* strain used is a reference strain used in antimicrobial testing (Clinical and Laboratory Standards Institute, M26-A). The cells were cultivated in Mueller-Hinton broth (MHB) (Fluka Analytical) at 37 ºC for 16 h.

VI.8.2. Antibacterial assay

Mueller-Hinton broth prepared double-strength was added (70 µL) to the round-bottomed wells of a sterile polystyrene 96-well microplate (Greiner Bio One International GmbH). Sweet whey before and after hydrolysis and the fractions collected from the SP-Sepharose XL™ column were all tested (70 µL) without serial dilution. Wells were then inoculated with 70 µL of mid log-phase culture of bacterial strain suitably diluted in MHB. Negative controls were the working and the elution buffers (70 µL), and they were also inoculated. The microplates were then incubated at 37 ºC for 24h. Positive controls were obtained using penicillin-streptomycin solution at a final concentration of 0.03x (prepared from a 100x stock solution, Sigma Aldrich) added to inoculated wells. This concentration inhibited all tested bacteria. After 24h of incubation microplates were read at 640 nm on a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). The percentage of inhibition was measured as indicated in the equation below.

$$Inhibition\ (\%) = \left[1 - \left(\frac{OD_{640nm} sample}{OD_{640nm} negative\ control}\right)\right] \times 100$$

Equation 3

Chapter VII – Results and Discussion

This chapter presents the results obtained throughout this investigation and has been organized according to the objectives proposed. Thus, the first subchapter outlines the bioinformatic evaluation of new antimicrobial peptides derived from whey proteins, followed by the obtainment of sweet whey and characterization of the peptides released upon it's hydrolysis with α-chymotrypsin, including determination of molecular weight, isoelectric point and prediction of the percentage of inhibition after a 24h incubation with the studied bacterial strains.

VII.1. Bioinformatic analysis

VII.1.1. Physicochemical characteristics of bovine milk AMPs

The theoretically calculated physicochemical properties and amino acid content of the 40 antimicrobial peptides originating from bovine milk proteins is summarized in Tables 12 and 13. Physicochemical properties of individual AMPs from bovine milk proteins are presented in Table A1 (Annexes pp. 83-84). More than 70% of all peptides contain Ala, Ile, Leu, Lys, Pro and Val. The Arg, Gln, Ser, Thr and Trp are present in at least 60% of the peptides, while Asn, Asp, Cys, Glu, Gly, His, Met, Phe and Tyr are their minor components. The content of amino acids in the sequences of the AMPs was calculated in reference to all of the examined peptides. The results indicate that certain amino acids are more common in the peptide sequences and can influence their biological activity. For instance, amino acids such as His, Pro, Arg, and Trp could be predominant in the sequences of biologically active peptides, such as antimicrobial ones[189].

The highest amino acid content in all analyzed antimicrobial peptides derived from bovine milk proteins was recorded for Lys, Asp, Tyr and Arg, whereas the lowest content was found for Met and His. However, evaluation of the amino acids content in the sequences where a given amino acid was found suggests that, in addition to the four predominant amino acids, Ala, Val and Pro may also play a certain role. Antimicrobial peptides generally have two common physical features: a cationic charge (high number of cationic Arg, Lys and His, and no or few acidic residues) and a significant proportion (≥30%) of hydrophobic residues (Ala, Val, Ile, Leu, Met, Phe, Tyr and Trp)[126].

The noted results are similar to those given by Wang, Z. and Wang, G. (2004)[181] for AMPs in the APD database, as well as the ones given by Hammami, R. *et al.* (2007)[190] in the Bactibase, with the exception of Gly and Leu that are also reported to be present in the antimicrobial peptides sequences in both databases.

Table 12. Amino acid content of the analyzed bovine milk-derived antimicrobial peptides.

Amino acid	Average amino acid content (%)		Number of peptides containing given amino acid
	a	b	
Ala (A)	10.6	8.6	29
Arg (R)	12.3	8.2	24
Asn (N)	11.0	2.1	7
Asp (D)	14.0	1.6	4
Cys (C)	8.9	2.7	11
Gln (Q)	10.8	7.2	24
Glu (E)	9.6	2.9	11
Gly (G)	7.3	2.4	12
His (H)	5.7	1.6	10
Ile (I)	8.1	5.8	26
Leu (L)	11.2	7.8	25
Lys (K)	14.5	10.4	26
Met (M)	3.7	1.6	15
Phe (F)	7.4	2.5	12
Pro (P)	10.9	8.2	27
Ser (S)	8.4	4.2	18
Thr (T)	9.1	5.6	22
Trp (W)	7.6	4.0	19
Tyr (Y)	12.5	4.2	12
Val (V)	11.3	8.5	27

a: For all antimicrobial peptides; and b: For peptides that contain given amino acid

The amino acid composition of given peptide it's often related to its specific properties. The minimum, maximum and mean values of selected physicochemical properties of the analyzed AMPs are presented in Table 13.

The molecular weight of the evaluated peptides ranged from 540 to 5500 Da, with an averaged of approximately 2100 Da. Molecules with a molecular weight in the range of 1000-3000 Da were predominant in approximately 80% of the peptides, whereas peptides in the range of 500-1000 Da were frequent in approximately 39%. This results are similar to the ones reported by Dziuba, B. and Dziuba, M. (2014)[179] for all AMPs derived from milk proteins (ovine, caprine and bovine).

Table 13. Physicochemical characteristics of antimicrobial peptides derived from bovine milk proteins in BIOPEP.

Index	AMPs from Bovine Milk Proteins		
	Mean Value	Minimum Value	Maximum Value
Molecular weight (Da)	2165.5	542.5	5451.6
pI	8.8	4.0	12.0
Instability index	45.7	-30.9	157.7
Aliphatic index	86.3	27.9	182.5
GRAVY index	-0.4	-2.0	1.3
Boman index (kcal/mol)	1.6	-1.4	4.2
Net charge at pH 7	3.3	-2.0	10.1

Out of the bovine milk-derived AMPs, 24 had pI > pH 7 and 12 had pI < pH 7. These findings correlate with peptide net charge in neutral pH and the content of various amino acids discussed earlier (cationic peptides, positively charged). In the analyzed group of peptides, 26 had a positive charge, 6 were negatively charged, and 4 were neutral. The average net charge was 3.3. Structure activity relationship analyses have yielded vital information relating to the structural features of effective antimicrobial peptides, indicating that antimicrobial activity is governed primarily by net positive charge and hydrophobicity, and that the initial target is the negatively charged bacterial cell membrane[191]. In addition to cationic AMPs, significant numbers of anionic peptides from eukaryotic organisms, including milk proteins, have been reported. Anionic peptides are generally abundant in Asp and Glu, they have a net charge of -1 or -2, and similarly to cationic peptides they can form amphiphilic structures, such as α-helix or β-sheet, that are crucial for their activity[192].

The value of the instability index calculated for 18 bovine milk AMPs (50%) was below 40, indicating their stability[193]. The aliphatic index, which is defined as the relative volume occupied by aliphatic side chains (Ala, Val, Ile and Leu), is regarded as a posivite factor for the increase of thermostability of globular proteins[194]. In all the analyzed peptides these amino acids were present, and for 78% of all peptides the aliphatic index was in the range of 60-180. The value of the GRAVY index, a measure of peptide solubilty[195], was negative for 26 bovine milk-derived AMPs (72%), and this is indicative of their hydrophobic nature. The value of the Boman index, which is a measure of peptide activity to proteins and its ability to establish biological interaction[196], ranged from -1.4 to 4.2 kcal/mol. The predominant value of the Boman index was 1-3 (72%). A higher Boman index value indicates that an AMP will be multifunctional or play a variety of different roles within the cell due to its ability to interact with a wide range of proteins. Moreover, a more hydrophobic peptide tends to have a negative index, while a more hydrophilic peptide tends to have a more positive index[196].

VII.1.2. *In silico* proteolysis of bovine milk proteins

Due to the fact that sequences of milk proteins and the cleavage sites of enzymes are known as well as the properties of antimicrobial peptides are well defined it is possible to apply bioinformatic tools in the search for new AMPs. This strategy is based on computer simulation of proteolysis and application of multivariate statistical methods to determine potentially antimicrobial motifs released from the analyzed proteins. *In silico* proteolysis can be performed with the use of several programs, such as PMAP (www.proteolysis.org/proteases) and PeptideCutter (www.expasy.org/tools/peptidecutter/), or a dedicated tool in the BIOPEP database. Briefly, the "Analysis" menu in BIOPEP "Bioactive peptides" section, contains the "Enzyme(s) action" option, which can be used to design the proteolytic process.

In this study, the enzyme chymotrypsin (EC 3.4.21.1) from the BIOPEP database was used to stimulate the proteolysis of β-LG (UniProtKB P02754), α-LA (UniProtKB P00711), LF (UniProtKB P24627), and also κ-CN (UniProtKB P02668) that can remain in whey after the destruction of casein micelles. The simulation produced hundreds of fragments, of which one example is given in Figure 8, but antimicrobial activity predictions were based solely on peptide chains containing 5 to 30 amino acids. In the case of fragments derived from β-LG, only 15 out of 38 fragments were analyzed. For fragments derived from α-LA, 12 out of 30 fragments were analyzed, for LF-derived fragments, only 57 out of 137 and for k-casein only 10 out of 31 peptide fragments were further evaluated. Additionally, the "Search for active fragments" command was used to determine released fragments whose structure corresponded to that of peptides with known antimicrobial activity. Those fragments were not further examined.

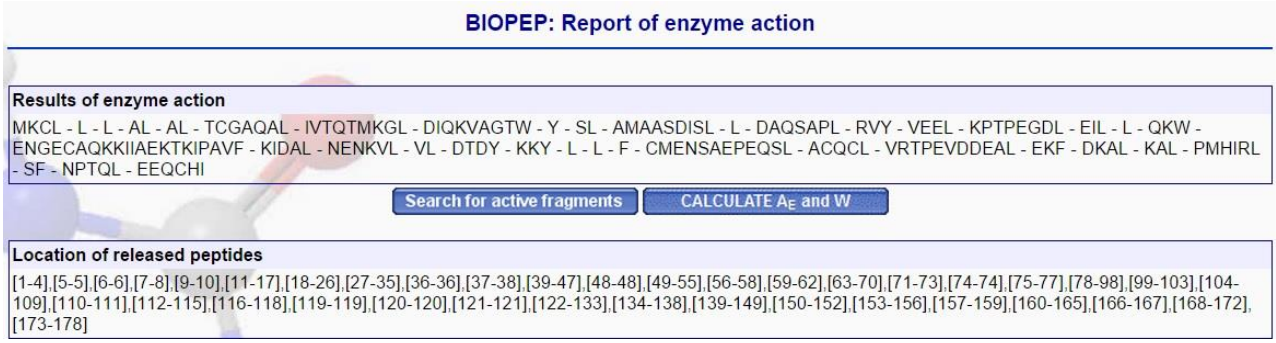

Figure 8. Results window of peptides released upon *in silico* hydrolysis of bovine β-lactoglobulin by chymotrypsin generated by BIOPEP database.

VII.1.3. Prediction of antimicrobial activity of peptides released during *in silico* proteolysis of whey proteins

The antimicrobial activity of peptides can be analyzed with the use of dedicated tools in several databases, such as APD[82,181], AMPA[197] and CAMP[83,180]. APD relies on the physicochemical properties of peptides, and if those parameters are within the APD-defined space for natural AMPs, the program will align their sequences with those present in the database. The AMPA algorithm uses the propensity scale to generate an antimicrobial profile and the calculation is based on antimicrobial indexes defined for individual residues. However, the AMPA algorithm is useful in analysis of whole proteins rather than their fragments. Moreover, the prediction tools available in CAMP, based on machine learning algorithms such as SVM, RF, ANN and DA, were most suitable for the purpose of this study.

In the first stage, the antimicrobial potential of the obtained fragments was evaluated with the use of four statistical models available in the CAMP database. The results were used to manually select peptides where a positive result was reported in at least two algorithms. Overlapping and partially overlapping peptides based on known AMPs from milk proteins, as well as peptides with poor water solubility (as predicted by Innovagen's Peptide Property Calculator), were excluded. A total of thirteen potentially antimicrobial peptides with scores higher than 0.45, or a positive recognition (in the case of ANN) for at least two statistical models (Table 14) were thus selected. The above procedure supported the identification of 88 fragments whose sequences and properties are presented in Table A2 (Annexes pp. 85-89). The peptides identified in CAMP were additionally analyzed with the use of the tools available in the APD database.

The general classification of AMPs include cationic peptides which can be divided in three subclasses: linear peptides forming helical structures, cysteine-rich open-ended peptides containg single or several disulfide bridges and molecules rich in specific amino acids, such as proline, glycine, histidine and tryptophan[198]. In the group of known AMPs derived from bovine milk proteins (Table A1, Annexes pp. 83-84), 30 have a positive charge ranging from 0.1 to 3. The second group contains anionic peptides that are generally rich in glutamic acid and aspartic acid[198]. Those peptides account for approximately 30% of all known bovine milk protein-derived AMPs. Another important feature characterizing AMPs is their ability to form amphipathic sructures[199]. This process involves hydrophobic amino acids such as tryptophan and valine, which account for 30%-50% of the peptide sequence[191].

Table 14. Characteristics of potential AMPs from bovine whey proteins released during *in silico* proteolysis and predicted by statistical models available in CAMP database (SVM, RF, ANN and DA).

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge	SVM	RF	ANN	DA
CKDDQNPHSSNICNISCDKF	α-LA f(80-99)	2268.4	5.3	83.31	39	-1.01	3.04	-1	0.354	0.5345	NAMP	0.594
ENGECAQKKIIAEKTKIPAVF	β-LG f(78-98)	2317.7	8.25	19.41	83.81	-0.352	1.33	1	0.176	0.3645	AMP	0.611
AAPRKNVRW	LF f(19-27)	1097.2	12.01	85.56	54.44	-1.233	3.56	3	0.936	0.4885	AMP	0.120
EAGRDPY	LF f(85-91)	806.8	4.37	8.57	14.29	-1.857	3.97	-1	0.848	0.5785	NAMP	0.000
GTKESPQTHY	LF f(102-111)	1147.2	6.75	126.91	0	-1.96	3.03	0.1	0.718	0.625	NAMP	0.007
AVAVVKKGSNF	LF f(113-123)	1119.3	10	-14.91	97.27	0.591	0.13	2	0.943	0.483	AMP	0.912
QGRKSCHTGL	LF f(129-138)	1086.2	9.51	58.17	39	-1.11	2.85	2	0.488	0.2205	AMP	0.007
GKNKSRSF	LF f(291-297)	923	11.17	106.74	0	-1.875	4.44	3	0.124	0.466	AMP	0.190
RPTEGY	LF f(443-446)	721.7	6	58.38	0	2	3.91	0	1.000	0.4985	NAMP	0.025
AVAPNHAVVSRSDRAAHVKQVL	LF f(594-608)	2325.6	10.84	45.77	106.36	0.036	1.73	2.2	0.662	0.319	NAMP	0.461
EACAF	LF f(701-705)	539.6	4	95.88	40	1.08	-0.21	-1	0.988	0.3665	AMP	0.006
MMKSF	k-CN f(1-5)	642.8	8.50	2.24	0.00	0.380	0.25	1	1.000	0.405	NAMP	0.914
AKPAAVRSPAQIL	k-CN f(83-95)	1321.5	11.00	115.48	113.08	0.292	0.63	2	0.527	0.428	AMP	0.193

[a] SVM: Support Vector Machines; [b] RF: Random Forest; [c] ANN: Artificial Neural Networks; [d] DA: Discriminant Analysis

An analysis of the selected peptides with the use of APD tools revealed that seven fragments may form amphipathic helices. Fragment AVAPNHAVVSRSDRAAHVKQVL (Table 14) contains five cationic (one lysine, two arginine and two histidine) and one anionic (aspartic acid) residues, one proline and it may have five residues on the same hydrophobic surface. Peptide ENGECAQKKIIAEKTKIPAVF has four positively charged (four lysines) and three negatively charged (three glutamic acids) residues, it has nine hydrophobic residues, but only two have been predicted on the same hydrophobic surface. Peptide AVAVVKKGSNF fragment is cationic due to the presence of two lysines with a high hydrophobic ratio of 54%, but only two residues have been predicted on the same hydrophobic surface. In the same way, fragment MMKSF is a cationic peptide due to the presence of one lysine with a high hydrophobic ratio of 60%, but also only two residues have been predicted on the same hydrophobic surface. Peptide AKPAAVRSPAQIL has two cationic amino acid residues (one lysine and one arginine), which makes it a cationic peptide. It also has two prolines, a hydrophobic ratio of 53%, and three residues on the same hydrophobic surface. There are two negatively charged peptides each with possibly two residues on the same hydrophobic surface. Peptide CKDDQNPHSSNICNISCDKF has three negatively charged residues (three aspartic acids) and three positively charged residues (two lysines and one histidine), and peptide EACAF has one negatively charged residue (glutamic acid), but it has four hydrophobic residues (two alanines, one lysine and on phenylalanine) that contribute for a hydrophobic ratio of 80%.

The remaining six peptides (AAPRKNVRW, EAGRDPY, GTKESPQTHY, QGRKSCHTGL, GKNKSRSF and RPTEGY) do not have residues on the same hydrophobic surface, according to the APD database. Three of them are cationic, two are neutral and one is anionic. Fragment AAPRKNVRW contains three positively charged residues (two arginines and one lysine) and four hydrophobic residues (two alanines, one valine and one tryptophan) that contribute for a total hydrophobic ratio of 44%. Fragment QGRKSCHTGL contains 3 cationic residues (lysine, histidine and arginine) and two glutamic acids. Fragment GKNKSRSF contains no anionic residues and two cationic residues (lysine and arginine). The hydrophobic ratio for both fragments is relatively low at 20% and 12%, respectively. Fragment GTKESPQTHY contains two cationic residues (one lysine and one histidine) and one anionic residue (glutamic acid), but due to the presence of glutamine, the overall charge is 0.1. Fragment RPTEGY is a neutral peptide with one positively charged residue, but it also contains one proline and one glycine. The latter two peptides have a hydrophobic ratio of 0%. Fragment EAGRDPY contains two negatively charged and one positively charged residue, and also one proline and one glutamic acid typical for anionic peptides. Despite not forming amphipathic structures, these peptides could potentially exhibit antimicrobial activity, since there are known antimicrobial peptides derived from milk proteins that do not form these structures either. Some examples are VLVLDTDYK, YYQQKPVA, IKHQGLPQE and LECIRA.

Another important feature of antimicrobial peptides is stability. Molecules with an instability index higher than 40 are considered as unstable, and therefore they are characterized

by lower bioavailability and shorter half-time. In the group of selected peptides (Table 14), 3 were predicted to be stable. Nevertheless, some processing techniques, such as chemical modification or incorporation of synthetic amino acids, could be applied to increase peptide stability.

VII.2. Enzymatic activity of α-chymotrypsin

VII.2.1. Estimation of a working enzyme concentration

In order to evaluate the enzymatic activity of α-chymotrypsin both in a casein solution and in ultra-high temperature (UHT) skimmed milk, several dilutions of an enzyme solution of 15 mg/mL and 7 mg/mL were prepared. The dilutions were also studied with the goal of determining the best range of enzyme solution working concentration for further assays. For this, a tyrosine standard curve was generated in order to determine the amount of tyrosine released upon hydrolysis of casein and UHT skimmed milk with different dilutions of the standard enzyme solutions. The resulting equation, with a R^2 of 0.9952, is as follows:

$$Absorbance_{660nm} = 1.2419\ \mu mol_{tyrosine} + 0.0052$$

Equation 4

Solving this equation in order to the amount of tyrosine released, in µmol, the enzyme activity and enzyme specific activity can be determined for the several enzyme dilutions (Tables 15 and 16).

Table 15. Summary of the amount of tyrosine released (µmoles), enzyme activity (U/mL) and enzyme specific activity (U/mg) for the different dilutions of a 15 mg/mL and a 7 mg/mL standard enzyme solution, with a 0.65% (w/v) casein solution as the substrate.

	15 mg/mL standard enzyme solution			7 mg/mL standard enzyme solution		
Dilutions	**Tyrosine released (µmoles)**	**Enzyme Activity (U/mL)**	**Enzyme Specific Activity (U/mg)**	**Tyrosine released (µmoles)**	**Enzyme Activity (U/mL)**	**Enzyme Specific Activity (U/mg)**
1:5	1.98	4.41	1.47	1.06	2.35	1.68
1:10	1.95	4.34	2.89	0.99	2.21	3.16
1:20	1.85	4.11	5.48	0.86	1.91	5.44
1:50	1.52	3.37	11.24	0.62	1.39	9.90
1:100	1.40	3.10	20.69	0.12	0.28	3.96
1:200	0.92	2.05	27.31	0.08	0.17	4.85
1:400	0.68	1.51	40.31	0.06	0.13	7.65

Table 16. Summary of the amount of tyrosine released (µmoles), enzyme activity (U/mL) and enzyme specific activity (U/mg) for the different dilutions of a 15 mg/mL and a 7 mg/mL standard enzyme solution, with UHT skimmed milk as the substrate.

	15 mg/mL standard enzyme solution			7 mg/mL standard enzyme solution		
Dilutions	**Tyrosine released (µmoles)**	**Enzyme Activity (U/mL)**	**Enzyme Specific Activity (U/mg)**	**Tyrosine released (µmoles)**	**Enzyme Activity (U/mL)**	**Enzyme Specific Activity (U/mg)**
1:5	1.36	3.01	1.00	1.50	3.33	2.38
1:10	1.31	2.92	1.94	1.46	3.25	4.64
1:20	1.16	2.57	3.42	1.12	2.48	7.08
1:50	0.85	1.90	6.32	0.85	1.89	13.48
1:100	0.63	1.40	9.36	0.55	1.23	17.51
1:200	0.55	1.23	16.34	0.29	0.63	18.14
1:400	0.51	1.13	30.05	0.09	0.20	11.53

In general, both the results from the hydrolysis of casein (Table 15) and UHT skimmed milk (Table 16) with several dilutions of enzyme solution standards of 15 and 7 mg/mL were expected, meaning that with the increase in the dilution factor, the less enzyme concentration was present, and the less tyrosine was released. Consequently, the enzyme activity decreased with the increasing dilution factor. Moreover, it was also expected that the enzyme dilutions from the 7 mg/mL standard enzyme solution translated into a lower enzyme activity, since less final enzyme concentration was present. In the case of the hydrolysis of casein this is verified, however, for the hydrolysis of UHT skimmed milk the activities are quite similar, with the exception of the last dilutions (1:100, 1:200, and 1:400, respectively). Nevertheless, the results show that even for the smallest concentration of enzyme in solution, the later exhibits activity towards both substrates. Also, comparing the enzyme dilutions from the 15 mg/mL standard enzyme solution for both substrates, it is possible to note that mostly the enzyme activity towards UHT skimmed milk is lower than the enzyme activity towards casein. This could be due to inhibitor agents in milk, such as the micellar structure of the casein itself that prevents full access to them, in comparison with soluble caseins.

Regarding the enzyme specific activity in particular (Tables 15 and 16), it increases with the decrease in enzyme concentration (increase in the dilution factor). Interestingly, the commercial enzyme specific activity (40 U/mg) was achieved when the enzyme concentration is 400 times lower than 15 mg/mL, respectively 0.0375 mg/mL when using casein as the substrate. However, the value of 40 U/mg is not achieved for any of the other dilutions, including when using UHT skimmed milk as the substrate. Even so, the closest one is also observed for the concentration of 0.0375 mg/mL of enzyme in the hydrolysis of UHT skimmed milk, where the value obtained was 30 U/mg.

In terms of the soluble protein concentration for both the hydrolysis of casein and UHT skimmed milk (Figure 9 A and B, respectively), it was also observed a decrease with the increase in the dilution factor. Furthermore, as expected, it was verified a smaller protein concentration for the dilutions of a stock 7 mg/mL enzyme solution. Also, in general, the protein concentration was higher for the hydrolysis of UHT skimmed milk for both the stock enzyme solution of 15 mg/mL and 7 mg/mL.

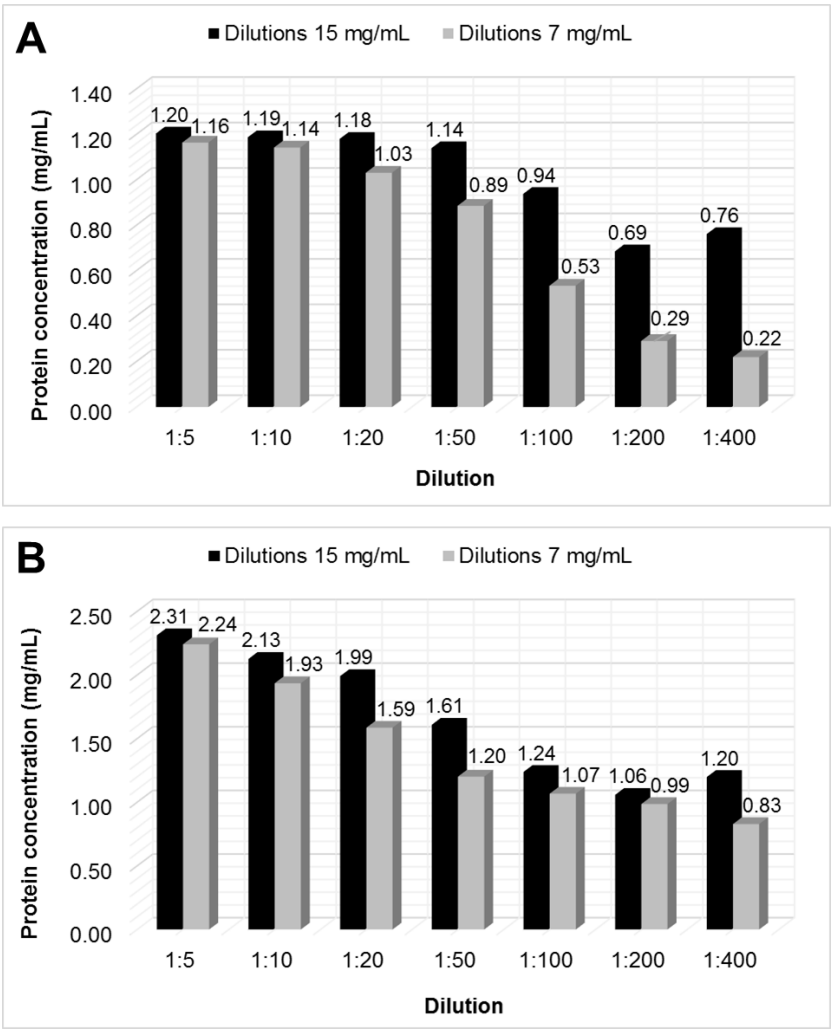

Figure 9. Soluble protein concentration, in mg/mL, for the hydrolysis of A) a casein solution (initial protein concentration 6.5 mg/mL) and B) UHT skimmed milk (initial protein concentration 21.3 mg/mL), using a series of dilutions of stock enzyme solutions of 15 and 7 mg/mL.

These results may suggest that the hydrolysis was more successful when using a casein solution as the substrate instead of UHT skimmed milk, because the soluble protein concentrations are lower in comparison with UHT skimmed milk. This means that the higher the enzyme activity (lower dilution factor), the more fragments are in the supernatant (proteins and peptides that do not precipitate with the addition of TCA) and the more protein in the given supernatant is expected. In the case of using UHT skimmed milk as the substrate, the results may be influence by milk inhibitors, for example casein micelles themselves that may prevent the action of the enzyme.

Furthermore, since the minimal enzyme concentration (enzyme diluted 400x) yielded the maximum enzyme specific activity for one of the substrates, it was studied the enzyme kinetics for the hydrolysis of different concentrations of substrate using an enzyme concentration of 0.0375 mg/mL (Figure 10).

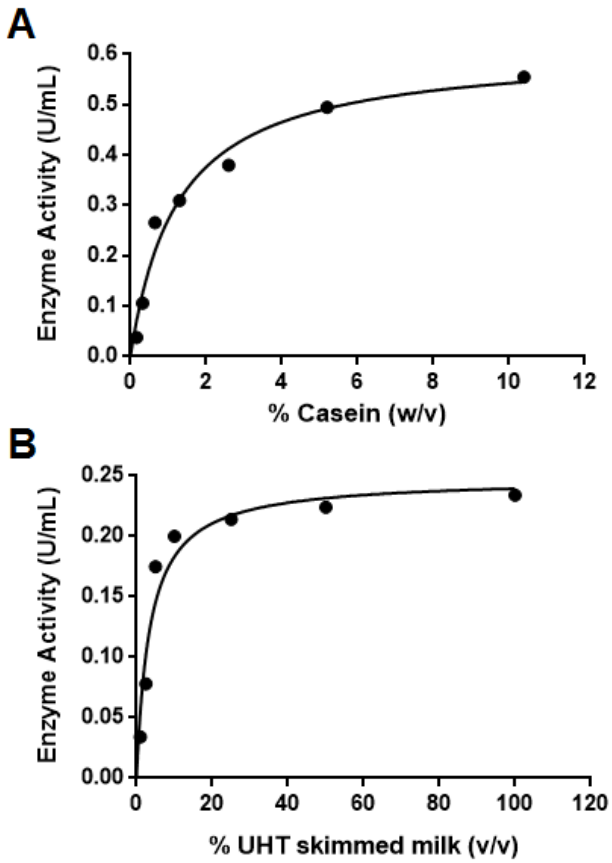

Figure 10. Michaelis-Menten enzyme kinetics of the hydrolysis of A) casein and B) UHT skimmed milk, using an enzyme concentration of 0.0375 mg/mL.

From the analysis of Figure 10, when a casein solution is used as the substrate, the rate of reaction when the enzyme is saturated (V_{max}) is 0.61 U/mL, whereas for UHT skimmed milk this value decreases to 0.25 U/mL. In relation to the Michaelis-Menten constant (K_m) the values are 1.2 and 3.5, respectively. This means that, in order to achieve half of the enzyme activity, the concentration required for casein is 1.2% (w/v) and for UHT skimmed milk is 3.5% (v/v). The enzyme kinetics constants were determined by the Lineweaver-Burk method.

Despite of displaying the best enzyme specific activity, the lower enzyme concentration tested (0.0375 mg/mL) showed the lowest results for the enzyme activity (Figure 11). Results from Figure 11 suggest that the best working concentration should be between 0.5 and 3 mg/mL, since they provide the best results in terms of enzyme activity (U/mL). For instance, for casein hydrolysis, the maximum enzyme activity achieved was 4.3 U/mL and 2.2 U/mL for the dilutions of a 15 mg/mL and 7 mg/mL standard solution, respectively. In the case of UHT skimmed milk

hydrolysis, the maximum enzyme activity obtained was 2.9 U/mL and 3.2 U/mL for the dilutions of a 15 mg/mL and 7 mg/mL standard solution, respectively. This values of maximum enzyme activity were achieved with enzyme concentration in the range of 1-3 mg/mL in the case of dilutions of a 15 mg/mL standard solution, and in the range of 0.5-1.5 in the case of dilutions of a 7 mg/mL standard solution. Therefore, the enzyme concentration chosen for further analysis was 2 mg/mL. This concentration allows a rapid hydrolysis reaction (10 min) and a high number of fragments can be produced.

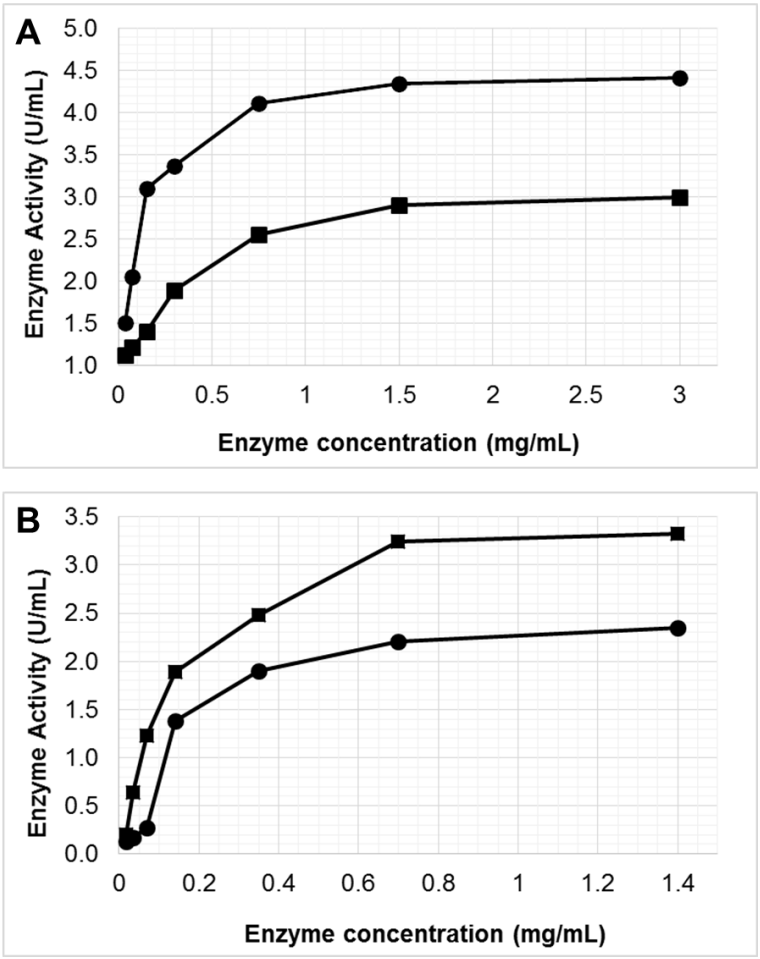

Figure 11. Graphical representation of the enzyme concentration, in mg/mL, *versus* the enzyme activity, in U/mL achieved by the different dilutions of a A) 15 mg/mL standard enzyme solution and B) 7 mg/mL standard enzyme solution for the hydrolysis of casein (●) and UHT skimmed milk (■).

VII.3. Casein precipitation

To obtain whey – the main substrate in this work – the precipitation of caseins from milk was performed via acidic and enzymatic hydrolysis. Milk, in its natural state, is negatively charged. The negative charge allows the dispersion of caseins in milk. When an acid is added to milk, in this case hydrochloric acid, the protons concentration neutralize the anionic charges of the casein micelles. When milk is acidified to pH 4.6, the isoelectric point of casein, a precipitate known as acid casein is formed (Figure 12). The remaining liquid is called acid whey.

Figure 12. Precipitated caseins with a tridimensional jellified appearance.

Caseins can also be precipitated by chymosin, which is the predominant milk-clotting component of rennet, a crude mixture of enzymes traditionally extracted from the stomachs of calves. This enzyme specifically cleaves the Phe_{105}-Met_{106} bond of κ-casein to give two macropeptides, a hydrophobic N-terminal extreme named para-kappa-casein (p-κ-CN) and a hydrophilic C-terminal moiety called caseinemacropeptide (CMP)[182]. This C-terminal region protrudes to the media, and provides electrostatic and steric stabilization for the protein suspension. When the chymosin causes the enzymatic destabilization of κ-CN, the second stage of spontaneous aggregation of p-κ-CN occurs, followed by the formation of a 3D network of gel[11]. The liquid obtained upon rennet-induced precipitation of caseins is called sweet whey.

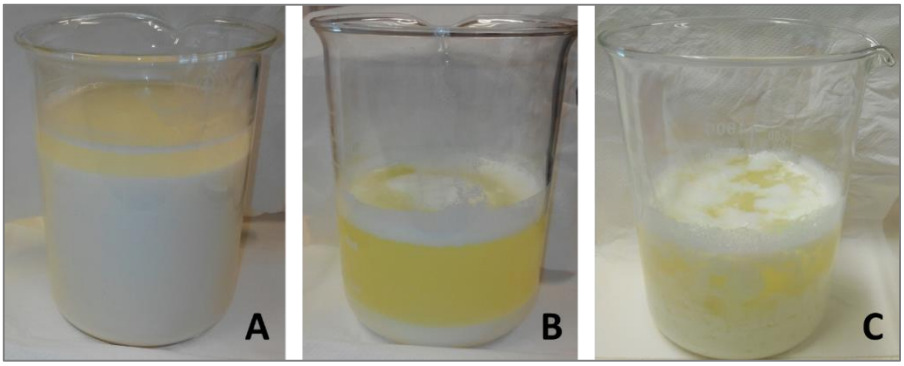

Figure 13. A) UHT skimmed milk and B) pasteurized skimmed milk after isoelectric precipitation of caseins with hydrochloric acid; C) pasteurized skimmed milk after enzymatic precipitation of caseins with liquid chymosin. The images were taken 30 minutes after the precipitation occurred.

For the purpose of this work, the interest was focused on whey, the yellowish liquid remaining after precipitation of UHT skimmed milk (Figure 13A and Figure 14A) and HTST skimmed milk with HCl (Figure 13B and Figure 14B) and HTST skimmed milk with liquid chymosin (Figure 13C and Figure 14C). A volume of approximately 300 mL of whey was obtained from an initial volume of 500 mL of skim milk (UHT or HTST). The results showed that 3/5 of milk (60%) is liquid whey.

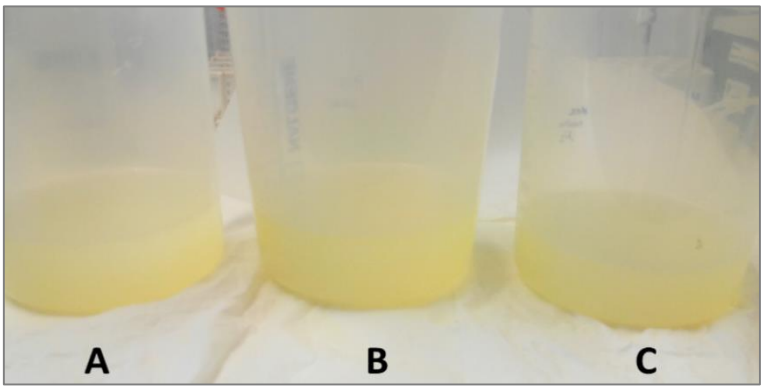

Figure 14. Acid whey obtained by isoelectric precipitation of caseins with hydrochloric acid of A) UHT skimmed milk and B) pasteurized skimmed milk; and sweet whey obtained by enzymatic precipitation of caseins with C) liquid chymosin. The images were taken after filtration and centrifugation, respectively.

Also, several samples were taken during the assays of casein precipitation in order to evaluate the variation in the amount of protein (Figure 15).

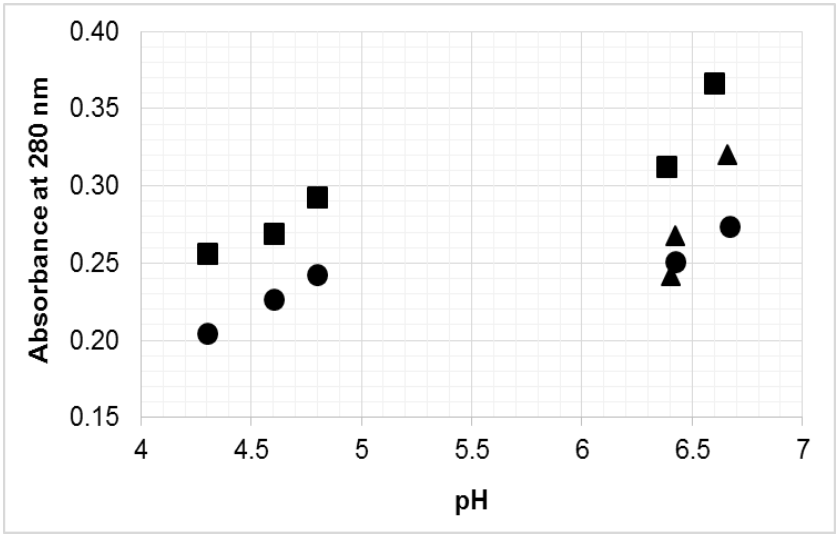

Figure 15. Protein variation as a measurement of absorbance at 280 nm with decreasing pH for the acid precipitation of caseins from UHT skimmed milk (■) and pasteurized skimmed milk (●), and for the precipitations of caseins from pasteurized skimmed milk with liquid rennet (▲).

Figure 15 shows that with decreasing pH (increasing casein precipitation), the absorbance at 280 nm is decreasing. At 280 nm, the aromatic amino acids phenylalanine, tryptophan, and tyrosine present in proteins, have a maximum of absorbance in the UV spectrum

and can be spectrophotometrically detected. With the increased precipitation of caseins, the less proteins are available and thus, the less absorbance of these amino acids is detected, as expected. Moreover, the protein concentration in the different whey obtained was assessed and the results are summarized in Table 17.

Table 17. Summary of the protein concentration, in mg/mL, of the different whey obtained either by isoelectric or enzymatic precipitation of caseins from UHT and HTST skimmed milk.

	Protein concentration (mg/mL)
Acid whey from UHT skimmed milk	1.79 ± 0.08
Acid whey from HTST skimmed milk	2.09 ± 0.04
Sweet whey from HTST skimmed milk	2.45 ± 0.09

From the obtained results, the highest protein concentration (2.45 mg/mL) was obtained for sweet whey, obtained from the chymosin-induced precipitation of caseins in pasteurized skimmed milk. This results are in agreement with Siso, M. (1996) that wrote that acid wheys typically have lower protein contents than sweet wheys[33]. In the other hand, this results can also be explained by the use of two types of milk (two different pasteurization processes). The most commonly used pasteurization processes today are the High Temperature Short Time (HTST) and Ultra-High Temperature (UHT) pasteurization. In the HTST process, milk is heated to a minimum of 72°C for 15 seconds, whereas the UHT pasteurization holds the milk at temperatures in the range of 135 to 140°C for a minimum holding period of 2 seconds[200]. It is now well established that high temperature processing, especially UHT, causes a series of effects on milk such as partial destruction of vitamins, caramelization of lactose and denaturation of proteins[201]. As a result, many chemical changes could also occur in addition to modifications on functional properties of milk proteins. These changes inevitably affect the renneting, emulsifying, and foaming properties of the dairy products based on the processed milk[200]. In this sense, the results summarized in Table 17 were expected as the protein concentration in whey derived from the acid precipitation of caseins from UHT milk is lower (1.79 mg/mL) than the protein concentration in whey derived from the acid precipitation of caseins from HTST milk (2.09 mg/mL). Furthermore, chymosin-induced precipitation of caseins didn't occur with UHT milk (results not shown). This may be due to the high temperatures used in this type of pasteurization and the protein alterations that consequently can occur.

In addition, comparing the total protein amount in the wheys obtained (Table 17) with the total protein content measured for both types of milk (Table 18), it is possible to estimate the percentage of whey proteins (Table 19) by the equation below.

$$\frac{Protein_{whey}(mg/mL)}{Protein_{milk}(mg/mL)} \times 100\%$$

Equation 5

Table 18. Summary of the protein concentration, in mg/mL, of the different types of milk used, UHT and HTST skimmed milk.

	Protein concentration (mg/mL)
UHT skimmed milk	21.3 ± 0.1
HTST skimmed milk	22.3 ± 0.4

Once again, the results in Table 18 corroborate the fact that the high temperatures applied in the pasteurization of UHT milk result in a loss of protein content. When compared to HTST milk, where a lower temperature is applied in the pasteurization process, the loss in protein is approximately 1 mg/mL.

Table 19. Summary of the whey to total protein in milk ratio, of whey obtained either by isoelectric or enzymatic precipitation of caseins from UHT and HTST skimmed milk.

	Whey composition (%)
Acid whey from UHT skimmed milk	8.43
Acid whey from HTST skimmed milk	9.15
Sweet whey from HTST skimmed milk	10.73

As mentioned before, whey proteins constitute 20% of the protein in bovine milk[9], but differences in processing conditions of milk may result in variability in composition of whey as far as both protein (IGs, LF, BSA, β-LG, α-LA) and non-protein (lipids, lactose, minerals) components are concerned[202]. The values obtained and summarized in Table 19 reveal that the whey protein to total protein in milk ratio varies with the type of milk, with lower values obtained from isoelectric precipitation of caseins (8.43 and 9.15%), when compared to rennet-driven precipitation (10.73%). The results also show that the ratio is lower when UHT skimmed milk is used, showing that the composition of proteins in whey is dependent on the intensity of the thermal treatment of milk.

In a study by Carbonaro, M. *et al.* (2000), they also measured the composition of acid whey from pasteurized (HTST) and UHT milk, obtaining a ratio of 10.99% and 6.00%, respectively[202]. The results summarized in Table 19 are close to what was expected, but the differences could be due to the methods used to precipitate the caseins (in this study it was used hydrochloric acid, instead of acetic acid), and the total protein was measured by the BCA method, instead of the Kjeldahl method used by Carbonero *et al.*.

VII.4. Characterization of Sweet Whey Hydrolysate

Sweet whey is one of the most frequent obtained type of whey since the precipitation of caseins in the cheesemaking industry is most commonly achieved through the action of liquid rennet, also known as chymosin. Also, due to the importance of this work in terms of obtaining peptides with potential antimicrobial properties with use in human health, it was given more relevance to the characterization of sweet whey instead of acid whey, mainly because of its non-toxic nature. Therefore, this section reflects solely the results obtained for the analysis of soluble sweet whey hydrolysate (SWH).

VII.4.1. Hydrolysis with α-chymotrypsin

The hydrolysis of sweet whey with α-chymotrypsin aimed to generate peptides to be further fractionated and purified and screened for their antimicrobial activity against *Escherichia coli (E. coli)*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Burkholderia contaminans* (*B. contaminans)* and *Staphylococcus aureus* (*S. aureus*). The concentration of α-chymotrypsin used was 2 mg/mL and the enzyme activity and enzyme specific activity were measured as well as the soluble total protein concentration (Table 20). It is worth noticing that after the enzyme action and the addition of TCA to stop the hydrolysis the pH of the sweet whey hydrolysate was registered as 2, whereas the initial pH for the sweet whey was registered at 6.7. Also, Tricine SDS-PAGE (Figure 16) and isoelectric focusing (Figure 17) were performed to samples of HTST skimmed milk, sweet whey (SW), and sweet whey hydrolysate (SWH) in order to characterize these samples in terms of molecular weight and isoelectric point.

Table 20. Summary of the amount of tyrosine released (μmoles), enzyme activity (U/mL) and enzyme specific activity (U/mg) upon hydrolysis of sweet whey obtained from HTST skimmed milk with a 2 mg/mL solution of α-chymotrypsin. The protein concentration, in mg/mL, was also measured.

Tyrosine released (μmoles)	Enzyme Activity (U/mL)	Enzyme Specific Activity (U/mg)	Soluble protein concentration (mg/mL)
0.528	1.173	0.5867	1.05 ± 0.02

In comparison with the initial amount of protein in sweet whey (2.45 mg/mL), the soluble protein concentration in the sweet whey hydrolysate decreased to 1.05 mg/mL. This corresponds to a sweet whey hydrolysate to sweet whey ratio of 42.86% (Equation 6). This means that less than 50% of the proteins were hydrolysed by α-chymotrypsin.

$$\frac{Soluble\ protein_{whey\ hydrolysate}(mg/mL)}{Total\ protein_{whey}(mg/mL)} \times 100\%$$

Equation 6

In fact, the characterization of the soluble SWH by Tricine SDS-PAGE (Lane 4, Figure 16), shows that soluble κ-casein and probably β-LG were not hydrolysed, when compared to α-LA, for instance. However, for a ten minute hydrolysis reaction time, the breakdown of whey proteins was successful, generating small proteins and peptides, varying from 16.7 to 2.8 kDa, and even lower molecular weight peptides, which are not visible by Coomassie staining.

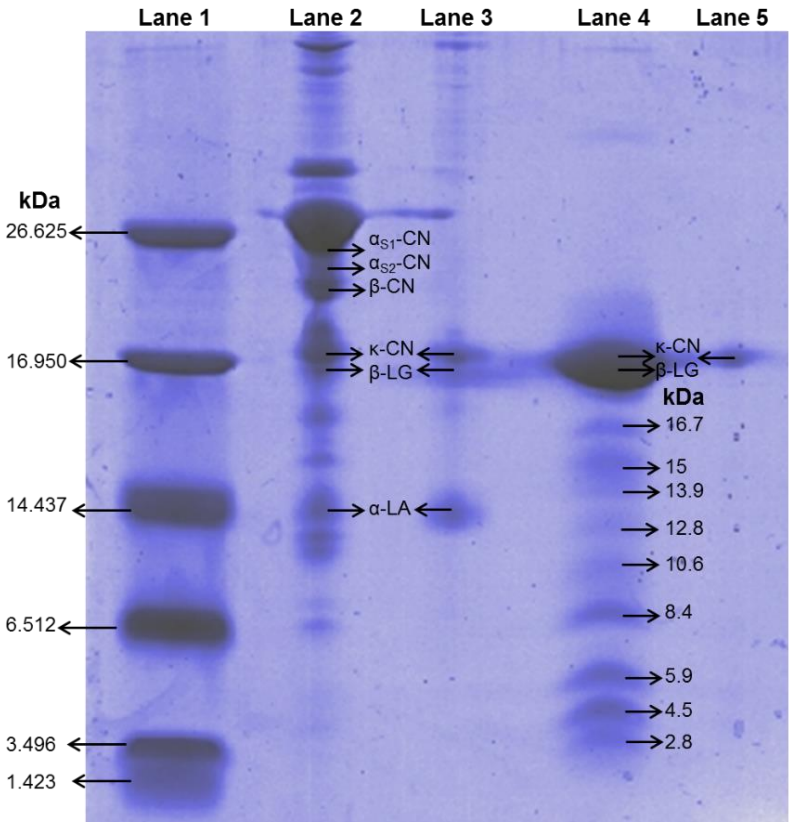

Figure 16. Tricine SDS-PAGE of samples of HTST skimmed milk (diluted 20x, Lane 2), sweet whey (diluted 20x, Lane 3), sweet whey hydrolysate supernatant (without dilution, Lane 4), and sweet hydrolysate supernatant corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 for the subsequent chromatographic separation (diluted 24x, Lane 5). Lane 1 represents the polypeptide SDS-PAGE molecular weight standards composed of

Moreover, it is possible to see that the precipitation of caseins was also successful by comparing the HTST skimmed milk composition (Lane 2) and the sweet whey composition (Lane 3), where the α_{S1}, α_{S2} and β-caseins are non-existent, and only the main proteins in whey, β-LG, α-LA, and soluble κ-casein are present. Additionally, LF (80 kDa), another whey protein, is not visible by Tricine SDS-PAGE since proteins with high molecular weight tend to be retained in the "spacer" gel. Also, its concentration in whey (1.5%) is quite low when compared to β-LG (52%) and α-LA (17%).

The characterization of soluble SWH in terms isoelectric point is highlighted in Figure 17. The IEP of proteins in this solution seem to be between 3.75 and 4.55.

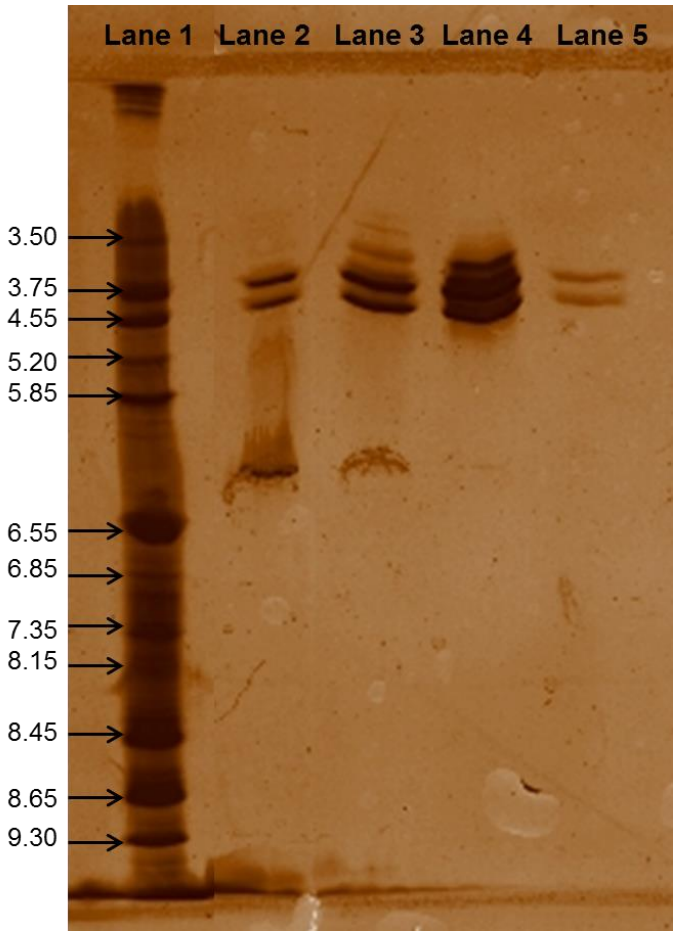

Figure 17. Isoelectric focusing of samples of HTST skimmed milk (diluted 20x, Lane 2), sweet whey (diluted 20x, Lane 3), sweet whey hydrolysate supernatant (without dilution, Lane 4), and sweet hydrolysate supernatant corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 for the subsequent chromatographic separation (diluted 24x, Lane 5). Lane 1 represents the Broad pI Kit, pH 3-10 (GE Healthcare).

Regarding the IEP of milk proteins (Lane 2), due to the proximity of pI values for some proteins, it was not possible to individualize them. Therefore, a summary of the isoelectric points of the main bovine milk proteins is given in Table 21.

Table 21. Summary of the isoelectric points for the major bovine milk proteins.

Protein (suggested abbreviation)	Isoelectric point
α_{S1}-Casein (α_{S1}-CN)	4.44-4.76[a]
α_{S2}-Casein (α_{S2}-CN)	4.8-5.1[b]
β-Casein (β-CN)	4.83-5.07[a]
κ-Casein (κ-CN)	5.3-5.8[a]
β-Lactoglobulin (β-LG)	5.13[a]
α-Lactalbumin (α-LA)	4.2-4.5[a]

[a] According to Farrell, H. *et al.* (2004)[203]

[b] According to Seibert, B., Erhardt, G., and Senft, B. (1985)[204]

VII.4.2. Purification in small-scale ion-exchange chromatography

In this study, 2 mL of the soluble sweet whey hydrolysate (SWH) were loaded into the SP-Sepharose XL™ 1 mL adsorbent (from now on referred to as SP-column), whose apparatus is outlined in Figure 18. The goal was to verify if this adsorbent had the capacity to adsorb some of the peptides effectively and releasing them during the elution step using increasing concentrations of the NaCl salt. Regarding the pI of the peptides in SWH (Figure 17, Lane 5), they seem to be around 3.75 and 4.55, which suggests that with a working pH of 3 the peptides and proteins in the solution should be positively charged and therefore should adsorb to the negatively charged SP-column. Unfortunately, for long-term experiments the pH stability for the column is between 4 and 13 and so the pH chosen for the experiment was 4.5. At this working pH, there should be partially positive charged peptides that would adsorb to the SP-column whereas the non-adsorbed peptides are supposed to be eluted in the washing steps.

Figure 18. Apparatus used for the small-scale purification of the sweet whey hydrolysate using the SP Sepharose XL™ 1 mL adsorbent.

The purification process, quantified in terms of relative soluble protein concentration in the sample loading, removal of non-attached peptides (washing step) and recovery of the desired peptides/proteins (elution step) in relation to the soluble total protein concentration of SWH is shown in Figure 19. Regarding the column used, it can be said that the adsorption was successful, as the percentage of protein in the sample loading was approximately 16±2%, which means that the reaming 84±2% of hydrolysate remained adsorbed in the column. In the washing step, the proteins/peptides that did not adsorb are eluted and this corresponds to a protein concentration of approximately 24±5%. Finally, regarding the elution steps, it is possible to observe that for higher concentrations of salt (250 and 350 mM), this step is more effective, since around 76±1% of the proteins/peptides adsorbed are eluted. In contrast, for the lower salt concentration (150 mM), only approximately 39% of proteins/peptides are eluted (Figure 19).

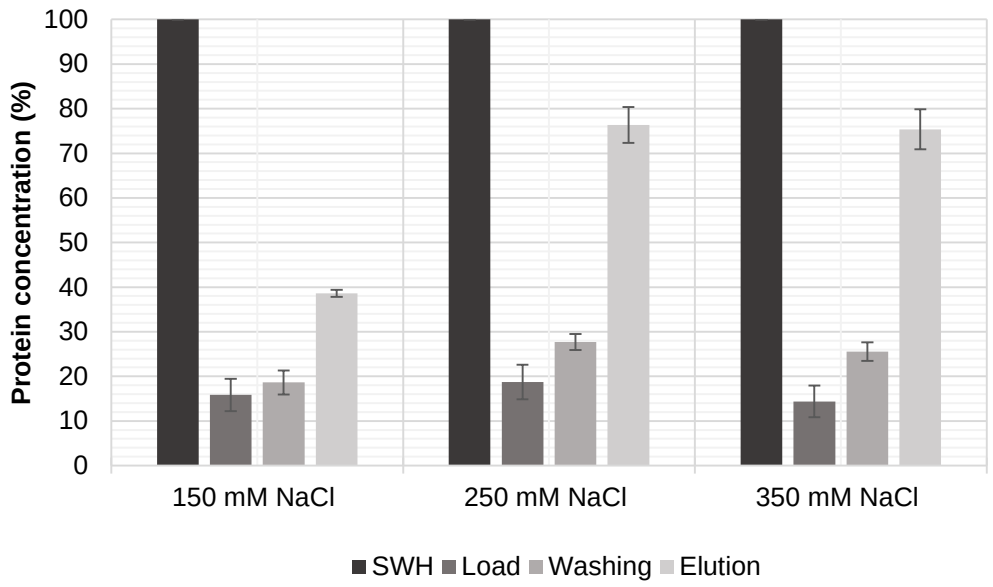

Figure 19. Relative percentage of soluble protein concentration obtained in each chromatographic step (Load, Washing and Elution) concerning the SP-Sepharose XL^{TM} adsorbent (1 mL) used to fractionate and purify the soluble sweet whey hydrolysate (SWH) corrected at pH 4.5 with citrate buffer 20 mM pH 4.5. The initial soluble total protein concentration was 1.05 mg/mL (100%).

The previous results were obtained when the SWH was corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 (diluted SWH). The effect of SWH corrected to pH 4.5 with NaOH 1M (concentrated SWH) was also studied in terms of adsorption to the SP-column (Figure 20) and consequently the fractions collected were also tested for their antimicrobial activity. When studying the effect of concentrated SWH the elution strategies chosen were elution with citrate buffer 20 mM pH 4.5 with NaCl 250 mM, because it showed the better results in the previous case, and elution with citrate buffer 20 mM pH 6.

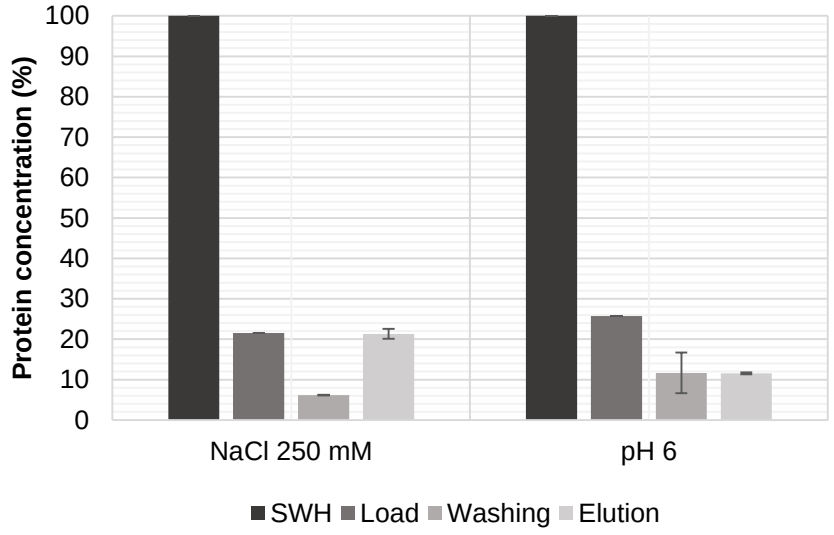

Figure 20. Relative percentage of soluble protein concentration obtained in each chromatographic step (Load, Washing and Elution) concerning the SP-Sepharose XL^{TM} adsorbent (1 mL) used to fractionate and purify the soluble sweet whey hydrolysate (SWH) corrected at pH 4.5 with NaOH 1M. The initial soluble total protein concentration was 1.05 mg/mL (100%).

For both the experiments with concentrated SWH the mean of the load, washing, and elution steps were calculated and yielded the respective values of 23.6±3%, 8.9±4%, and 16.4±7%. Particularly, in the case if the elution step with NaCl 250 mM, the percentage of protein in the sample loading was approximately 21.5%, which means that the reaming 78.5% remained adsorbed in the column, whereas for the elution with citrate buffer 20 mM pH 6, 76.4% remained adsorbed. In terms of the washing steps, 6.1% and 11.7% of the non-attached proteins/peptides were eluted with the working buffer, respectively. Finally, regarding the elution steps, these represent 21.3% and 11.6% of the total protein for elution with salt and citrate buffer pH 6, respectively, suggesting that not sufficient fractions were collected, since approximately 50% of the proteins are still to be collected.

Nonetheless, these preliminary results show a fairly good capacity of these adsorbent, at pH 4.5, to adsorb and purify hydrolysates of sweet whey obtained with α-chymotrypsin. In order to better understand the adsorption capacity of peptides to the negatively charged SP-column, an isolation step prior to the purification, for example nanofiltration, could be eventually assessed, since in the whey hydrolysate remained proteins that were not hydrolysed by α-chymotrypsin. Even so, the isolated fractions of the different purification strategies were analyzed for their antimicrobial activity.

VII.4.3. Assessment of antimicrobial activity

As a visual aid, Figure 21 represents the layout of the 96-well microplate used for the determination of antimicrobial activity. The scheme color helps differentiate between the bacterial strains tested – *E. coli* (orange), *P. aeruginosa* (yellow), *B. contaminans* (blue), and *S. aureus* (green). Each sample was tested in triplicate. For each elution tested 3 microplates were used. The first one is illustrated in Figure 21 and the two others are similar in design but represent the samples eluted from the column (load, washing and elution fractions). NC1 is the negative control with citrate buffer 20 mM pH 4.5, NC2 is the negative control with citrate buffer 20 mM pH 4.5 with 150, 250 or 350 mM NaCl, NC3 is the negative control with ultra-pure water, PC is the positive control with penicillin-streptomycin 0.03x, SW is sweet whey, SWH1 is the soluble sweet whey hydrolysate at pH ~2, SWH2 is the soluble sweet whey hydrolysate corrected to pH 4.5 (either with citrate buffer 20 mM pH 4.5 or NaOH 1M). A total of 13 microplates were inoculated to address the antimicrobial activity of the isolated fractions of the different purification strategies.

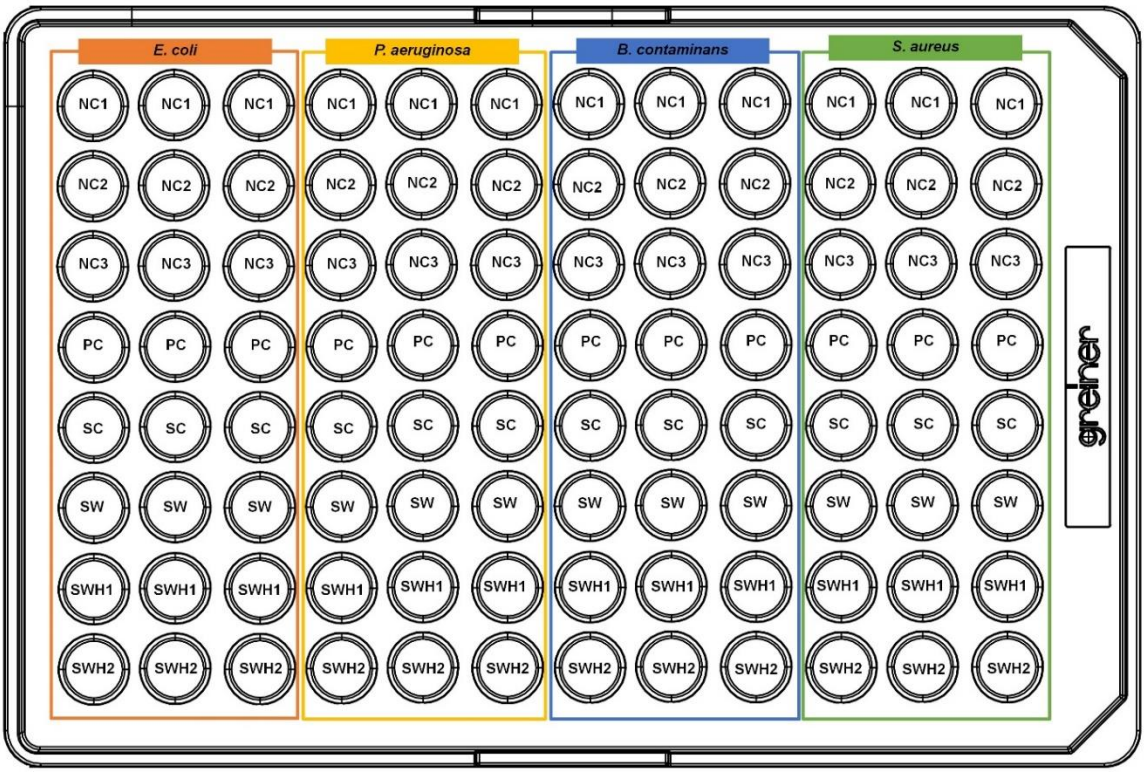

Figure 21. Microplate scheme showing the organization of the different reactions. The wells were inoculated with *Escherichia coli* (orange), *Pseudomonas aeruginosa* (yellow), *Burkholderia contaminans* (blue) and *Staphylococcus aureus* (green). NC stands for negative control, PC for positive control, SC for sterility control, SW for sweet whey, and SWH for sweet whey hydrolysate. The other microplates were used to evaluate the antimicrobial activity of the fractions eluted from SP-Sepharose XL™ column. A total of 13 microplates were used in order to evaluate the effect of the different elution strategies in the purification process.

In terms of the percentage of inhibition of the positive control (Table 22), the results show that the antibiotic mixture of penicillin-streptomycin (0.03x) inhibited approximately 99% of the *E. coli* and *S. aureus* strains, whereas for *P. aeruginosa* the percentage of inhibition was 98%. The less inhibited strain was *B. contaminans* with a mean percentage of inhibition of 88%.

Table 22. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with the positive control penicillin-streptomycin 0.03x.

	Inhibition after 24 hours (%)			
	E. coli	***P. aeruginosa***	***B. contaminans***	***S. aureus***
Penicillin-streptomycin 0.03x	100.0	97.6	99.5	99.6
	98.7	97.4	92.7	100.0
	99.7	99.5	72.2	96.8
Mean Value	99.5	98.2	88.1	98.8

Moreover, it was also addressed the potential of sweet whey and the different soluble sweet whey hydrolysates to inhibit the tested bacterial strains (Table 23). For more detailed results please see Table A3 (Annexes, p.90).

Table 23. Summary of the mean percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey (SW), soluble sweet whey hydrolysate at pH 2 (SWH1), soluble sweet whey hydrolysate corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 (SWH2), and soluble sweet whey hydrolysate corrected to pH 4.5 with NaOH 1M (SWH3).

	Inhibition after 24 hours (%)			
Sample	***E. coli***	***P. aeruginosa***	***B. contaminans***	***S. aureus***
SW	-79.5	-236.1	-104.9	-418.9
SWH1	-21.8	36.7	21.4	-31.4
SWH2	-5.0	-29.2	-7.8	-128.6
SWH3	-12.5	-4.0	17.5	-86.4

In general, the results summarized in Table 23 show that sweet whey is able to promote the cellular growth of all the studied bacterial strains. This was quite expected since whey contains more than half of the solids present in the original whole bovine milk, including whey proteins (20% of the total protein) and most of the lactose, water-soluble vitamins and minerals[205]. Moreover, lactose can act as an alternative carbon source and it has already been proposed as cultivation additive for microbial cultivations[206]. Additionally, when sweet whey is hydrolysed with α-chymotrypsin, an inhibition starts to occur, especially in the *P. aeruginosa* (36.7%) and *B. contaminans* (21.4%) strains. However, when the soluble sweet whey hydrolysates are corrected to pH 4.5 either with citrate buffer 20 mM pH 4.5 (SWH2) or with NaOH 1M (SWH3) the inhibition tends to get smaller. This may suggest that the inhibition was due to the very acidic pH of the initial soluble sweet whey hydrolysate (pH ~ 2), but pH stability tests were performed (Table A4, Annexes p.90) with all the sample controls (buffers) and both sweet whey and sweet whey hydrolysates. At the antimicrobial testing conditions, the pH stability tests showed that no significant alterations to the final pH. Concluding, the observed inhibition of SWH1 for *P. aeruginosa* and *B. contaminans* appears to be due to a synergetic effect among the mixture of fragments present in this supernatant. This also corroborates the fact that in order to manifest their bioactivity, peptides need to be released from their parent molecule.

On the other hand, after the fractionation and purification through the SP-column, some fractions showed a considerable satisfactory capacity of inhibiting some of the bacterial strains tested after 24 hours (Table 24). For full details in the results of the antimicrobial activity please see the Tables A5 to A9 in the Annexes section, pp. 91-93).

Table 24. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours, at 37ºC with fractions collected from the SP-Sepharose XL™ 1 mL adsorbent with different elution strategies and using sweet whey hydrolysate (SWH) corrected to pH 4.5 either with citrate buffer 20 mM pH 4.5 or with NaOH 1M.

			Inhibition after 24 hours(%)			
	Elution strategy	**Fraction**	***E. coli***	***P. aeruginosa***	***B. contaminans***	***S. aureus***
SWH corrected to pH 4.5 with citrate buffer (Diluted SWH)	[NaCl] 150 mM	Load 1	4.8	-	13.7	24.4
		Load 2	4.56	9.2	10.2	-
		Wash 1	5.7	-	13.3	-
		Wash 2	9.4	-	7.8	20.0
		Wash 3	14.7	1.0	11.6	3.5
		Wash 4	19.1	-	2.6	5.8
	[NaCl] 250 mM	Elution 1	16.2	-	13.8	-
		Elution 2	-	-	13.0	-
		Elution 8	4.7	-	-	16.7
	[NaCl] 350 mM	Load 2	6.7	-	-	21.5
		Wash 1	5.2	12.4	4.1	26.5
		Elution 4	-	18.4	7.2	-
		Elution 5	-	18.6	-	-
SWH corrected to pH 4.5 with NaOH 1M (Concentrated SWH)	[NaCl] 250 mM	Wash 1	6.5	-	-	41.7
		Wash 2	5.6	-	-	32.9
		Elution 3	-	-	-	15.9
	pH 6	Wash 1	6.9	-	-	33.5
		Wash 2	4.3	-	-	30.3

In a first look, when comparing the antimicrobial activities of the fractions selected with the positive control penicillin-streptomycin, the results don't look very promising. However, the mixture of these broad-spectrum antibiotics is commonly used in extreme situations, such as when multiple type of bacteria are causing infection or when dealing with drug-resistant bacteria. In this sense, when studying natural possible antibiotics, such as peptides derived from whey proteins, the results appear to be promising. In particular, when concentrated SWH is used, the fractions of non-adsorbed peptides appear to have an inhibitory effect mainly on *S. aureus,* with a mean value of 38±6% for washed fraction 1, and 32±2% for washed fraction 2. In the other hand, the elution strategy with pH 6 did not showed any fraction with potential inhibitory effect, whereas in the elution strategy with NaCl 250mM, eluted fraction 3 exhibited an inhibitory capacity of approximately 16%.

When comparing the results for the diluted SWH, *P. aeruginosa* seems to be the strain less affected by the antimicrobial effect of the fractions, followed by *E. coli,* and *B. contaminans.* More so, independently of the elution strategies, some peptides in the loaded and washed fractions exhibited potential inhibitory effects. This is the case of loaded fraction 2 and washed

fraction 1, which yielded 21.5% and 26.5% inhibition against *S. aureus*, respectively. Also for *S. aureus*, loaded fraction 1 and washed fraction 2 respectively showed an inhibitory capacity of 24.4% and 20%. Regarding *B. contaminans*, the antimicrobial effect was around 12-13% for loaded fraction 1 and washed fractions 1 and 3. Finally, the washed fractions 3 and 4 showed an inhibitory capacity of 14.7% and 19.1%, respectively, against *E. coli*.

Regarding the different elution strategies applied when using diluted SWH, the most effective were the ones using 250mM and 350mM of NaCl. For instance, when a salt concentration of 350mM is used, eluted fractions 4 and 5 showed approximately 18.5% of inhibition against *P. aeruginosa*. Moreover, when using a salt concentration of 250 mM, eluted fraction 1 showed an inhibitory capacity of 16.2% and 13.8% against *E.coli* and *B. contaminans*, respectively. Eluted fraction 2 also acted against *B. contaminans*, revealing an inhibitory effect of approximately 13%. Finally, eluted fraction 8 showed only an inhibitory capacity of 16.7% against *S. aureus*.

Regardless of the elution strategy or the effect of diluted or concentrated SWH, the results summarized in Table 24 showed that whey-derived peptides have a greater inhibitory capacity towards *S. aureus*, the only gram-positive bacteria tested. This could be due to the absence of an outer membrane, when in comparison with gram-negative bacteria, which may difficult the protection of gram-positive bacteria to the environment.

Concluding, the soluble fragments concentration were in the order of 20 to 157 µg/mL. Although copper-ion based assays, such BCA, are the best option for detecting peptides, due to its detection limits, problems may arise with very small peptides or very dilute solutions. Therefore, the concentration measured may not correspond to the real peptide concentration, and thus this results are not comparable with the positive control. Nonetheless, in a study performed by Théolier, J. *et al* (2013) they found inhibitory fractions with concentrations estimated at below 40 µg/mL by the µBCA method, when using a whey protein isolate as the substrate and pepsin as the enzyme for its hydrolysis[207]. Additionally, in the same study, they also tested chymotrypsin as the hydrolysis agent, but no fraction showed antimicrobial activity both against the gram-negative *E. coli* MC4100 and the gram-positive *Listeria ivanovii* HPB28. Still, the results are encouraging, especially in the case of *S. aureus*, a recognized important pathogen in patients with liver diseases[208] and that chronically infects the airways of patients with cystic fibrosis[209].

VII.4.4. Assessment of molecular weight

The assessment of the molecular weight of the peptides present in the fractions that showed antimicrobial activity was performed by Tricine-SDS/PAGE. This is currently one of the most effective electrophoretic methods to analyze peptides and small proteins on gels[185]. The presence of a “spacer” gel between the separating and the stacking gels considerably sharpens the bands for proteins and peptides between 1-5 kDa, and the use of an acrylamide/bis-acrylamide mixture of 49.5%T, 6%C for the separating gel increases the resolution of such peptides.

In order to assess the molecular weight of the peptides the gels were stained always with silver nitrate after staining with Coomassie blue. This suggests that the majority of the peptides in the fractions collected were at least at concentrations below 100 µg/mL, the detection limit for Coomassie blue staining[210]. Moreover, if peptides are visible with silver staining this suggests that they have at least a concentration higher than 1 µg/mL[210]. Additionally, the fact that silver staining is a multistep procedure and that the staining reaction has no endpoint is the main disadvantage of the technique. Furthermore, silver staining is not suitable for quantitative studies, because the proteins can be easily overstained, whereas the centers of the protein spots become lighter than the edges, and no supporting computer software for quantitative analysis are yet available to overcome this difficulty[211]. Tricine SDS-PAGE electrophoresis gel was then performed to the fractions exhibiting antimicrobial activity in order to estimate the molecular weight of the peptides and also to evaluate the presence of single or multiple fragments (Figures 22 and 23).

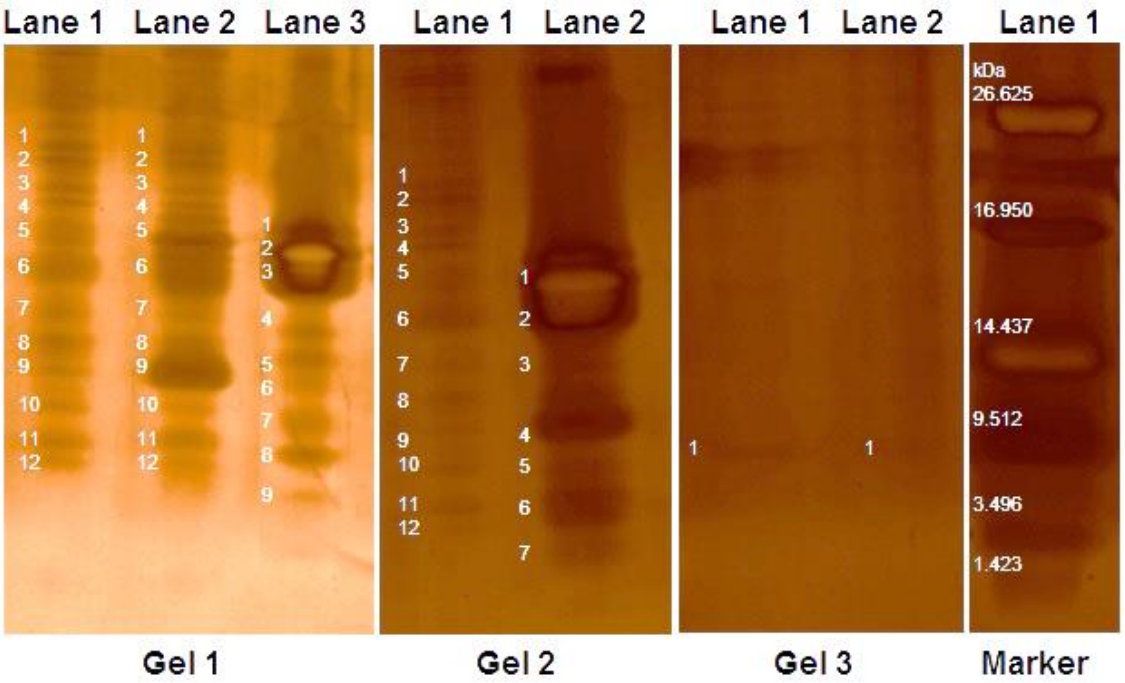

Figure 22. Tricine SDS-PAGE of washed fractions 1 and 2, and eluted fraction 3 from concentrated sweet whey hydrolysate eluted with NaCl 250 mM (Gel 1, Lanes 1, 2 and 3, respectively); washed fractions 1 and 2 from concentrated sweet whey hydrolysate eluted with citrate buffer 20 mM pH 6 (Gel 2, Lanes 1 and 2, respectively; and loaded fraction 1 and washed fraction 1 from diluted sweet whey hydrolysate eluted with NaCl 350 mM (Gel 3, Lanes 1 and 2, respectively). The marker represents the polypeptide SDS-PAGE molecular weight. All the lanes are at the same scale.

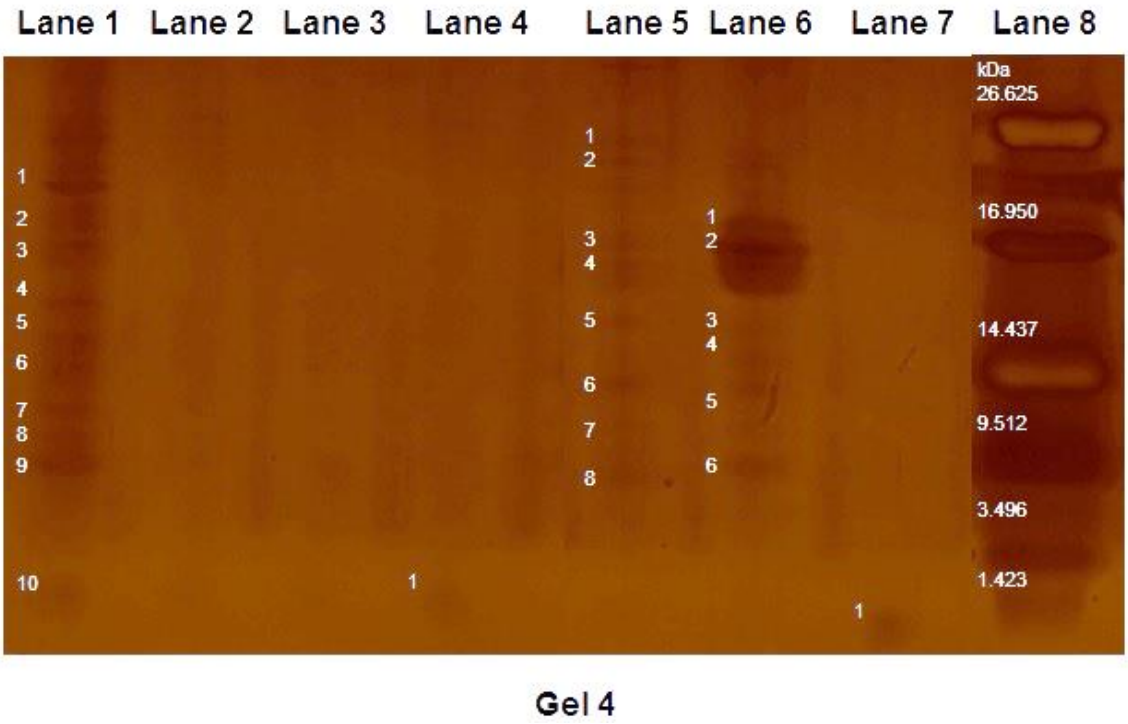

Figure 23. Tricine SDS-PAGE of washed fractions 1 to 4 from diluted sweet whey hydrolysate eluted with NaCl 150 mM (Lanes 1 to 4, respectively), and the eluted fractions 1, 2, and 8 from diluted sweet whey hydrolysate eluted with NaCl 250 mM (Lanes 5, 6, and 7, respectively. Lane 8 represents the polypeptide SDS-PAGE molecular weight. All the lanes are at the same scale.

Table 25 summarizes the approximate molecular weights for the peptides found in each chromatographic fraction that showed antimicrobial activity.

Table 25. Summary of the predicted molecular weight of the peptides encountered in the chromatographic fractions that showed antimicrobial activity.

	Predicted Molecular Weight (kDa)											
	Gel 1			**Gel 2**		**Gel 3**		**Gel 4**				
Lane / Strip	**1**	**2**	**3**	**1**	**2**	**1**	**2**	**1**	**4**	**5**	**6**	**7**
1	25.4	25.4	20.9	24.5	19.4	6.5	6.5	22.2	0.8	15.7	21.4	0.4
2	24.6	24.6	19.6	23.6	17.6			20.2		12.6	20.0	
3	23.3	23.3	18.3	22.2	15.3			17.7		7.8	14.4	
4	22.2	22.2	17.2	21.3	12.0			16.2			12.6	
5	20.0	20.0	15.3	19.9	10.0			14.4			8.3	
6	18.8	18.8	14.0	17.6	7.0			12.1				
7	16.6	16.6	10.6	15.3	5.3			10.8				
8	14.7	14.7	8.6	13.9				8.9				
9	13.2	13.2	6.5	12.0				7.1				
10	11.2	11.2		10.0				0.8				
11	9.7	9.7		8.3								
12	8.4	8.4		6.7								

In a first analysis, Lanes 1 and 2 from Gel 3 (Figure 22), corresponding to the loaded fraction 2 and washed fraction 1 from the chromatographic separation of diluted SWH, appear to have a single peptide with an approximate molecular weight of 6.51 kDa. The same happens with Lanes 4 and 7 from Gel 4 (Figure 23), which single band matches to a peptide with a molecular weight of around 800 and 400 Da, respectively.

In Gel 1, Lanes 1, 2, and 3 (Figure 22), corresponding to the washed fractions 1 and 2, and eluted fraction 3 from the chromatographic separation of concentrated SWH and eluted with a salt concentration of 250mM, showed that a mixture of peptides is present, with molecular weights ranging from 25 to 6 kDa, approximately. The same happens to Lanes 1 and 2 from Gel 2 (Figure 22) that correspond respectively to the washed fractions 1 and 2 from concentrated SWH; and Lanes 1, 5, and 6 from Gel 4 (Figure 23) that correspond to washed fractions 1 to 4, and eluted fractions 1 and 2 from diluted SWH eluted with a salt concentration of 250 mM.

Finally, comparing this results with the ones predicted by the bioinformatic analysis, the molecular weights calculated herein are bigger that the ones predicted, in general. Nonetheless, the bioinformatic analysis showed potential antimicrobial peptides with sizes of 0.807 Da (EAGRDPY), 0.923 Da (GKNKSRSF) and 0.722 Da (RPTEGY) and the analysis of the tricine SDS-PAGE showed a peptide (or mixture) with a molecular weight around 0.8 Da, present in the washed fraction 1 from the separation of diluted SWH. Other predicted peptide by the bioinformatic analysis had a molecular weight of 0.539 Da (EACAF), comparable with the 0.4 Da peptide (eluted fraction 8 following elution of diluted SWH with NaCl 250 mM) obtained by tricine SDS-PAGE.

VII.4.5. Assessment of isoelectric point

The assessment of the isoelectric point (IEP) of the peptides present in the antimicrobial fractions was performed by isoelectric focusing. The gels were stained with silver nitrate, which average sensitivity limit is estimated to be 1 to 5 ng protein per band for IEF[212]. As mentioned before, the peptides concentrations may be lower than the detection limit of this method and thus some bands were not visible. Therefore, Figure 24 highlights the fractions which bands were visible.

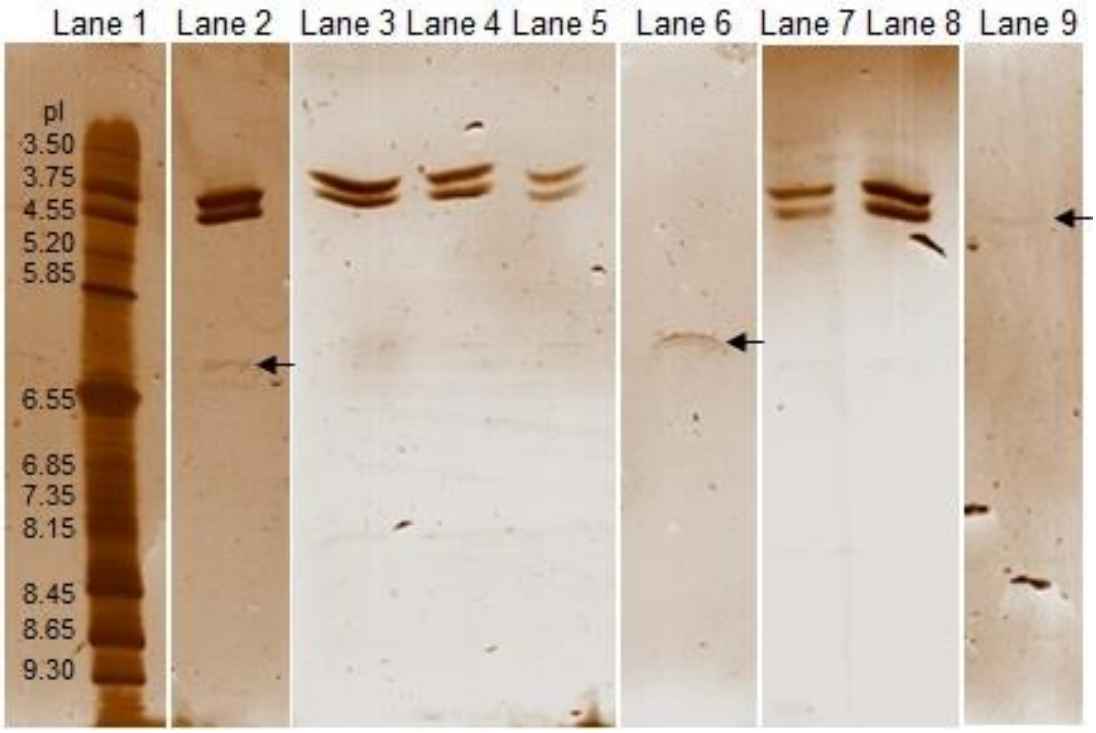

Figure 24. Isoelectric focusing of washed fraction 1 from concentrated sweet whey hydrolysate (Lane 2), eluted fractions 3, 4 and 5 from concentrated sweet whey hydrolysate eluted with NaCl 250 mM (Lanes 3, 4, and 5, respectively), washed fraction 1 from diluted sweet whey hydrolysate (Lane 6), loaded fractions 1 from concentrated sweet whey hydrolysate (Lanes 7 and 8, respectively), and eluted fraction 2 with NaCl 250 mM from diluted sweet whey hydrolysate. Lane 1 represents the Broad pI Kit, pH 3-10 (GE Healthcare).

As visible in Figure 24, the IEP for the majority of the peptides is around 3.75 and 4.55, with the exception of the washed fraction 1 from diluted SWH (Lane 6), with a IEP of approximately 6.12. Moreover, the existence of more than two bands per lane also suggests a mixture of peptides in the chromatographic fractions, as expected from the previous results.

Comparing the results obtained herein with the ones predicted by the bioinformatic analysis, some of the IEP are in agreement with the ones predicted. For example, the peptide EAGRDPY has a theoretical pI of 4.37, peptide RPTEGY a pI of 6 and the peptide EACAF had a predicted pI of 4. This may suggest that one or more of these predicted antimicrobial peptides is present in one of the mixture of peptides in the fractions selected.

Chapter VIII – Conclusions and Future perspectives

The main conclusions from this work are that, in fact, the employment of α-chymotrypsin as the protease for the hydrolysis of whey proteins originates a complex mixture of peptides and small proteins with potential antimicrobial activity. Also, that this mixture of peptides is more effective against the *S. aureus* strain when compared to the *E.coli*, *P. aeruginosa* and *B. contaminans* strains. Although the percentages of inhibition are not comparable to the ones obtained with penicillin-streptomycin (0.03x) for the same bacterial strains, as demonstrated by the tricine SDS-PAGE and IEF techniques, the peptides seem to be in very low concentration (≥1 µg/mL and ≤100 µg/mL), when compared to the BCA results.

Comparing the results obtained with the ones predicted by the bioinformatic analysis, it appears to be at least one peptide identified, RPTEGY. The bioinformatic analysis predicted a molecular weight of 722 Da and a pI of 6 for this peptide, whereas by tricine SDS-PAGE and IEF the molecular weight is around 830 Da and the pI 6.12, respectively. This peptide corresponds to the fragment f(443-446) derived from lactoferrin.

In general, the results obtained are considered positive, since it was demonstrated that low concentrated peptides derived from whey proteins have the potential to act as antimicrobial aids. Moreover, it was also possible to correlate the results obtained with a bioinformatic analysis with an *in vitro* approach.

Nonetheless, further work is necessary, mainly in terms of concentrating the peptides, to better study their capability of inhibiting the studied bacterial strains. Also, since antimicrobial peptides can act also as biopreservatives other strains of interest may be studied, for instance *Salmonella* or *Listeria*. Additionally, the concentration of peptides would help with their identification by the techniques employed herein. Moreover, more sophisticated techniques such as reverse-phase HPLC (RP-HPLC) would facilitate the quantification of peptides and also it would be a comparable technique in terms of identifying the presence of a single peptide or mixture of peptides in the collected fractions.

The results presented herein contribute to the transformation of whey from a waste material to a valuable dairy stream for use in the agro-food, biotechnology, medical and related markets. Whey is currently a major by-product of the modern cheese and casein production industries and represents an important source of environmental pollution due to its large volume, production rate and high organic matter content[213] and therefore it would interesting to have a partnership between academia and a cheese producing company, with the main goal of researching the potential of whey in generating peptides with bioactive activities, in this case antimicrobial. Indeed, a growing body of evidence exists that whey peptides exhibit physiological activities on specific components of the immune system and, consequently, many new products with bioactive peptides have reached the market[214].

Moreover, several studies have suggested that milk protein-derived bioactive peptides may be used as preventive/prophylactic agents to alleviate symptoms of various diseases in humans. Although various drugs exist to cure or slow down the progress of specific diseases in humans, their side-effects may sometimes overshadow their benefits. In this context, food protein-derived peptides, in particular milk protein-derived bioactive peptides have potential as natural alternatives to drugs for disease management[215].

However, in order to fully verify the potential of whey peptides in immunomodulation, conclusions from *in vitro* models need to be constantly corroborated with physiological data obtained *in vivo*[216]. Additionally, for a candidate peptide to be labeled as bioactive, its resistance to gastrointestinal conditions must be determined in advance. The exact mechanisms by which whey peptides exert their bioactivities upon reaching the intestine need further elucidation. For instance, there is a need to assess whether their effect is mediated directly in the gut lumen or through receptors on the intestinal cell wall. Therefore, *in vivo* studies are essential not only to validate the potential bioactive peptides, but also to confirm whether they will require protection from gastrointestinal enzymes when orally administered[217].

Concluding, future research should focus on novel hydrolysis pathways for breakdown of whey proteins and peptides, brought about by unusual proteases aimed at releasing unique amino acid sequences. These may include enzymes from the native microbiota of dairy products or from plant rennets[218]. Additionally, molecular studies concerning the mechanisms by which bioactive peptides exert their activities are to be undertaken[219]. In the specific case of antimicrobial peptides, although neither their effectiveness as food preservatives nor their safety for use as food additives has been sufficiently demonstrated, they offer the advantage of being derivable from generally recognized as safe (GRAS) substances, such as whey. Consequently, their use in medicine and in the food industry should meet with less consumer resistance and easier regulatory approval, on the basis of their natural occurrence[207].

Chapter IX – References

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Table A1. Characteristics of known AMPs derived from bovine milk proteins collected from the BIOPEP database.

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge at pH 7
VYQHQKAMKPWIQPKTKVIPYVRY	α_{S2}-casein f(183-206)	3002.6	10.12	2.25	72.92	-0.783	1.37	5.1
APRKNVRW	LF f(1-8)	1026.2	12.01	95	48.75	-1.613	4.23	3
FKCRRWQWRMKKLGAPSITCVRRAFA	LF f(17-42)	3196.9	11.84	75.31	52.69	-0.485	2.57	7.9
FKCRRWQWRMKKLGAPSITCVRRAFAL	LF f(17-43)	3310.0	11.84	72.89	65.19	-0.326	2.29	7.9
APRKNVRWCTISQPEW	LF f(1-16)	1971.2	9.51	98.42	48.75	-1.056	2.72	2
FKCRRWQWRMKKLGAPSITCVRRAFALECIR	LF f(17-47)	3811.6	11.37	78.95	69.35	-0.316	2.5	7.9
APRKNVRWCTI	LF f(1-11)	1343.6	10.86	101.45	70.91	-0.6	2.74	3
VAGTWY	β-LG f(15-20)	695.7	5.49	-30.87	65	0.45	-1.06	0
CRRWQWRMKKLGAPSITCV	LF f(19-37)	2319.8	10.93	77.14	61.58	-0.468	2.16	4.9
FKCRRWQWRMKKLG	LF f(17-30)	1923.3	11.74	74.31	27.86	-1.421	3.55	6
VYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-casein f(183-207)	3115.7	10.12	2.56	85.6	-0.6	1.12	5.1
LKKISQRYQKFALPQY	α_{S2}-casein f(164-179)	2011.4	10.17	25.45	79.38	-0.938	2.02	4
QELLLNPTHQYPVTQPLAPVHNPISV	B-casein f(184-210)	2906.3	5.98	49.23	112.31	-0.185	0.62	-0.8
GLPQE	α_{S1}-casein f(10-14)	542.5	4	123.56	78	-1.04	1.29	-1
RPKHPIK	α_{S1}-casein f(1-7)	875.0	11.17	19.84	55.71	-2.029	3.68	3.1
IPAVFK	β-LG f(78-83)	673.8	8.75	10.58	130	1.3	-1.36	1

Table A1. Continued.

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge at pH 7
AASDISLLDAQSAPLR	β-LG f(25-40)	1627.8	4.21	60.91	122.5	0.256	1.32	-1
VLVLDTDYK	β-LG f(92-100)	1065.2	4.21	-7.81	151.11	0.344	0.86	-1
KTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-casein f(181-207)	3345.0	10.22	3.11	79.26	-0.726	1.33	6.1
LKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-casein f(180-207)	3458.2	10.22	0.32	90.36	-0.564	1.11	6.1
ALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-casein f(175-207)	4030.8	10.1	11.18	91.52	-0.503	0.91	6.1
QKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-casein f(172-207)	4434.3	10.17	11.08	83.89	-0.589	1.06	7.1
LKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{S2}-casein f(164-207)	5451.6	10.35	9.69	86.36	-0.7	1.44	10.1
YYQQKPVA	κ-casein f(42-49)	996.1	8.5	62.9	48.75	-1.137	1.38	1
VQVTSTAV	κ-casein f(162-169)	803.9	5.49	-11.29	121.25	1.087	0.01	0
STVATL	κ-casein f(141-146)	590.6	5.24	8.33	130	1.267	-0.37	0
FSDKIAK	κ-casein f(18-24)	807.9	8.59	-15.69	70	-0.429	1.93	1
VESTVATL	κ-casein f(139-146)	818.9	4	32.83	133.75	1.038	0.06	-1
PAAVRSPAQILQ	κ-casein f(64-75)	1250.4	10.18	157.72	114.17	0.2	0.84	1
IKHQGLPQE	α_{S1}-casein f(21-29)	1049.1	6.75	63.66	86.67	-1.256	1.92	0.1
VLNENLLR	α_{S1}-casein f(30-37)	970.1	5.97	32.83	182.5	0.075	2.02	0
SDIPNPIGSENSEK	α_{S1}-casein f(195-208)	1486.5	4.14	32.69	55.71	-1.314	2.89	-2
LECIRA	LF f(43-48)	703.8	5.99	81.57	146.67	0.767	1.46	0
APRKNVRWCTISQPEWFKCRRWQWRMKKLGAPSITCVRRAFA	LF f(1-42)	5150.1	11.63	84.35	51.19	-0.702	2.63	9.9
FKCRRWQWRMKKLGAPSITCVRRAFAL	LF f(17-43)	3310.0	11.84	72.89	65.19	-0.326	2.29	7.9
FKCRRWQWRMKKLGAPSITCVRRAFALECIRA	LF f(17-48)	3882.7	11.37	76.8	70.31	-0.25	2.36	7.9

Table A2. Characteristics of potential AMPs from bovine whey proteins released during *in silico* proteolysis according to positive prediction scores of two or more statistical models available in CAMP database (SVM, RF, ANN and DA).

Sequence	Origin and position	Molecular mass (Da)	pI	Instability índex	Aliphatic índex	GRAVY índex	Boman índex (kcal/mol)	Net charge	SVM	RF	ANN	DA
HATQAEQL	α-LA f(15-22)	896.9	5.24	23.4	73.75	-0.875	2.07	-0.9	0.000	0.3125	NAMP	0.000
TKCEVF	α-LA f(23-28)	725.8	5.66	8.33	48.33	0.233	1.1	0	0.001	0.2665	NAMP	0.000
GGVSL	α-LA f(38-42)	431.4	5.52	32.68	136	1.28	-1.48	0	0.000	0.3445	AMP	0.018
VCTTF	α-LA f(46-50)	569.6	5.49	97.88	58	1.62	-0.63	0	0.014	0.377	NAMP	0.003
HTSGY	α-LA f(51-55)	563.5	6.74	-24.06	0	-1.28	1.96	0.1	1.000	0.254	NAMP	0.003
DTQAIVQNNDSTEY	α-LA f(56-69)	1597.6	3.49	14.61	55.71	-1.25	3.32	-3	0.171	0.2745	NAMP	0.005
QINNKIW	α-LA (73-79)	915	8.75	30.27	111.43	-0.9	1.74	1	0.284	0.374	AMP	0.939
CKDDQNPHSSNICNISCDKF	α-LA f(80-99)	2268.4	5.3	83.31	39	-1.01	3.04	-1	0.354	0.5345	NAMP	0.594
TDDIMCVKKIL	α-LA f(105-115)	1278.5	5.62	9.41	132.73	0.536	0.78	0	0.146	0.358	NAMP	0.013
DKVGINY	α-LA f(116-122)	807.9	5.83	-39.94	97.14	-0.557	1.59	0	0.013	0.3055	NAMP	0.008
AHKAL	α-LA f(125-129)	538.6	8.8	38.38	118	0.06	0.33	1.1	0.001	0.4145	AMP	0.082
CSEKL	α-LA f(130-134)	578.6	5.99	29.54	78	-0.38	1.91	0	0.000	0.333	AMP	0.001
TCGAQAL	β-LG f(11-17)	662.7	5.18	-3.56	84.29	0.757	-0.37	0	0.001	0.3445	NAMP	0.007
IVTQTMKGL	β-LG f(18-26)	990.2	8.75	-27.79	118.89	0.578	-0.1	1	0.626	0.403	AMP	0.173
DIQKVAGTW	β-LG f(27-35)	1017.1	5.84	-26.68	86.67	-0.267	0.92	0	0.005	0.2375	AMP	0.030
AMAASDISL	β-LG f(39-47)	878	3.8	22.6	120	1.167	-0.23	-1	0.049	0.2765	NAMP	0.015
DAQSAPL	β-LG f(49-55)	700.7	3.8	98.86	84.29	-0.286	1.3	-1	0.002	0.3655	AMP	0.000
KPTPEGDL	β-LG f(63-70)	855.9	4.37	21.05	48.75	-1.425	2.22	-1	0.979	0.335	NAMP	0.006

Table A2. Continued.

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge	SVM	RF	ANN	DA
ENGECAQKKIIAEKTKIPAVF	β-LG f(78-98)	2317.7	8.25	19.41	83.81	-0.352	1.33	1	0.176	0.3645	AMP	0.611
KIDAL	β-LG f(99-103)	558.6	5.84	-8.98	176	0.54	0.52	0	0.944	0.3955	NAMP	0.021
NENKVL	β-LG f(104-109)	715.8	6	33.65	113.33	-1.067	2.78	0	0.993	0.409	NAMP	0.073
CMENSAEPEQSL	β-LG f(122-133)	1337.4	3.67	119.53	40.83	-0.892	2.42	-3	0.000	0.099	NAMP	0.023
ACQCL	β-LG f(134-138)	536.6	5.55	104.24	98	1.42	-0.75	-0.1	0.014	0.397	NAMP	0.156
VRTPEVDDEAL	β-LG f(139-149)	1243.3	3.92	11.23	97.27	-0.618	3.06	-3	0.011	0.0655	NAMP	0.000
PMHIRL	β-LG f(160-165)	765.9	10.18	164.47	130	0.15	1.23	1.1	0.058	0.467	AMP	0.192
NPTQL	β-LG f(168-172)	571.6	5.52	-12.84	78	-1.1	1.96	0	0.000	0.3315	NAMP	0.208
EEQCHI	β-LG f(173-178)	757.8	4.51	209.77	65	-1.117	2.93	-2	1.000	0.302	NAMP	0.018
AAPRKNVRW	LF f(19-27)	1097.2	12.01	85.56	54.44	-1.233	3.56	3	0.936	0.4885	AMP	0.120
CTISQPEW	LF f(28-35)	963	4	100.59	48.75	-0.5	1.22	-1	0.062	0.2805	NAMP	0.004
KCRRW	LF f(37-41)	747.9	10.86	237.12	0	-2.26	6.35	3	0.999	0.555	NAMP	0.118
RMKKL	LF f(44-48)	674.9	11.17	-8.98	78	-1.32	3.75	3	0.900	0.536	NAMP	0.926
GAPSITCVRRAF	LF f(49-60)	1277.5	10.35	75.64	73.33	0.425	1.5	2	0.682	0.451	AMP	0.749
ECIRAIAEKKADAVTL	LF f(63-78)	1731	6.27	26.22	116.25	0.2	1.47	0	0.054	0.368	AMP	0.043
DGGMVF	LF f(79-84)	624.7	3.8	28.9	48.33	0.767	-0.42	-1	0.998	0.644	AMP	0.001
EAGRDPY	LF f(85-91)	806.8	4.37	8.57	14.29	-1.857	3.97	-1	0.848	0.5785	NAMP	0.000
RPVAAEIY	LF f(94-101)	918	6	80.97	110	0.175	1.16	0	0.346	0.3235	NAMP	0.001
GTKESPQTHY	LF f(102-111)	1147.2	6.75	126.91	0	-1.96	3.03	0.1	0.718	0.625	NAMP	0.007

Table A2. Continued.

Sequence	Origin and position	Molecular mass (Da)	pI	Instability índex	Aliphatic index	GRAVY índex	Boman índex (kcal/mol)	Net charge	SVM	RF	ANN	DA
AVAVVKKGSNF	LF f(113-123)	1119.3	10	-14.91	97.27	0.591	0.13	2	0.943	0.483	AMP	0.912
QGRKSCHTGL	LF f(129-138)	1086.2	9.51	58.17	39	-1.11	2.85	2	0.488	0.2205	AMP	0.007
GRSAGW	LF f(139-144)	632.6	9.75	102.13	16.67	-0.867	2.04	1	0.000	0.26	AMP	0.018
IIPMGIL	LF f(145-151)	756	5.52	9.07	222.86	2.457	-3.28	0	0.001	0.452	AMP	0.017
QGAVAKF	LF f(165-171)	719.8	8.75	-3.56	70	0.4	-0.07	1	0.012	0.3485	AMP	0.090
SASCVPCIDRQAY	LF f(173-185)	1412.6	5.55	46.54	67.69	0.1	1.61	-0.1	0.185	0.37	NAMP	0.020
CKGEGENQCACSSREPY	LF f(192-208)	1861	4.79	46.51	5.88	-1.288	3.08	-1.1	0.195	0.3075	NAMP	0.019
QDGAGDVAF	LF f(219-227)	878.8	3.56	20.86	54.44	-0.078	1.16	-2	0.431	0.3745	NAMP	0.002
VKETTVF	LF f(228-234)	822.9	5.97	4.46	82.86	0.343	0.92	0	0.016	0.2835	NAMP	0.000
PEKADRDQY	LF f(238-246)	1121.1	4.56	2.01	11.11	-2.611	5.39	-1	0.111	0.5975	NAMP	0.012
NNSRAPVDAF	LF f(252-261)	1090.1	5.84	51.75	49	-0.68	2.96	0	0.027	0.2565	NAMP	0.003
KECHL	LF f(262-266)	628.7	6.74	161.08	78	-0.86	2.16	0	0.969	0.289	NAMP	0.000
AQVPSHAVVARSVDGKEDL	LF f(267-285)	1978.1	5.38	46.66	97.37	-0.168	1.8	-0.9	0.162	0.11	NAMP	0.008
SKAQEKF	LF f(291-297)	836.9	8.31	36.09	14.29	-1.571	3.15	1	0.000	0.3125	NAMP	0.004
GKNKSRSF	LF f(298-305)	923	11.17	106.74	0	-1.875	4.44	3	0.124	0.466	AMP	0.190
GSPPGQRDL	LF f(309-317)	926	5.84	79.11	43.33	-1.389	2.86	0	0.000	0.4295	AMP	0.001
KDSAL	LF f(320-324)	532.5	5.84	46.52	98	-0.52	2.18	0	1.000	0.3585	NAMP	0.007
RIPSKVDSAL	LF f(328-337)	1085.2	8.75	21.12	117	-0.08	2.03	1	0.105	0.2105	NAMP	0.008

Table A2. Continued.

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge	SVM	RF	ANN	DA
RETAEEVKARY	LF f(351-361)	1351.4	6.23	29.25	44.55	-1.6	4.62	0	0.000	0.1895	NAMP	0.000
TRVVW	LF f(362-366)	659.7	9.41	8	116	0.46	1.41	1	0.000	0.3355	NAMP	0.015
CAVGPEEQKKCQQW	LF f(367-380)	1633.8	6.13	61.05	27.86	-1.229	2.11	-0.1	0.111	0.328	NAMP	0.153
SQQSGQNVTCATASTTDDCIVL	LF f(381-402)	2242.4	3.56	39.31	70.91	-0.059	1.64	-2.1	0.035	0.1825	NAMP	0.001
KGEADAL	LF f(405-411)	702.7	4.37	-26.46	84.29	-0.557	1.65	-1	0.998	0.38	NAMP	0.000
TAGKCGL	LF f(420-426)	648.7	7.89	-3.56	70	0.386	-0.25	1	0.005	0.3585	AMP	0.061
AENRKSSKHSSL	LF f(431-442)	1343.4	9.99	41.27	40.83	-1.675	4.24	2.1	0.361	0.3095	AMP	0.019
RPTEGY	LF f(447-452)	721.7	6	58.38	0	2	3.91	0	1.000	0.4985	NAMP	0.025
AVAVVKKANEGL	LF f(454-465)	1198.4	8.64	6.77	130	0.55	0.09	1	0.339	0.355	NAMP	0.128
KDKKSCHTAVDRTAGW	LF f(471-486)	1803	9.2	33.71	30.63	-1.225	3.12	2	0.046	0.268	NAMP	0.004
NIPMGL	LF f(487-492)	643.8	5.52	64.2	130	0.783	-1.08	0	0.000	0.3815	AMP	0.012
IVNQTGSCAF	LF f(493-502)	1039.1	5.52	17.08	78	0.69	0.21	0	0.020	0.249	AMP	0.156
SQSCAPGADPKSRL	LF f(507-520)	1416.5	7.94	93.1	42.14	-0.821	2.44	1	0.007	0.1775	NAMP	0.002
CAGDDQGL	LF f(524-431)	777.8	3.56	8.75	61.25	-0.4	1.63	-2	0.186	0.339	NAMP	0.001
DKCVPNSKEKY	LF f(532-542)	1310.4	8.16	12.03	26.36	-1.745	3.36	1	0.345	0.2545	NAMP	0.025
AEDVGDVAF	LF f(553-561)	921.9	3.49	20.86	86.67	0.433	0.95	-3	0.946	0.347	NAMP	0.001
VKNDTVW	LF f(562-568)	860.9	5.81	-17.01	82.86	-0.586	1.86	0	0.006	0.317	NAMP	0.000
ENTNGESTADW	LF f(569-579)	1223.1	3.57	-23.02	9.09	-1.745	3.55	-3	0.000	0.3095	NAMP	0.002
NREDF	LF f(584-588)	679.6	4.37	31.44	0	-2.44	6.82	-1	1.000	0.3675	NAMP	0.004

Table A2. Continued.

Sequence	Origin and position	Molecular mass (Da)	pI	Instability index	Aliphatic index	GRAVY index	Boman index (kcal/mol)	Net charge	SVM	RF	ANN	DA
DGTRKPVTEAQSCHL	LF f(594-608)	1641.8	6.74	91.43	52	-0.933	2.78	0	0.054	0.0215	NAMP	0.001
AVAPNHAVVSRSDRAAHVKQVL	LF f(609-630)	2325.6	10.84	45.77	106.36	0.036	1.73	2.2	0.662	0.319	NAMP	0.461
HQQAL	LF f(632-636)	595.6	6.74	46.52	98	-0.92	1.8	0.1	0.002	0.292	NAMP	0.058
GKNGKNCPDKF	LF f(638-648)	1207.3	9.2	-19.69	0	-1.755	2.95	2	0.024	0.1915	AMP	0.274
NREDF	LF f(584-588)	679.6	4.37	31.44	0	-2.44	6.82	-1	1.000	0.3675	NAMP	0.004
DGTRKPVTEAQSCHL	LF f(594-608)	1641.8	6.74	91.43	52	-0.933	2.78	0	0.054	0.0215	NAMP	0.001
AVAPNHAVVSRSDRAAHVKQVL	LF f(609-630)	2325.6	10.84	45.77	106.36	0.036	1.73	2.2	0.662	0.319	NAMP	0.461
HQQAL	LF f(632-636)	595.6	6.74	46.52	98	-0.92	1.8	0.1	0.002	0.292	NAMP	0.058
GKNGKNCPDKF	LF f(638-648)	1207.3	9.2	-19.69	0	-1.755	2.95	2	0.024	0.1915	AMP	0.274
KSETKNL	LF f(652-658)	818.9	8.59	36.09	55.71	-1.786	3.65	1	0.000	0.3685	NAMP	0.082
NDNTECL	LF f(661-667)	807.8	3.67	114.24	55.71	-1.2	3.59	-2	0.999	0.309	NAMP	0.019
GGRPTY	LF f(671-676)	649.7	8.75	61	0	-1.483	2.62	1	0.460	0.3535	AMP	0.050
VTAIANL	LF f(685-691)	700.8	5.49	-3.56	181.43	1.7	-1.18	0	0.036	0.3995	NAMP	0.227
KKCSTSPL	LF f(692-699)	863	9.31	63.67	48.75	-0.675	1.78	2	0.858	0.3395	NAMP	0.202
EACAF	LF f(701-705)	539.6	4	95.88	40	1.08	-0.21	-1	0.988	0.3665	AMP	0.006
MMKSF	k-CN f(1-5)	642.8	8.50	2.24	0.00	0.380	0.25	1	1.000	0.405	NAMP	0.914
VVTIL	k-CN f(8-12)	543.7	5.49	29.54	272.00	3.200	-3.07	0	0.000	0.4695	AMP	0.000
AKPAAVRSPAQIL	k-CN f(83-95)	1321.5	11.00	115.48	113.08	0.292	0.63	2	0.527	0.428	AMP	0.193

Fragments of known AMPs are marked in red. Fragments with low solubility are marked in blue.

Table A3. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey (SW), sweet whey hydrolysate pH 2 (SWH1), sweet whey hydrolysate corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 (SWH2), and sweet whey hydrolysate corrected to pH 4.5 with NaOH 1M (SWH3).

	% Inhibition after 24 hours			
Sample	***E.coli***	***P.aeruginosa***	***B. contaminans***	***S. aureus***
SW	-56.88	-131.98	-34.27	-183.57
	-80.72	-247.40	-107.93	-439.98
	-80.04	-252.90	-95.76	-397.82
	-77.74	-208.00	-110.93	-235.04
SWH1	-53.18	36.22	24.46	-48.86
	-27.58	43.29	18.30	-31.97
	2.36	31.84	38.93	-30.80
	-8.80	35.37	-10.86	16.44
SWH2	3.46	-10.74	6.65	4.97
	-13.46	-47.71	-22.32	-262.19
SWH3	-8.29	-4.70	19.88	-122.51
	-16.72	-3.37	15.06	-50.32

Table A4. Summary of the pH stability test in the conditions for antimicrobial testing (70µL MH broth, 70µL mid log-phase bacterial strain diluted in MH broth, 70µL sample).

Sample	**pH**
MH broth	7.09
MH broth + citrate buffer 20 mM pH 4.5	6.45
MH broth + citrate buffer 20 mM pH 4.5 w/ NaCl 150 mM	6.45
MH broth + citrate buffer 20 mM pH 4.5 w/ NaCl 250 mM	6.45
MH broth + citrate buffer 20 mM pH 4.5 w/ NaCl 350 mM	6.45
MH broth + citrate buffer 20 mM pH 6	6.93
MH broth + sweet whey	6.87
MH broth + sweet whey hydrolysate pH 2	5.86
MH broth + sweet whey hydrolysate corrected to pH 4.5 with citrate buffer 20 mM pH 4.5	6.47
MH broth + sweet whey hydrolysate corrected to pH 4.5 with NaOH 1M	6.81

Table A5. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey hydrolysate (SWH) corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 and eluted with 150 mM NaCl in the SP-Sepharose XL™ 1 mL adsorbent.

	% Inhibition after 24 hours			
Sample	***E.coli***	***P.aeruginosa***	***B. contaminans***	***S. aureus***
Loaded fraction 1	4.84	-3.13	13.70	24.43
Loaded fraction 2	4.56	9.18	10.18	-195.65
Washed fraction 1	5.71	-73.26	13.31	-5.43
Washed fraction 2	9.44	-48.73	7.82	20.04
Washed fraction 3	14.69	1.03	11.56	3.46
Washed fraction 4	19.11	-90.97	2.62	5.83
Eluted fraction 1	11.88	-9.10	-19.75	-8.08
Eluted fraction 2	3.95	-62.95	-17.13	-4.83
Eluted fraction 3	6.43	-2.08	-14.75	-20.92
Eluted fraction 4	-3.07	-22.75	-11.13	-69.90
Eluted fraction 5	-22.30	-1.10	-4.95	-79.80
Eluted fraction 6	4.79	-15.25	-4.57	-23.69
Eluted fraction 7	7.68	-18.71	-12.56	0.45
Eluted fraction 8	3.11	-19.10	-14.51	6.97
Eluted fraction 9	0.88	-22.42	-8.99	0.27
Eluted fraction 10	-0.99	-14.58	-9.37	-1.25

Table A6. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey hydrolysate (SWH) corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 and eluted with 250 mM NaCl in the SP-Sepharose XL™ 1 mL adsorbent.

	% Inhibition after 24 hours			
Sample	***E.coli***	***P.aeruginosa***	***B. contaminans***	***S. aureus***
Loaded fraction 1	0.28	-8.00	-13.19	-29.41
Loaded fraction 2	8.52	-12.11	-15.37	-67.07
Washed fraction 1	5.88	-11.58	-15.33	-23.53
Washed fraction 2	9.70	-13.07	-14.80	-29.20
Washed fraction 3	5.00	-16.89	-19.69	-8.050
Washed fraction 4	0.47	-11.69	-20.16	-36.22
Eluted fraction 1	16.23	-0.010	13.82	-25.84
Eluted fraction 2	0.65	-15.56	12.96	-21.70
Eluted fraction 3	-18.21	-54.51	-0.32	-24.91
Eluted fraction 4	-22.32	-55.60	3.18	-35.45
Eluted fraction 5	-0.25	-62.47	1.21	-5.82
Eluted fraction 6	7.19	-22.86	-1.38	-22.04
Eluted fraction 7	-5.60	-43.74	1.45	-5.12
Eluted fraction 8	4.72	-66.02	-0.75	16.70
Eluted fraction 9	7.93	-61.01	8.52	-20.83
Eluted fraction 10	1.14	-53.53	4.08	-4.82

Table A7. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey hydrolysate (SWH) corrected to pH 4.5 with citrate buffer 20 mM pH 4.5 and eluted with 350 mM NaCl in the SP-Sepharose XL™ 1 mL adsorbent.

	% Inhibition after 24 hours			
Sample	***E.coli***	***P.aeruginosa***	***B. contaminans***	***S. aureus***
Loaded fraction 1	3.97	-11.61	0.41	8.37
Loaded fraction 2	6.70	-0.93	-5.37	21.46
Washed fraction 1	5.20	12.36	4.07	26.54
Washed fraction 2	-0.71	-71.31	0.55	-87.12
Washed fraction 3	-18.60	0.50	0.82	-55.93
Washed fraction 4	9.78	-20.02	1.29	-46.20
Eluted fraction 1	4.34	-12.04	3.73	-109.77
Eluted fraction 2	-13.56	-9.55	-9.96	-183.30
Eluted fraction 3	-21.32	5.73	-8.94	-103.05
Eluted fraction 4	-8.21	18.45	7.20	-136.81
Eluted fraction 5	-4.09	18.59	-37.08	-181.67
Eluted fraction 6	-11.12	-6.98	10.94	-69.03
Eluted fraction 7	-2.91	-3.21	8.98	-75.32
Eluted fraction 8	-1.71	-82.64	7.47	-62.93
Eluted fraction 9	-6.02	-81.57	-1.79	-71.67
Eluted fraction 10	-13.06	-6.98	-0.41	-53.14

Table A8. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey hydrolysate (SWH) corrected to pH 4.5 with NaOH 1M and eluted with 250 mM NaCl in the SP-Sepharose XL™ 1 mL adsorbent.

	% Inhibition after 24 hours			
Sample	***E.coli***	***P.aeruginosa***	***B. contaminans***	***S. aureus***
Loaded fraction 1	-6.11	-3.55	-0.96	1.43
Washed fraction 1	6.51	-21.69	-15.79	41.72
Washed fraction 2	5.58	-31.70	-4.32	32.94
Eluted fraction 1	-69.02	-5.60	-28.97	-32.49
Eluted fraction 2	-71.03	-5.86	-30.53	-58.30
Eluted fraction 3	-4.95	-7.40	-19.39	15.91
Eluted fraction 4	-12.10	4.13	-9.58	-3.97
Eluted fraction 5	-19.77	5.80	-16.53	-29.64

Table A9. Summary of the percentage of inhibition of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia contaminans* (*B. contaminans*) and *Staphylococcus aureus* (*S. aureus*) after a period of incubation of 24 hours at 37ºC with sweet whey hydrolysate (SWH) corrected to pH 4.5 with NaOH 1M and eluted with citrate buffer 20 mM pH 6 in the SP-Sepharose XL™ 1 mL adsorbent.

	% Inhibition after 24 hours			
Sample	***E.coli***	***P.aeruginosa***	***B. contaminans***	***S. aureus***
Loaded fraction 1	-4.81	-13.22	-25.96	-1.25
Washed fraction 1	6.88	-23.18	-8.64	33.53
Washed fraction 2	4.30	-22.12	-9.66	30.26
Eluted fraction 1	-18.17	-8.90	-11.27	-45.19
Eluted fraction 2	-90.18	-19.03	-39.40	-77.27
Eluted fraction 3	-10.00	0.11	-2.90	-14.69
Eluted fraction 4	2.85	2.03	0.68	-7.47
Eluted fraction 5	-7.71	-49.62	-7.99	-7.52