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Extraction, purification, and sequence identification of bioactive peptides from bovine milk with beneficial effects in cellular models

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Abstract:

Bioactive peptides are short fragments of proteins (2-20) that exhibit various physiological effects and have gained significant attention in recent years. These compounds are derived from different natural sources such as plants (soy, oat, etc.) and animals (egg, meat, milk, etc.). Bioactive peptides possess the ability to modulate biological processes in the human body, exerting antihypertensive, opioid, antioxidant, and anti-inflammatory effects. One of the most studied sources for bioactive peptides is milk. In this thesis, peptides from bovine milk of both mastitis-affected (M) and healthy (H) cows were extracted, identified, and studied to understand their physiological effects. Firstly, two different peptide extraction techniques were compared aiming to identify a more effective method for obtaining peptides. In particular, this study focuses on using a nanotechnological approach taking advantage of Surface Active Maghemite Nanoparticles (SAMNs) in order to extract peptides from M and H milk. Secondly, solid phase extraction (SPE) was employed in comparison with SAMNs to separate the milk-derived peptides. Moreover, mass spectrometry (MS) analysis was utilized to identify the sequences of the peptides derived from M and H milk in order to understand if the inflammatory process can lead to the release of different peptides in milk. In addition, this analysis aimed to determine the specificity of peptide sequences that were specifically bound to the surface of SAMN nanoparticles and those that were separated using solid surface extraction (SPE). In the end, the pool of peptides isolated with the two different methods was tested in human cells (Caco-2 cells). The peptides showed to exert protective effects against oxidative stress and inflammation induced by $TbOOH$ and $TNF-\alpha$, respectively. In conclusion, understanding the properties and functions of bioactive peptides opens up new strategies for the development of novel therapeutic interventions and functional products aimed at promoting human health and well-being.

Keywords: Bioactive Peptides, Surface Active Maghemite Nanoparticles (SAMNs), Solid Phase Extraction (SPE), mastitis, antioxidant, anti-inflammatory

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1. Introduction

1.1 Bovine mastitis and related concerns

Mastitis refers to the inflammation of mammary glands, a condition triggered by both physical trauma and microbial infection. This issue holds significant recognition within the dairy farming sector due to its far-reaching economic implications, including decreased milk yield, compromised reproductive success, adverse effects on animal well-being, and financial setbacks. The primary contributors to bovine mastitis cases encompass *Staphylococcus aureus*, other *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*, and *Bacillus* spp. Among these, *S. aureus* stands out as the most prevalent gram-positive pathogen, responsible for a spectrum of clinical and sub-clinical mastitis presentations in dairy cow [1]. In terms of pathophysiology and clinical presentation, disparities arise between infections caused by *Escherichia coli* and *Staphylococcus aureus*. *E. coli* prompts clinical mastitis, triggering a rapid and pronounced inflammatory reaction that could potentially lead to fatality or significant damage to mammary tissues [2]. On the other hand, *S. aureus* infections typically initiate as an acute phase but tend to transition into a state of persistent subclinical inflammation. Furthermore, *S. aureus* gives rise to contagious mastitis, where infected mammary quarters function as the primary bacterial reservoir, while *E. coli* leads to environmental mastitis, tied to bacterial strains primarily originating from the cows' surrounding environment [2]. The immune response of the bovine mammary gland (MG) primarily targets the lobule-alveolar tissue and epithelial cells. Pathogens responsible for mastitis invade the MG by entering through the teat canal and subsequently spreading through the teat sinus, gland cistern, and large ducts, thereby disseminating within the MG [2]. Initial signs of inflammation become evident in the teat sinus tissue, which is equipped with distinct anatomical and physiological attributes that serve as the first line of defense against infections. Therefore, it is essential to pay special attention to the immune response mechanisms occurring in this specific region of the udder [2]. Clinical mastitis is easily identifiable through observable anomalies such as a swollen and reddened udder, along with fever in dairy cows. The cow's milk may exhibit a watery consistency with the presence of flakes and clots. Clinical mastitis can be further divided into per-acute, acute, and sub-acute stages depending on the severity of inflammation. In severe instances, clinical mastitis can even lead to fatality. On the contrary, sub-clinical mastitis does not manifest any apparent abnormalities in the udder or milk, yet it contributes to decreased milk production as indicated by an elevated somatic cell count (SCC). Quantifying the losses attributed to sub-clinical mastitis is challenging, but experts concur that it inflicts greater financial burdens on the herd than clinical cases do. In contrast, chronic mastitis

denotes an extended inflammatory process spanning multiple months, marked by sporadic clinical exacerbations occurring irregularly [3].

1.2 Biochemical characteristics of milk proteins

Milk proteins are an essential component of milk and play a crucial role in providing vital nutrients to mammals, including humans. They can be broadly classified into two major groups, caseins and whey proteins. Each group consists of several individual protein types, each with its unique biochemical characteristics.

Milk proteins and their breakdown products exhibit notable levels of biological activity. The positive impact on health attributed to proteins, peptides, and amino acids from cow's milk arises from their abilities to act as antioxidants, reduce inflammation, and enhance immune responses [4]. Within the human body, milk proteins, peptides, and enzymes contribute to maintaining a balance between pro-oxidant and antioxidant factors by neutralizing free radicals, binding to metals, and activating antioxidant enzymes [4]. Certain components like lactoperoxidase, lysozyme, immunoglobulins, lactoferrin, and specific milk-derived peptides possess antimicrobial characteristics [4]. Lactoferrin demonstrates anti-inflammatory and immune-stimulating effects by boosting the synthesis of IgA and IgG antibodies while also stimulating the production of T and B cells [4]. Bovine lactoferrin has shown potential in counteracting cancer, as observed in various cancer cell lines including highly metastatic breast cancer, prostate cancer, osteosarcoma, lung cancer, and gastric adenocarcinoma [4]. The beneficial effects of milk proteins and peptides on health encompass an increased ability of the immune system to detect and eliminate cancerous cells [4].

Caseins

Caseins are the primary proteins in milk, accounting for about 80% of the total protein content. Bovine milk's casein is composed of four fractions - α s1, α s2, β , and κ -casein - which carry essential minerals like calcium, phosphorus, iron, zinc, and copper [4]. Moreover, these fractions play integral roles in metabolic regulation and the facilitation of antioxidant enzyme activities. Notably, casein molecules exhibit an absence of a distinct three-dimensional structures and exist within milk as intricate assemblies termed casein micelles [4]. The casein proteins exhibit a distinctive composition characterized by high proline content and an open, flexible structure. This allows them to adopt multiple energetically favorable shapes when in solution, possessing both hydrophobic and hydrophilic regions. Within this group, α s1-casein, α s2-casein, and β -casein possess serine-phosphate centers that aid in calcium binding, while κ -casein is a glycoprotein featuring

intermolecular disulfide bridges formed by two cysteine residues. A notable feature is that approximately 95% of caseins naturally self-assemble into micellar structures, known as casein micelles [5]. These micelles are spherical colloidal particles, averaging around 150 nm in diameter and having a molecular mass of between 10^6 and 3×10^9 Da [5]. Comprising about 94% protein and 6% low molecular weight compounds, collectively referred to as colloidal calcium phosphate, these micelles have an affinity for water, with approximately 2 grams of water per gram of protein. Milk contains an impressive quantity of these micelles, ranging in the order of 10^{14} to 10^{16} micelles per milliliter [5]. These micelles play a vital role in concentrating, stabilizing, and transporting essential nutrients such as calcium, phosphate, and protein from the mother to the neonate during evolution. The unique structure of caseins, marked by their proline-rich nature, makes them easily susceptible to enzymatic breakdown. Together with their ability to form acid-soluble calcium-phosphate bonds, this property establishes an effective mechanism for targeted release of their cargo in the stomach. Thus, the casein micelle stands as a remarkable example of a natural nanocarrier designed for efficient nutrient delivery [5].

The entire sequences of bovine α s1-caseins have been identified [6]. There are five genetic variants, A, D, B, C, and E of α s1-caseins. The B variant consists of 199 amino acids with a calculated weight of 23,614. This protein possesses more acidic than basic amino acids, resulting in a negative charge at pH 6.5. Around 8.5% proline causes limited secondary structure. Notably, seven phosphoserine residues cluster in the hydrophilic region, and three hydrophobic segments are identified. A variant, differing in a deletion of 13 residues, and C and D variants, characterized by specific amino acids, contribute to the genetic variability [6].

Four variants, denoted A, B, C, and D, of α s2-caseins are known [6]. The amino acid sequence encompasses 207 residues, resulting in a calculated molecular weight of 24,350 kDa. This polypeptide chain stands out due to its abundance of positively charged side chains, particularly in the C-terminal section, and contains two Cysteine (Cys) residues. Remarkably, it presents an extensive level of phosphorylation and a relatively low proline content [6].

β -casein makes up a substantial portion, around 30 to 35%, of the overall casein content [6]. The specific variant b-CN A1-5P consists of a single chain with 209 amino acids, resulting in a calculated molecular weight of 23,982 Da. A distinctive feature is the prominent negative net charge, mainly concentrated in the N-terminal segment (residues 1 to 43). Seven genetic variants (A1, A2, A3, B, C, D, and E) feature a cluster of five phosphoserine residues. The other half of the protein (residues 136 to 209) displays a notable hydrophobic character [6].

The γ -caseins, mentioned in a literature [6], are derived from proteolytic cleavage of β -casein. κ -Casein accounts for approximately 15% of total casein content and contains cysteine. The major variant, κ -CN B-1P, comprises 169 amino acids with a calculated molecular weight of 19,023 Da. κ -Casein forms polymers through disulfide bonds, with sizes ranging from 60 to 600 kDa. The combination of uneven carbohydrate distribution, high Asp and Glu content, and net negative charges (3.5 at pH 6.8) contribute to κ -casein stabilization [6].

Whey proteins

Whey proteins constitute approximately 20% of the total protein content in milk. The category of whey proteins comprises a range of proteins such as β -lactoglobulin (β -LG), α -lactalbumin (α -LA), lactoferrin (LF), immunoglobulins, lactoperoxidase (LPO), lysozyme, and serum albumin (BSA). These whey proteins are rich in essential amino acids, notably sulfur-containing amino acids (SAA), and branched-chain amino acids (BCAA), surpassing the levels found in casein [4].

Lactoglobulin

β -Lactoglobulin (β -LG) is the most abundant whey protein, constituting about 58% of the total protein content. It was first identified in 1934 [7]. Despite its globular structure, isolated β -LG has low solubility and weak ionic strength. This protein is synthesized in the mammary glands of various animals, particularly ruminants, and is a major component of milk [7]. The prevalent genetic variant is β -LG A. Comprising mainly of beta-sheet patterns, β -LG consists of 162 amino acids, resulting in a molecular weight of around 18,277 Da. β -LG has multiple roles, including aiding in transferring passive immunity to newborns and regulating phosphorus metabolism in mammary glands [7]. Its amino acid composition is significant for promoting muscle growth and providing an essential amino acid, cysteine, crucial for glutathione (GSH) synthesis. Due to its stability at low pH, β -LG safeguards hydrophobic molecules during stomach transit, assisting their delivery to specific receptors in the intestines of suckling neonates [7].

Lactalbumin

α -lactalbumin (α -LA) takes up the role of the second most significant whey protein, comprising approximately 20% of the total whey protein content. This compact protein is entirely produced within the mammary gland and functions as a coenzyme for the synthesis of lactose – a vital energy source for newborns [5]. With a sequence quite akin to lysozyme, α -LA consists of 123 amino acids, resulting in a molecular weight of 14,175 Da. It exhibits three genetic variants, namely A, B, and C. Stabilized by four disulfide bonds, its globular structure remains intact within pH values spanning 5.4 to 9.0. Operating as a regulatory agent, it forms an integral component of the lactose synthase

enzyme system within the mammary gland. α -lactalbumin (α -LA) has diverse effects, including inhibiting cell division in intestinal cell lines, acting as a calcium-raising and apoptosis-inducing agent, and displaying antiproliferative properties in colon adenocarcinoma cells. This protein has also been associated with addressing chronic stress-induced cognitive decline through its impact on brain serotonin function. With its notable tryptophan content, α -LA contributes to elevated plasma tryptophan and larger neutral amino acids [7].

Lactoferrin

Bovine lactoferrin (BLF), a monomeric glycoprotein, stands out with a molecular weight of 80,000 kDa, adorned with two attached carbohydrate groups. Operating as an iron-chelating agent, it originates from glandular epithelial cells and mature neutrophils, pervading bodily fluids such as milk, saliva, tears, nasal and intestinal secretions, pancreatic juice, and seminal fluid. BLF is also housed within neutrophils' secondary granules. In the bovine milk, BLF content fluctuates between 0.02 and 0.35 mg/ml, contingent on lactation stages. Beyond its anti-inflammatory role, BLF showcases an array of other biological properties, encompassing antimicrobial, antiviral, and anticarcinogenic activities [7].

Immunoglobulins

Immunoglobulins (IG) constitute a multifaceted group of elements synthesized by B-lymphocytes, contributing significantly to whey protein content while playing a crucial role in immunity, particularly during colostrum production [7]. These proteins are present in the serum and bodily fluids of all mammals, functioning as both surface receptors and antibodies released into the bloodstream and lymph [7]. In terms of their quaternary structure, IG exist as either monomers or polymers of a four-chain molecule. This arrangement consists of two light polypeptide chains, weighing approximately 25,000 kDa, and two heavy chains, ranging in molecular weight from 50,000 to 70,000 kDa. The IG family comprises three core classes: IGG, IGA, and IGM, with IGG further divided into subclasses IGG1 and IGG2. IGG, which can make up to 80% (w/w) of all IG in milk or whey, stands out prominently [7]. Furthermore, immune milk, with its antimicrobial and antiviral attributes, coupled with its role in immune system modulation, holds promise in potentially reducing blood pressure [7]. A notable study involved a double-blind clinical trial that explored the effects of immune milk from hyper-immunized dairy cows, resulting in reduced plasma cholesterol and blood pressure. Subjects with hypercholesterolemia who consumed 90 g of immune milk daily displayed potential benefits in managing their condition [7].

Lactoperoxidase

Lactoperoxidase (LP) is found in various animal secretions like tears, saliva, and milk. As a member of mammalian peroxidases, it's notably abundant in plain milk, constituting about 1% (w/w) of whey's total protein content [7]. LP's enzymatic activity relies on a complex system involving multiple factors, including animal species, breed, and lactation cycle [7]. LP's significance extends to its crucial role in the natural defense system of mammals, protecting against invading microorganisms. Lactoperoxidase plays a multifaceted role as a key component of the immune defense system in mammals. Its antimicrobial properties, mediated through its interaction with hydrogen peroxide and thiocyanate ion, contribute to inhibiting bacterial growth and pathogen-related activities, making it an essential element in safeguarding health and aiding potential therapeutic applications [7].

Bovine Serum Albumin (BSA)

Bovine Serum Albumin (BSA) doesn't originate from the mammary gland but enters milk through passive leakage from the bloodstream. Comprising 582 amino acid residues, it has a molecular weight of 66,267 Da and features 17 intermolecular disulfide bridges, with a thiol group at residue. Due to its substantial size and complex structure, BSA has the capacity to bind to various compounds, including free fatty acids and flavor components, a characteristic that diminishes upon denaturation. BSA plays a multifaceted role in milk, with its binding properties, potential anticarcinogenic activity, and antioxidant abilities contributing to its significance in various biological processes [7].

1.3 Bioactive Peptides

While milk proteins, such as caseins and whey proteins, play a crucial role in providing nutrients, milk also contains a diverse range of bioactive peptides that have been shown to have potential health benefits. These peptides are short chains of amino acids derived from the enzymatic breakdown of milk proteins during digestion or processing.

1.3.1 General characteristic of bioactive peptides (BPs)

Bioactive peptides derived from food proteins exhibit specific roles that positively impact bodily functions and conditions, garnering attention for their potential to enhance health quality and contribute to the production of beneficial products [8]. These peptides possess antioxidative, anti-inflammatory, anticancer, antimicrobial, immunomodulatory, and antihypertensive effects within the body, benefiting various bodily systems. Most bioactive peptides typically consist of 2 to 20

amino acid residues and possess a molecular mass falling within the range of 0.4 to 2 kDa. In exceptional cases, longer peptides have also been documented [9]. As an example, lunasin, a 43-amino-acid peptide derived from soy, showcases both anti-cancer and hypocholesterolemic properties. The potential of food-derived bioactive peptides for produce functional foods and nutraceuticals to prevent or manage certain chronic conditions is noteworthy [9]. Numerous articles have been published that delve into the characterization of bioactive peptides, encompassing attributes like antimicrobial, anti-inflammatory, antihypertensive, anti-obesity, and antioxidant effects [9–14]. Bioactive peptides exist in are usually concealed within the structure of their native protein. The constituent chains of BPs commonly consist of amino acids such as proline, arginine, and lysine, in addition to hydrophobic residues. Structurally, the architecture of BPs lacks a unified consensus [8].

Bioactive peptides, derived from various sources, such as plant (pulses, cereals, fruit, and fruit seeds), animal (milk, meat, and eggs), marine (fish, prawns, lobsters) and food by-products [15]. In recent years, extensive research has illuminated the role of bovine milk and its derivatives as abundant sources of bioactive compounds. Among these compounds, lysozyme, lactoferrin, immunoglobulins, growth factors, and hormones have gained prominence for their inherent presence in milk [16]. However, the hydrolysis of native milk proteins can also yield other bioactive components. These bioactive peptides, often latent within the primary structure of milk proteins, can be unlocked through the proteolysis of casein (α -, β -, and κ -casein) and whey proteins (β -lactoglobulin, α -lactalbumin, serum albumin, immunoglobulins, lactoferrin, and protease-peptone fractions). With an expanding spectrum of health-enhancing effects attributed to these milk-derived bioactive peptides, they hold potential as valuable ingredients for the formulation of functional foods [16].

Bioactive peptides can be generated through processes like chemical hydrolysis, enzymatic hydrolysis, or microbial fermentation, and can also be produced using innovative techniques [17].

1.3.2 Bioactive peptides production

1.3.2.1 Chemical hydrolysis

Chemical hydrolysis, an economical and straightforward method for peptide production, involves the use of various alkalis and acids. However, this approach leads to diminished nutritional quality and reduced biofunctionality in the final product due to the solid nature of the chemicals, extreme pH levels, and high temperatures [17]. Acid hydrolysis has been attempted on fish-derived proteins,

yielding unsatisfactory outcomes when compared to enzymatic hydrolysis. Similarly, alkali has been employed to create peptides from food proteins, but the resulting lysine alanine derivative poses potential harm to food products. Consequently, although chemical hydrolysis has been employed by certain researchers, it is not the preferred method for peptide generation [17].

1.3.2.2 *In vitro enzymatic hydrolysis*

In vitro enzymatic hydrolysis, as compared to traditional chemical methods, is the preferred technique for producing bioactive peptides [15]. It offers benefits like reduced allergenicity and improved physiochemical, nutritional, and techno-functional properties of the protein, along with the production of hydrolysates containing specific peptides. This method employs commercially available proteolytic enzymes to catalyze the breakdown of peptide bonds, resulting in low molecular weight peptones, peptides, and amino acids [15]. The effectiveness of hydrolysis depends on factors like the nature of the parent protein, type of protease enzyme, enzyme specificity, and degree of hydrolysis. Proteases from various sources, such as plants, animals, and microbes, are employed. The choice of proteolytic enzymes is critical for generating peptides with well-defined bioactive properties. For instance, alcalase-hydrolyzed bambara bean protein showed the highest ACE inhibitory potential, while pepsin was effective in hydrolyzing camel milk [15]. Factors like pH, temperature, and duration influence the degree of hydrolysis and amino acid release from protein sources. Controlled hydrolysis conditions ensure reproducible bioactive peptide production, known for its safety and absence of toxic chemicals or solvents in the final products [15].

1.3.2.3 *Microbial fermentation*

Using food-grade microbial strains like lactic acid bacteria for fermentation is an economical way to produce protein hydrolysates and bioactive peptides with enhanced functionality and nutrition. This approach is safe for humans and lacks pathogenicity and toxicity [18]. Microbes, particularly *Bacillus spp* with exopeptidase activity, transform protein-rich substrates into a mix of bioactive peptides. Microbial fermentation is especially useful in dairy products, driven by the choice of microorganisms. *Lactobacillus* species, notably *Lactobacillus. helveticus*, yield diverse bioactive peptides suited for dairy [18]. Beyond *Lactobacillus*, strains like *Bacillus subtilis*, *Bacillus natto*, and fungi like *Aspergillus* contribute to enhance peptide production. A study highlight microbial fermentation's potential, generating ACE-inhibitory and antioxidant peptides from tomato and celery seeds through *Bacillus subtilis* [18]. Compared to enzymatic hydrolysis, microbial

fermentation is cost-effective and safe due to microorganisms' affordability. In summary, employing food-grade microbes for fermentation offers an economical and secure method for bioactive peptide and protein hydrolysate production [15].

1.3.2.4 Innovative techniques

Traditional methods employed in peptide production are effective, yet alongside these, innovative techniques have emerged, referred to as novel technologies [17]. Examples of such novel methods include high hydrostatic pressure, ultrasonic extraction, microwave-assisted extractions, pulsed electric fields, ohmic heating, and subcritical water hydrolysis, which have been developed to modulate the extent of hydrolysis [17]. While the application of these approaches in peptide production remains limited, growing interest among scientists is evident due to their promising peptide generation. However, certain techniques, like ohmic heating and pulsed electric fields, have not undergone extensive investigation in the field of peptide production. Consequently, it becomes imperative to substantiate that peptides generated via novel technologies exhibit enhanced effectiveness and safety compared to conventional methods. This validation can be accomplished through further research, thereby dispelling uncertainties surrounding the utilization of these novel technologies and amplifying their practical implementation [17].

1.3.3 Biological effects of BPs

A wide range of rich protein sources has been extensively utilized to create bioactive peptides, aimed at enhancing human health and preventing illnesses. As mentioned before, a multitude of research findings highlights the versatile functionalities offered by food proteins and peptides, including antioxidant, antimicrobial, immunomodulatory, anticancer, antihypertensive, anti-inflammatory, mineral binding, opiate, and lipid-lowering activities [9–14]. The activities exhibited by BPs against specific molecular disease targets are strongly related to their structural characteristics, such as chain length and physicochemical properties of amino acid residues, including composition, sequence, and molecular charge [15]. In the paragraphs below the most studied effects of bioactive peptides are reported. Since, the aim of this work is to evaluate antioxidant and anti-inflammatory properties of bioactive peptides derived from milk, the peptides with these specific properties will be discussed more in detail.

1.3.3.1 Immunomodulatory and antimicrobial BPs

Milk proteins, including caseins (α s, β , κ , and γ) and whey proteins (α -lactalbumin, β -lactoglobulin, and lactoferrin), were examined as potential sources of immunomodulatory peptides [19]. The immunomodulatory impact of whey proteins, enzymatically hydrolyzed using a combination of enzymes (trypsin/chymotrypsin), was assessed in murine splenocyte cultures, both in the absence and presence of concanavalin A (ConA). The outcomes revealed significant secretion of IL-2 and interferon (IFN)- γ . These findings were ascribed to acidic or neutral peptide fractions that prompted splenocyte proliferation and the release of cytokines [19]. Antimicrobial BPs show a wide range of abilities against bacteria, yeasts, fungi, viruses, and parasites. They can also affect cancer cells, either by targeting their membranes or using other methods. These versatile BPs find uses in medical fields like dental surgery, wound healing, and eye care, as well as in the food industry for preserving and packaging, animal farming, and aquaculture to replace antibiotics [20].

1.3.3.2 Antihypertensive BPs

Antihypertensive peptides have demonstrated the ability to lower blood pressure by inhibiting the angiotensin-converting enzyme (ACE). This property is particularly beneficial for individuals with hypertension [21]. Hypertension, often labeled the "silent killer," affects around 1 billion people globally, resulting in approximately 9 million deaths annually. A key approach to managing hypertension is inhibiting Angiotensin-converting enzyme (ACE) activity, which blocks the conversion of angiotensin I to angiotensin II and bradykinin to bradykinin receptor B2, both contributing to elevated blood pressure. ACE inhibitory peptides show great potential as nutraceuticals or pharmaceuticals [20]. Exploring different pathways could further help lower blood pressure, such as peptides that inhibit renin action to prevent the conversion of angiotensinogen to angiotensin I. While numerous ACE inhibitory peptides are discovered yearly, much of the research focuses on initial *in vitro* tests. The increasing use of computational techniques streamlines the identification of antihypertensive peptides, avoiding time-consuming trial-and-error. Additionally, aside from directly using purified peptides, employing protein hydrolysates offers an alternative method for initial screening, avoiding the high costs linked to purified peptides [20].

1.3.3.3 Anticancer BPs

Cancer is characterized by abnormal cell growth and proliferation resulting DNA mutations. The primary mechanisms to combat cancer involve triggering cell death (apoptosis), preventing tumor development, and regulating the immune system. When searching for bioactive peptides (BPs) with anti-cancer effects, all three key mechanisms need to be considered. Initial assessments of anti-cancer BPs often involve cellular experiments, with human hepatocarcinoma (HepG2) cells being a common choice. However, the lack of comprehensive information about target proteins in tumor cells presents a challenge for using bioinformatics to screen for anti-cancer BPs. The connection between anticancer peptides and their structure-activity relationship emphasizes that these peptides often have short sequences of 3 to 25 amino acids. Shorter sequences enhance mobility and interaction with cancer cell components. The effectiveness of these peptides against cancer is influenced by factors such as amino acid composition, sequence, length, overall charge, and hydrophobic properties. Certain specific amino acids, like hydrophobic ones (Ala, Leu, Pro, Gly) and others (Arg, Lys, Tyr, Thr, Glu, Ser), have a vital role. They contribute to the potent cytotoxic effects against cancer cells by increasing interaction with the outer membrane layers of tumor cells. Overall charge and the presence of heterocyclic amino acids are also key contributors to the anticancer activity of peptides from food sources. Although the exact mechanisms of anticancer action remain incompletely understood, current studies suggest that food protein hydrolysates or peptides might trigger cancer cell apoptosis, halt the cell cycle, damage cell membranes, impede cell adhesion, influence topoisomerases, adjust immune responses, and disrupt intracellular signaling in cancer cells [15,20].

1.3.3.4 Antioxidant Peptides

Reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($\bullet OH$), and hydrogen peroxide (H_2O_2), are highly reactive molecules that form within the body (physiologically) or from external sources [22]. Naturally, ROS are generated as by-products during metabolic oxidation processes in living organism, through the action of specific enzymes, for example NADPH oxidases and lipoxygenases. ROS can also arise unnaturally due to exposure to various environmental factors such as pollutants, ionizing radiation, and ultraviolet light [22]. Endogenous ROS serve vital physiological roles, including cell protection against infections, modulation of intercellular signaling pathways, and support for reproduction. However, excessive ROS

accumulation leads to oxidative stress when organisms are unable to mount effective antioxidant defenses. Moreover, ROS-induced lipid peroxidation (LPO) significantly contributes to the degradation of oxidation-sensitive components in foods [22].

Antioxidants play a crucial role in the food industry, serving as essential inhibitors against lipid oxidation processes. By incorporating antioxidants, the emergence of lipid peroxidation products is effectively delayed, preserving the authentic tastes and textures of formulated food products [23]. These antioxidants operate through a variety of mechanisms that hinder oxidative pathways. They act by deactivating peroxides and other reactive oxygen species, eliminating free radicals, binding to or removing metal catalysts, and neutralizing secondary products of oxidation [23]. While well-established antioxidants like phenolic compounds are recognized, a growing body of scientific research highlights the assessment and characterization of potentially valuable bioactive peptides derived from food proteins, showing promising antioxidant properties both in laboratory settings and in living organisms. Some of these peptides operate through mechanisms similar to other antioxidants present in the food matrix. Consequently, these antioxidant peptides can be incorporated into food systems to extend the overall shelf life and health-promoting attributes of the final food products [23].

Antioxidant peptides stand out for their significant contributions to enhancing human health by aiding in the prevention and management of chronic degenerative diseases like cardiovascular issues, cerebrovascular disorders, cancer, rheumatoid arthritis, and diabetes [24]. Furthermore, peptides possessing antioxidant qualities demonstrate effective abilities to chelate metal ions (such as Fe) and inhibit lipid peroxidation, making them promising candidates for use as additives in food processing. A particularly appealing attribute of peptides lies in their minimal adverse effects on human health, attributed to their natural origins [24]. In order to create an effective approach for designing and producing new natural-derived peptides with potent antioxidant abilities, a thorough understanding of the fundamental mechanism behind their antioxidant activity is crucial. However, a successful achievement needs a comprehensive exploration of the connections between the structure and function of antioxidant peptides. Equally important is gaining insight into the origins of these antioxidant sequences, tracing their derivation from precursor proteins [24].

Milk and its derivatives, including whey, stand as excellent protein sources for producing bioactive peptides, particularly those with antioxidant properties, and were among the earliest animal proteins employed for crafting such peptides [25]. Within milk, two inherent protease enzymes play a role in breaking down milk proteins, giving rise to peptides characterized by the amino acid

sequence VLPVPQK, which are recognized for their antioxidant capabilities [25]. Sheep's milk also hosts antioxidant peptides that emerge due to the enzymatic action of *Bacillus spp.* on s1-casein and s2-casein. Camel milk, subjected to enzymatic hydrolysis using pepsin, papain, and alcalase, yields antioxidant peptides with sequences like RLDGQGRPRVWLGR, TPDNIDIWLGGIAEPQVKR, and VAYSDDGENWTEYRDQGAVEGK, showing strong antioxidant ability. Buffalo milk casein, when influenced by pepsin, trypsin, and chymotrypsin, generates bioactive peptides carrying the sequence VLPVPQK, which exhibit robust antioxidant potential as evidenced by assessments using DPPH radical scavenging and Trolox equivalent antioxidant capacity (TEAC) tests [25]. Bovine and ovine milk proteins also contribute antioxidative peptides through hydrolysis processes; for instance, pepsin-treated bovine casein yields the peptide YFYPEL, while hydrolyzing bovine κ -casein with pepsin, trypsin, and chymotrypsin results in the production of antioxidant peptides. Notably, milk peptides including sequences ARHPHPLSFM, AVYPYQR, NPYVPR, and KVLPVPEK display significant antioxidant activity and impede lipid peroxidation within Caco-2 cells by activating the Keap1-Nrf2 system [25].

In another study, four synthetic peptides (NPYVPR, AVYPYQR, KVLPVPEK, and ARHPHPLSFM), related to milk-derived peptides, were explored [16]. Despite having distinct characteristics, as evidenced by RP-HPLC chromatography and MS analysis, these peptides demonstrated cellular uptake by Caco-2 cells organized in an epithelial monolayer arrangement. All four peptides effectively maintained cell viability against induced oxidative stress, suggesting their potential role in managing oxidative stress [16]. Consequently, an assessment of total thiols and glutathione content was conducted following cell exposure to oxidants such as hydrogen peroxide (H_2O_2) or tert-butyl hydroperoxide (TbOOH). The peptides exhibited the ability to prevent the depletion of both total thiols and glutathione caused by H_2O_2 or TbOOH. Additionally, they displayed a protective influence on thiol-associated antioxidant enzymes, namely thioredoxin reductase and glutathione reductase. Notably, these peptides led to a reduction in ROS production induced by TbOOH in Caco-2 cells. These findings underscore the significant antioxidant role played by bioactive peptides within cells, complementing their previously recognized properties [16]. Some examples of antioxidant peptides have summarized in Table 1.

The identification of casein-derived bioactive peptides by LC-MS/MS revealed that β -casein is the main source of milk-derived antioxidant peptides, which is likely due to its composition rich in highly hydrophobic residues. These results indicated the important potential of well-characterized β -

casein-derived AIPs obtained *in vitro* for the elaboration of therapeutics and dietary supplements [26].

Table 1. Examples of peptides with antioxidant activity

Source of the proteins	Name of the protein	Hydrolysis method	Sequences of proteins and peptides	Biological activity
Human milk	κ -casein	Milk-derived Synthetic peptides	NPYVPR	Antioxidant peptides[16]
Bovine milk	β -casein and κ -casein	Fermentation	AVPYPQR, KVLPVPEK, and ARHPHPLSFM	Antioxidant peptides [16]
Buffalo milk	Casein	Enzymatic hydrolysis (pepsin, trypsin, and chymotrypsin)	VLPVPQK	Antioxidant peptides [25]
Sheep milk	s1-casein and s2-casein	fermentation (<i>Bacillus spp</i>)	-	Antioxidant peptides [25]
Camel milk		Enzymatic hydrolysis (pepsin, papain, and alcalase)	RLDGQGRPRVWLGR, TPDNIDIWLGGIAEPQVKR, VAYSDDGENWTEYRDQGAVEGK	Antioxidant peptides [25]
Bovine milk	κ -casein	Enzymatic hydrolysis (pepsin, trypsin, and chymotrypsin)	YFYPEL	Antioxidant peptides [25]
Milk-derived	-	-	ARHPHPLSFM, AVPYPQR, NPYVPR, KVLPVPEK	Antioxidant peptides [25]
Milk-derived	-	-	K-8-K (KVLPVPEK)	Antioxidant peptides [27]
Soy	-	-	S-10-S (SLVNNDDRDS)	Antioxidant peptides [27]

1.3.3.4 Anti-inflammatory peptides

Inflammation is a natural immune response triggered by harmful stimuli, aiming to restore tissue balance during infection, injury, or exposure to toxins. This defense mechanism involves altering blood vessel permeability, enlisting immune cells, and controlling the release of pro-inflammatory agents at sites of immune activity [28]. Uncontrolled, prolonged acute inflammation can gradually evolve into chronic inflammation, leading to various chronic inflammatory diseases. Chronic inflammation is linked to an elevated risk of chronic diseases such as asthma, inflammatory bowel disease, cancer, cardiovascular disease, obesity, and type-2 diabetes [28]. Chronic inflammatory diseases are a prevalent cause of morbidity and mortality worldwide, accounting for over 50% of all deaths. Consequently, considerable efforts are directed toward developing alternative and more targeted anti-inflammatory therapies using natural sources. A significant focus is on deriving bioactive peptides with anti-inflammatory properties from sustainable protein sources like edible insects, agroindustry leftovers, and fishing by-products [28].

Milk stands as one of the earliest explored sources of food proteins whose peptides were investigated for their biological functions. Milk proteins, containing whey and casein proteins, have been thoroughly examined to uncover peptides with inherent bioactive properties [14]. Notably, VPP (Val-Pro-Pro) and IPP (Ile-Pro-Pro), derived from bacterial fermentation of casein, exhibit versatile bioactive potentials. Recent studies have highlighted their capacity to counter inflammation, as evidenced by their ability to impede the activation of the NF- κ B pathway and reduce adipokine levels in pre-adipocytes of murine origin [14]. Furthermore, these peptides have demonstrated the potential to mitigate the progression of atherosclerosis in mice deficient in apolipoprotein E. Another milk casein-derived peptide, QEPV (Gln-Glu-Pro-Val), has been shown to modulate the release of nitric oxide (NO) while enhancing the production of anti-inflammatory mediators IL-4 and IL-10. Also, whey protein hydrolysates treated with high hydrostatic pressure exhibited potential anti-inflammatory effects on intestinal epithelial and respiratory cells. However, the specific peptides responsible for this activity within the crude hydrolysate remain unidentified [14].

According to a study, the hydrolysis of β -casein using cod trypsin, TPCK-treated porcine trypsin, and a combination of porcine trypsin and chymotrypsin preparation yielded potentially effective anti-inflammatory peptides [26].

In a recent study, two bioactive peptides with potent antioxidant properties were investigated: K-8-K (KVLVPVEK) sourced from milk, and S-10-S (SLVNDDRDS) from soy [27]. These peptides

exhibited robust Nrf2 stimulation capability. Considering the interplay between Nrf2 and NF- κ B pathways, these Nrf2-activating bioactive peptides, especially S-10-S, hold the potential to mitigate inflammation by inhibiting the NF- κ B pathway. This dual effect involves both the direct impact of activated Nrf2 and the influence of phase II enzymes under Nrf2 regulation, with a focus on HO-1. These findings could offer promising avenues for enhancing human health [27].

Research on anti-inflammatory peptides from milk proteins is ongoing, and their potential therapeutic applications are being explored in various inflammatory conditions, such as inflammatory bowel disease (IBD), arthritis, and other chronic inflammatory disorders. Some anti-inflammatory peptides reported in previous literatures have categorized with some details in Table 2.

Table 2. Examples of peptides with anti-inflammatory activity

Source of the proteins	Name of the protein	Peptide production method	Sequences of proteins and peptides	Biological activity
Milk-derived	Casein	Bacterial fermentation	VPP (Val-Pro-Pro) and IPP (Ile-Pro-Pro)	Anti-inflammatory peptides [14]
Milk-derived	Casein	-	QEPV (Gln-Glu-Pro-Val) (Modulate the release of nitric oxide (NO))	Anti-inflammatory peptides [14]
Milk-derived	β -casein	TPCK-treated porcine trypsin, and a combination of porcine trypsin and chymotrypsin	-	Anti-inflammatory peptides [26]
Milk-derived	-	-	K-8-K (KVLPVPEK)	Anti-inflammatory peptides [27]
Soy	-	-	S-10-S (SLVNNDDRDS)	Anti-inflammatory peptides [27]

1.3.4 Possible application of BPs

The diverse physiological functions exhibited by peptides render them a favorable option for developing therapeutic agents. Various physiological impacts associated with bioactive peptides have been cataloged, influenced by factors such as their categorization, abundance, sequence, and amino acid properties [8]. From a nutritional perspective, peptides exhibit higher bioavailability compared to proteins. Additionally, smaller peptides typically provoke fewer allergic reactions than intact proteins, thus driving the extensive adoption of protein hydrolysates in infant formulas[8]. From a therapeutic standpoint, peptides offer numerous advantages that elevate their utility compared to conventional medications. For instance, bioactive peptides exhibit distinct and focused actions within target tissues, minimizing or even eliminating toxic repercussions. Moreover, their efficacy remains pronounced even at low dosages, making them well-suited for addressing chronic ailments [8]. In contrast, synthetic chemical compounds commonly employed as drugs exert a cumulative impact on the body, potentially leading to environmental concerns through their excretion. On the other hand, bioactive peptides do not accumulate within organisms and are readily expelled and broken down. Although initially, this characteristic might appear disadvantageous, it gains significance due to the potential harmful effects arising from drug toxicity within the body. Consequently, the timely elimination or degradation of these drugs post-use can be regarded as a highly favorable attribute [8].

1.4 Nanoparticles

Nanoparticles are tiny particles with dimensions in the nanometer range (1-100 nanometers). They exhibit unique properties and behaviors compared to their bulk counterparts due to their small size, high surface area-to-volume ratio, and quantum effects. There are various types of nanoparticles, including metal nanoparticles (e.g., gold, silver), semiconductor nanoparticles (e.g., quantum dots), magnetic nanoparticles (e.g., iron oxide), polymer nanoparticles, carbon-based nanoparticles (e.g., carbon nanotubes, fullerenes), and more. Each type of nanoparticle has unique properties that make them suitable for different applications [29].

In recent years, nanoparticles have garnered significant attention for their versatile applications in the fields of pharmaceuticals, food science, and natural product chemistry. Moreover, they exhibit exceptional properties that make them highly attractive for a wide range of uses. In the pharmaceutical industry, nanoparticles are employed for targeted drug delivery, enhancing

therapeutic efficacy while minimizing side effects [30]. Additionally, they have proven valuable in imaging and diagnostic applications, enabling early disease detection and monitoring. In the food science field, nanoparticles contribute to novel food packaging materials with enhanced barrier properties and prolonged shelf life. Moreover, they are utilized as food additives for improving texture, stability, and bioavailability of nutrients [31].

The field of nanotechnology holds great promise for revolutionizing future medical diagnoses and treatments. Extensive research has been devoted to various types of nanoparticles, exploring their potential across a wide spectrum of applications in both biochemical and biomedical contexts. In the domain of natural product chemistry, nanoparticles play a vital role in the extraction and purification of bioactive compounds from various natural sources, unlocking their full potential for medicinal and nutritional applications [32]. The growing interest in nanoparticle applications in these fields highlights their immense potential to revolutionize healthcare, food technology, and the understanding of natural products' therapeutic benefits. The unique properties of nanoparticles, such as their high surface area, tunable surface chemistry, and enhanced adsorption capacity, make them excellent candidates for bioactive compound extraction [32].

Notably, magnetic nanoparticles have emerged as prominent nanomaterials, boasting precise control over their size, responsiveness to external magnetic fields, and the ability to enhance contrast in magnetic resonance imaging [32]. As a result, these nanoparticles offer a versatile range of possibilities, including the detection of bacteria, refinement of proteins, immobilization of enzymes, contamination management, drug delivery, and hyperthermia induction. To meet the demands of these multifaceted applications within the field of biochemistry and biomedicine, these nanoparticles must adhere to specific criteria, encompassing characteristics such as high magnetization, robust stability, biocompatibility, and degradability [32]. Acknowledging the potential benefits of multifunctional attributes in biomedical contexts, this discussion outlines overarching strategies for designing multifunctional nanostructures using magnetic nanoparticles as building blocks. Moreover, the adaptability of these magnetic nanoparticles extends significantly into biochemical domains, particularly in critical tasks like enzyme immobilization and protein purification [32].

Upon interaction with biological fluids, nanoparticles actively attract biomolecules such as proteins and lipids, resulting in the creation of a "corona" on their surface [33]. In the subsequent part, protein corona formation will be discussed in details.

1.4.1 Protein corona formation

Proteins are biopolymers with a wide range of structural variations that are involved in practically every action in living cells [34]. A coating of biomolecules, primarily proteins that have adhered to the surfaces, surrounds nanoparticles (NPs) when they interact with proteins in biological settings. The term "protein corona" refers to the coating of protein that forms around NPs [34]. The features of the corona control the subsequent interactions between NPs and proteins. The characteristics of NPs, such as their size, surface chemistry, and charge, have a significant impact on how protein corona behaves [34]. The cellular absorption of NPs is impacted by molecular characteristics and protein corona makeup. In studies on bio-related nanotechnology, understanding and assessing protein corona formation in relation to protein-NP characteristics and interpreting its biological ramifications are crucial [34].

Varied proteins exhibit distinct binding affinities towards NPs, thereby dictating the sequence in which proteins adhere to the surface. Proteins with higher binding affinities compose the "hard" corona, adhering more tightly to the surface compared to proteins with lower binding affinities that contribute to the "soft" corona [34]. The initial stages of PC formation witness the adsorption of the most abundant proteins, followed by replacement with higher-affinity proteins over time. This phenomenon is termed the Vroman effect, influencing the biodistribution of NPs, a factor critical for *in vivo* applications. Presently, research in the realm of NPs and proteins is primarily confined to *in vitro* environments, whereas the actual PC formation process in physiological contexts differs considerably [34].

The fundamental distinction between the hard and soft corona revolves around the inherent structure of adsorbed proteins. In the soft corona, proteins maintain their original configuration, whereas in the hard corona, structural modifications occur. These conformational alterations raise concerns for two primary reasons: (i) potential compromise of protein activity, heavily reliant on tertiary/quaternary structure, and (ii) the creation of novel epitopes that could incite an immune response. Thus, it becomes imperative to examine not just the composition, but also the conformation of proteins adhered to NPs [35]. This investigation is compounded by the continual interplay of protein adsorption and release governed by two phenomena: the Vroman effect, encompassing competitive protein adsorption and detachment on NP surfaces. Initially, the most prevalent proteins attach to NPs, subsequently being supplanted by proteins with greater affinities, irrespective of their concentrations (Figure 1a). Conversely, cooperative adsorption underscores the capability of previously adhered proteins to serve as scaffolds for additional proteins; intriguingly,

certain proteins may exhibit heightened adhesion to the corona in the presence of other proteins (Figure 1b) [35].

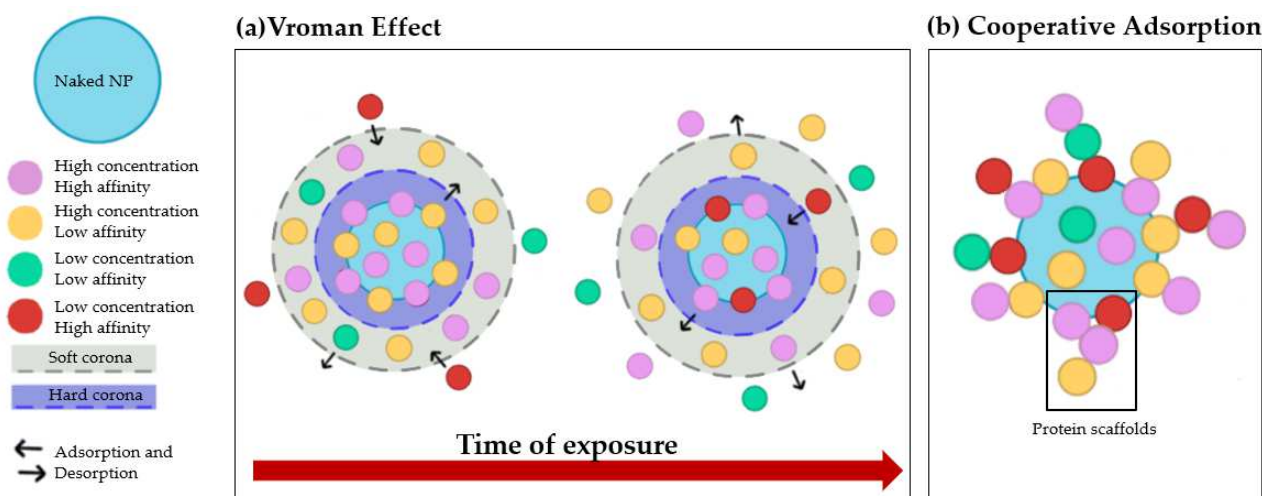


Figure 1. Summary of Factors Impacting Protein Binding to Nanoparticles. (a) The Vroman phenomenon: proteins of higher affinity progressively replace those with lower affinity over time. (b) Cooperative adhesion: proteins previously attached serve as a scaffold for additional proteins on the surface of NPs [35].

Both intrinsic and extrinsic factors can affect protein corona formation. Intrinsic factors encompass the physicochemical attributes of NPs, including their size, surface curvature, shape, charge, hydrophobicity, and surface structure, which collectively influence the behavior of the protein corona. Alongside these intrinsic closely linked to the composition and characteristics of NPs, a range of extrinsic factors also exert their impact on the *in vitro* development of the protein corona. Notably, conditions within the solution, dynamic interactions, and biomolecular circumstances assume essential roles within this context [35].

1.4.2 Surface Active Maghemite Nanoparticles (SAMNs)

Magnetic nanoparticles composed of iron oxides, specifically maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and magnetite (Fe_3O_4), play a crucial role in modern applications within the fields of biomedicine and biotechnology [36]. Among the various magnetic species available, nanoscale iron-oxide-based magnetic particles exhibit numerous exceptional properties. These include magnetic traits like superparamagnetism, high saturation magnetization, and the ability to be easily manipulated using small magnetic fields. Additionally, they possess favorable biochemical attributes such as being non-toxic, biodegradable,

and biocompatible. These advantageous characteristics have positioned them as leading contenders for widespread applications across diverse medical disciplines [36].

Superparamagnetic nanoparticles ranging in size from 20 to 40 nm, composed of well-defined maghemite ($\gamma\text{-Fe}_2\text{O}_3$) with precise stoichiometric properties, are synthesized through the borohydride reduction of ferric chloride at a high temperature of 100°C. After the borohydride reduction of ferric chloride at a high temperature of 100°C, the resulting product undergoes subsequent thermal treatment [36]. The resulting maghemite nanoparticles exhibit remarkable colloidal stability over an extended period without requiring any additional surface modification. This colloidal stability is attributed to the presence of OH^- groups on the surface, which serve as charge barriers, preventing particle aggregation and facilitating the reversible binding of various oppositely charged organic substances. This binding interaction, such as with rhodamine B isothiocyanate, leads to the creation of fluorescent magnetic nanocarriers. These nanocarriers also function as spacer arms, allowing for the covalent immobilization of other biological substances, including enzymes [36].

1.4.2.1 Applications of SAMNS

Due to their distinct physical and chemical attributes, surface-active maghemite nanoparticles (SAMNs) have found applications across a diverse array of research domains. These applications range from the elimination of harmful pollutants to the biomedicine field [37]. Surface Active Maghemite Nanoparticles (SAMNs) set themselves apart among iron oxide nanomaterials due to their remarkable colloidal stability even without the need for coatings or surface modifiers. This attribute results in effective cellular uptake, minimal toxicity, and outstanding capabilities as contrast agents for magnetic resonance imaging (MRI). Furthermore, SAMNs find utility as carriers for biomolecules due to these distinctive features. These exceptional qualities present an opportunity to enhance our understanding of the impacts of metal oxide nanomaterials on biological systems, free from any influence caused by organic or inorganic shells [38]. Importantly, previous studies have already established that SAMNs exhibit a marked preference for binding to proteins. This binding selectivity is contingent upon the composition of the protein environment, thereby resulting in distinct profiles of associated proteins. Consequently, SAMNs can efficiently gather proteins and peptides possessing strong affinities for their surfaces. In an initial investigation, SAMNs were employed to specifically isolate casein fragments originating from milk obtained from cows afflicted with mastitis [37].

1.4.2.2 Interaction with biomolecules and corona formation

The uncoated Surface Active Maghemite Nanoparticles (SAMNs) exhibit the unaltered surface chemistry of pristine iron oxide and are distinguished by their strong colloidal stability in aqueous environments. Notably, they exhibit a remarkable affinity for protein binding. Upon exposure to biological fluids, the self-regulating property of SAMNs control the absorption of proteins, giving rise to the formation of a corona predominantly composed of a select group of biomolecules [39]. In a research study aimed at showcasing the viability of the concept of analyzing the hard protein corona, surface-active maghemite nanoparticles (SAMNs) were tested on bovine milk samples potentially influenced by mastitis. This choice of sample was made due to its potential to illustrate a correlation between the proteome and pathological conditions [37]. By employing MALDI-TOF analysis to study the composition of the hard protein corona, a comparison was drawn with milk from healthy cows with low somatic cell counts (SCC). This analysis revealed an elevated presence of a α s1-casein fragment. The structure of this specific peptide biomarker provided insights into the distinct selectivity of SAMNs, particularly with regard to the arrangement of iron(III) sites on the surface of SAMNs, which serves as the binding interface for proteins [35]. Ultimately, a specific threshold value for the peak intensity of this peptide biomarker as determined by MALDI-TOF analysis was proposed as a potential means for swiftly diagnosing mastitis in bovine milk [37].

2. Aim of the thesis

The objective of this research was to extract, purify, and identify bioactive peptides from both healthy and mastitis bovine milk, with the aim of investigating their potential beneficial effects on human cells, particularly their antioxidant and anti-inflammatory properties.

Mastitis, an inflammatory condition of the mammary gland in cows, can alter the composition of milk. During mastitis, the immune response in the udder can lead to the release of various bioactive peptides into the milk. These peptides exhibit a range of biological activities, including antimicrobial, immunomodulatory, antioxidant, antihypertensive, and anti-inflammatory properties. As a result, there is growing interest in exploring the potential health benefits of these bioactive peptides extracted from mastitis bovine milk.

To achieve our research goal, we employed two efficient methods for extracting and purifying the bioactive peptides from healthy and mastitis cow milk. The first method involved using Surface Active Maghemite Nanoparticles (SAMNs). In this process, milk serum obtained using Vivaspin® 20 centrifugal concentrator (10.000 MWCO PES) was incubated with SAMNs. Subsequently, a 2 M ammonia solution was used to extract the peptides that had been incubated with SAMNs. The second method involved employing a Solid Phase Extraction (SPE) column specifically designed (C18) for peptide extraction from milk whey proteins. The comparison between these two methods allowed us to assess their extraction efficiency.

Following the successful extraction and purification, the bioactive peptide sequences were determined using mass spectrometry (MS). This step was crucial for identifying the exact structure of the bioactive peptides and confirming their specific biological activities. Armed with this knowledge, we were able to synthesize these bioactive peptides experimentally, further enhancing our understanding of their potential health benefits.

By unraveling the properties of these bioactive peptides from mastitis bovine milk and exploring their antioxidant and anti-inflammatory effects on human cells, this research opens up exciting possibilities for the development of new therapeutic approaches and functional foods that could positively affect human health.

3. Material and methods

The research work focused on extracting bioactive peptides with molecular weights below 10 kDa from mastitis and healthy bovine (*Bos taurus*) milk, utilizing two distinct methods, Surface Active Maghemite Nanoparticles (SAMNs) and Solid Phase Extraction (SPE). The ultimate goal was to investigate the potential health benefits, particularly the antioxidant and anti-inflammatory effects, of these peptides on human cells.

For the SAMNs-based extraction, the process commenced with centrifugation to remove milk fats, followed by ultracentrifugation to separate whey proteins. To obtain peptides within the desired molecular weight range, we determined the correct protein concentration using a UV-Vis (Cary 60) spectrophotometer through the Bradford assay. Subsequently, Vivaspin® 20 centrifugal concentrator (10.000 MWCO PES) was employed to isolate proteins with molecular weights below 10 kDa. The resulting milk serum contained the peptides that could then be incubated with SAMNs for extraction.

In the case of SPE, whey proteins were directly used for peptide extraction. The method involved using three different concentrations of acetonitrile (Acn) solutions (5%, 30%, 50%) prepared from a 100% acetonitrile solution. The Acn 30% solution was the primary one, as it effectively allowed the desired peptides to wash and move along the column for collection.

Once the peptides were lyophilized, they were used to treat cell models to observe their potential biological effects, especially their antioxidant and anti-inflammatory properties. Notably, the cells were also treated with peptides incubated with SAMNs to compare the efficiency of their effects. To determine the exact amino acidic sequence of the bioactive peptides, mass spectrometry (MS) was utilized. This crucial step enabled us to synthesize the precise peptides with antioxidant and anti-inflammatory effects.

3.1 Preparation of the whey protein samples

Milk samples obtained from both healthy and mastitis-affected bovines from “Azienda agraria sperimentale Lucio Toniolo” (Università degli Studi di Padova). The fresh milk samples were subjected to centrifugation at 4000 g for 15 minutes at 4 °C. This centrifugation process separated the fat from the aqueous part of the milk, than the skim milk samples was collected. The skimmed milk samples were further processed using ultracentrifugation for 30 minutes at 100 000 g and 4 °C. This step allowed the isolation of the supernatant, which contained whey proteins. To ensure purity,

the obtained supernatant was carefully filtered through paper filters to eliminate any potential unwanted substances, such as casein.

Following this meticulous procedure, we successfully collected the supernatant enriched with whey proteins, which was then stored at -80 °C.

3.2 Extraction approach with SAMNS

3.2.1 Protein Content Determination

Protein content of the isolated milk whey obtained from both mastitis-affected (M) and healthy (H) cows was determined using Bradford method [40] in order to properly dilute the samples for the next step. Coomassie G250 dye is the main component of Bradford reagent and when binds proteins present in the solution it changes color in the visible spectrum, with a peak of absorbance at 595 nm.a. Briefly, 0.01 mL of whey samples reacted with 0.09 mL of Bradford reagent for 5 min at RT. Then, as reported in Table 3, the absorbance of the samples was analysed using a UV-Vis spectrophotometer (Cary 60, Varian).

Table 3. Experimental scheme of Bradford assay. Whey from both mastitic affected (M) and healthy (H) cows was analyzed to determine protein content.

	Blank	Standard 1	Standard 2	Standard 3	Whey sample (M)	Whey sample (H)
H ₂ O	0.1 mL	0.0975 mL	0.095 mL	0.09 mL	0.9 mL	0.9 mL
BSA (1 mg/mL)	-	0.0025 mL	0.005 mL	0.01 mL	-	-
Whey Sample	-	-	-	-	0.01 mL	0.01 mL
Bradford reagent	0.9 mL	0.9 mL	0.9 mL	0.9 mL	0.9 mL	0.9 mL
5 min at RT and absorbance reading at 595 nm						

The obtained values were fitted with a standard curve, performed using increasing concentrations of BSA, and protein concentrations was accurately determined.

After concentration determination of the samples, they were dilute appropriately for subsequent filtration.

3.2.2 SAMNS@peptides

3.2.2.1 The enrichment approach for bioactive milk peptides

A specialized approach was implemented to effectively eliminate high molecular weight proteins from the samples while concurrently enriching them with the desired peptides. The enrichment process involved the use of Vivaspin® 20 centrifugal concentrator (10.000 MWCO PES). This technique allowed for the selective retention of the bioactive peptides of interest, while larger proteins were efficiently separated and removed from the samples.

Milk whey samples were dilute with PBS at a 0.5X reaching the final concentration of 2.26 mg/mL. The diluted solutions were transferred to Vivaspin® 20 centrifugal concentrator (10.000 MWCO PES) and subjected to centrifugation at 4000 g and 4 °C for 40 minutes. This step facilitated the removal of excess material and further enriched the concentration of the desired bioactive milk peptides in the samples. Upon completion of the centrifugation process, we collected the milk serums in 15 ml falcon tubes, which were subsequently stored at -20 °C until they were ready for use.

3.2.2.2 Incubation of the peptides with SAMNs

Under sterile hood, both healthy and mastitis milk samples were added into the SAMNs.

10 mL of each milk sample, was added to 0.5 mg/mL of SAMNs suspension in 15 ml falcon tubes and incubated for 1 hour in orbital rotation at 4 °C (Figure 2a). During the incubation the bioactive peptides interacted with the SAMNs, allowing for efficient binding and enrichment (Figure 2b). Subsequently, three washes with PBS 1X were carried out to remove the peptides or small proteins that were not bind the nanoparticles.

After the wash steps, the supernatant was removed, and 2 M ammonia solution was added to the SAMNs@peptides. The samples were then placed in orbital rotation at 4°C for 1 hour to extract the peptides that are bound to SAMNs. After the 1-hour extraction period, the supernatant containing the isolated bioactive peptides was collected using the magnet. Finally, the supernatants, which are enriched in the isolated peptides, were lyophilized to obtain the peptides in white powder form, facilitating their storage and use in subsequent analyses (Figure 2c). To obtain more pure powder of peptides, we dissolved the peptide powder in ammonia to remove SAMNs residues with magnet again. Then, lyophilization process was repeated to reach pure white powder.

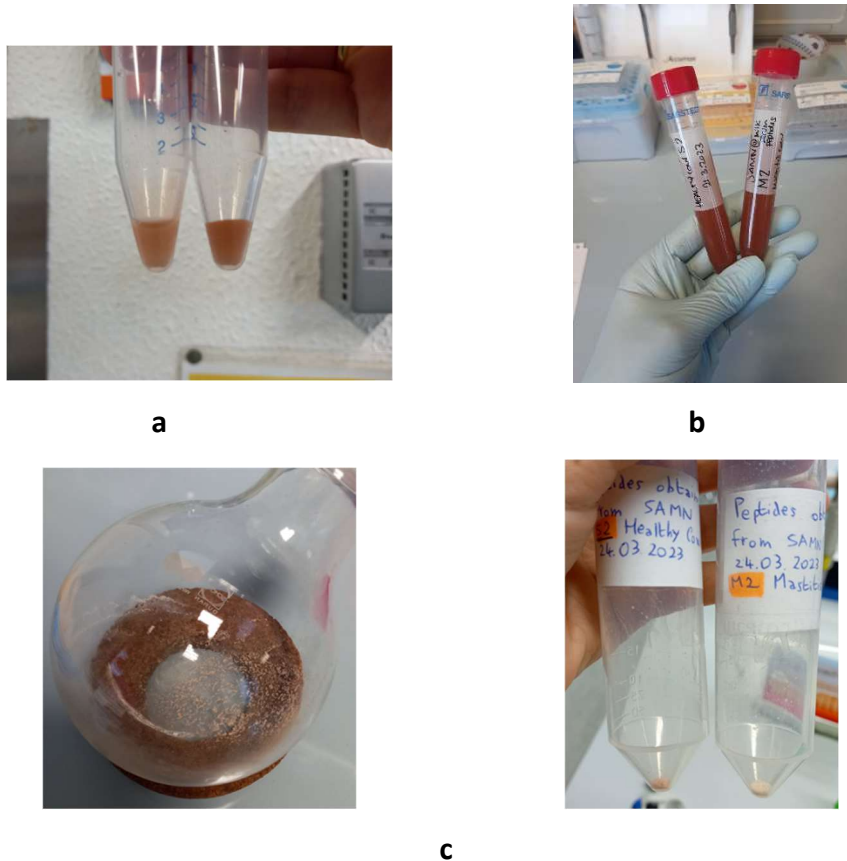


Figure 2. From SAMNs suspension to peptides powder: **a)** SAMNs suspension, **b)** incubated peptides with SAMNs, **c)** SAMNs@peptides (Left), extracted peptides powder with ammonia (Right)

The entire process, starting with milk and leading to lyophilized peptide powder, is comprehensively outlined in Figure 3.

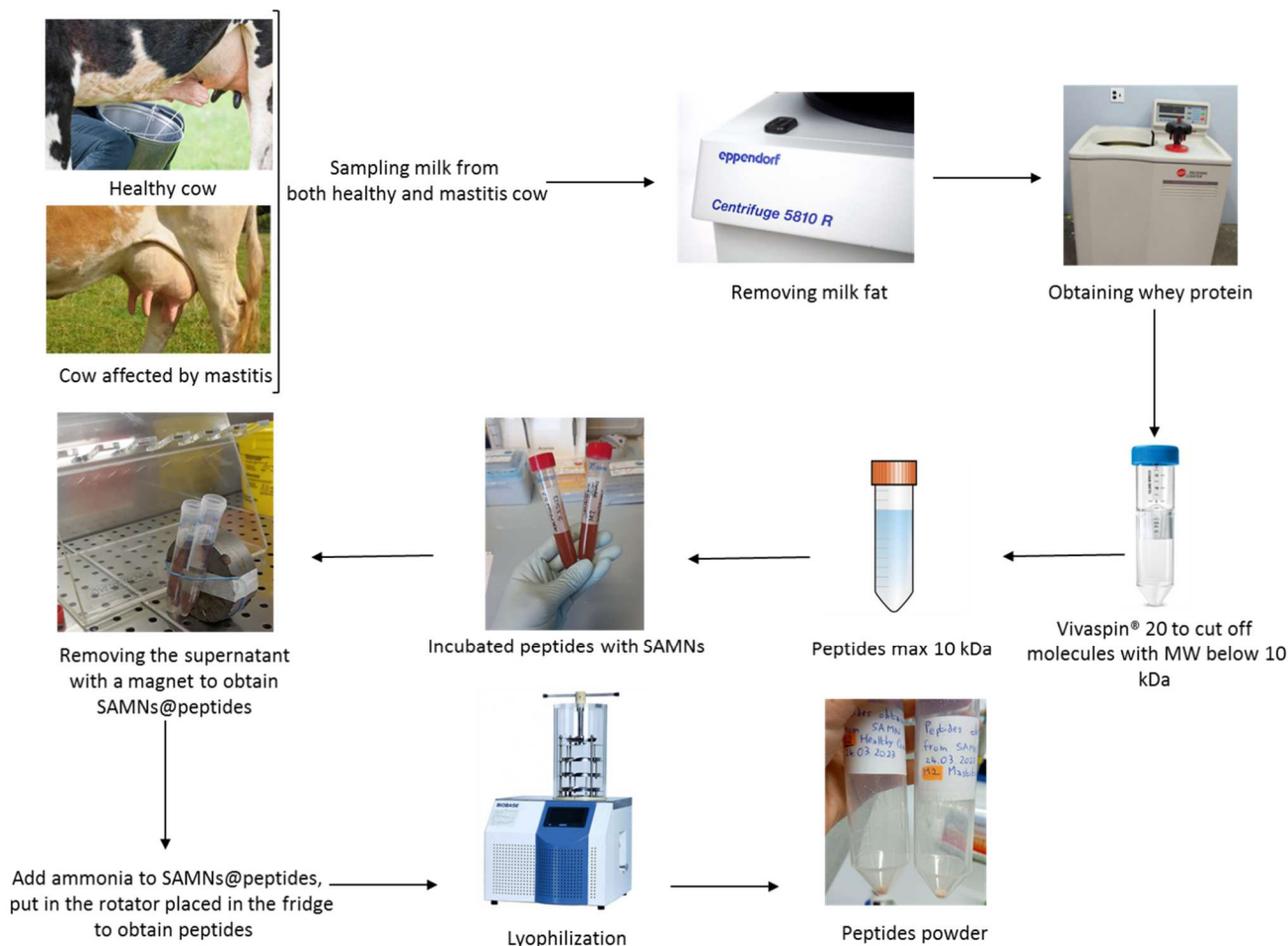


Figure 3. Peptides extraction process with SAMNs

3.3 Extraction approach with SPE

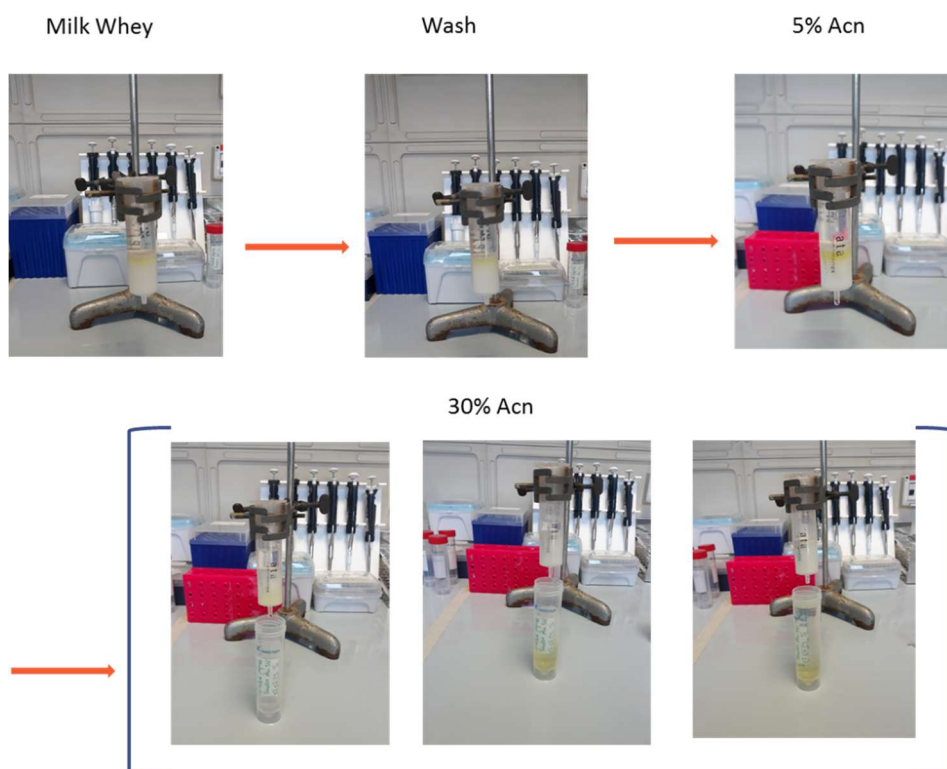
Precise analysis of small amounts of biological samples such as proteins or peptides demands the precise choice of enrichment and purification procedures before performing HPLC and MS analyses. Among the frequently employed techniques for sample preparation, solid-phase extraction (SPE) stands out. In the field of peptide purification, silica-based sorbents modified with C18 chains are widely used as the preferred stationary phase [41].

Reverse-phase SPE operates by separating salts and low-molecular-weight carbohydrates (such as simple sugars and oligosaccharides) from peptides, as only peptides are retained due to hydrophobic interactions [42]. Solid-Phase Extraction (SPE) relies on partitioning the analyte between a solid phase, typically a sorbent within a column, and a liquid phase consisting of either the sample matrix or a solvent containing analytes. This process establishes an equilibrium where molecules adsorb or penetrate the solid surface, causing the analyte to adhere to the solid surface based on the distribution coefficient [43].

In this study, we utilized STRATA column (Phenomenex) with a C18 solid phase that is specific to isolate peptides. To achieve this, we employed acetonitrile (Acn) solutions at various concentrations (5%, 30%, and 50%), which were prepared using 100% Acn with 1% trifluoroacetic acid (TFA). The goal was to facilitate the elution process, enabling the separation of whey proteins and the isolation of the target peptides.

First, we diluted 30 mL of ultracentrifuged M and H whey samples with a 0.01% TFA solution and water, resulting in a total volume of 50 mL. This crucial step was taken to counteract the high protein concentration, preventing potential column blockage. Prior to each use, the column underwent activation through rinsing with 30 mL of 100% Acn, followed by a wash step using 100 mL of the 0.01% TFA solution. The presence of TFA ensured an acidic environment, thereby enhancing peptide stability throughout the process. Once the column was activated and washed, we commenced the elution procedure for each sample. During this phase, the desired peptides selectively adhered to the column. Subsequently, a thorough wash with 100 mL of the 0.01% TFA solution and water was performed to eliminate any remaining substances.

To elute the peptides, acetonitrile solutions of varying concentrations (5%, 30%, and 50%) were employed as shown in Figure 4.



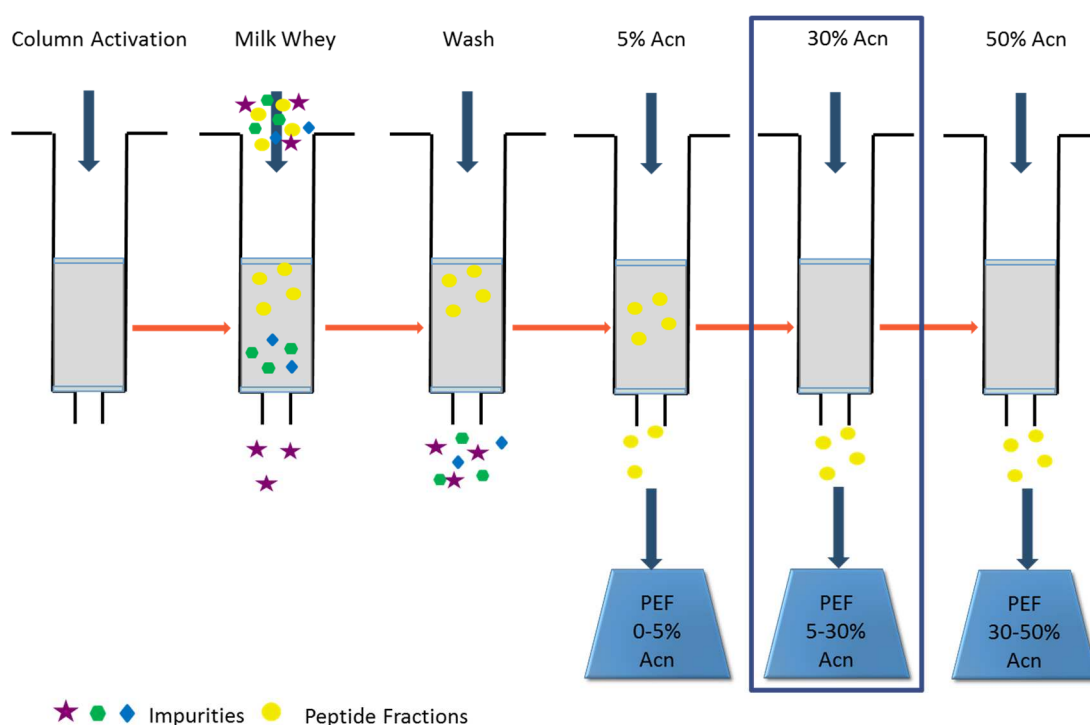


Figure 4. The procedure of peptide extraction with Solid Phase Extraction (SPE)

3.4 Identification of peptides through LC-MS analysis

The MS analyses was performed using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled online with a nano-HPLC Ultimate 3000 (Dionex-ThermoFisher Scientific, Waltham, MA, USA). The lyophilized samples were dissolved in H₂O/0.1% formic acid and 1 µg of the latter was subjected to LC-MS/MS analysis. A linear gradient from 3% to 40% of ACN, 0.1% FA in 20 min at a flow rate of 250 nL/min was chosen for peptide elution from the original sample. Data obtained through LC-MS/MS analysis were elaborated using Mascot software.

3.5 Effects of peptides in cellular model

3.5.1 Caco-2 cells

The Caco-2 cell line, derived from human intestinal adenocarcinoma, serves as a valuable tool for studying the absorption and transport of nutrients and drugs across the intestinal epithelium. This model closely mimics the characteristics of enterocytes, including brush borders, tight junctions, and transporters for efficient movement of substances. Indeed, as the cells grow, they undergo

phases of confluence, proliferation, and differentiation, eventually resembling mature enterocytes. The polarized structure of these cells, with brush borders on the luminal side and basolateral membrane on the opposite side, resembles the human gut's mucosa [44].

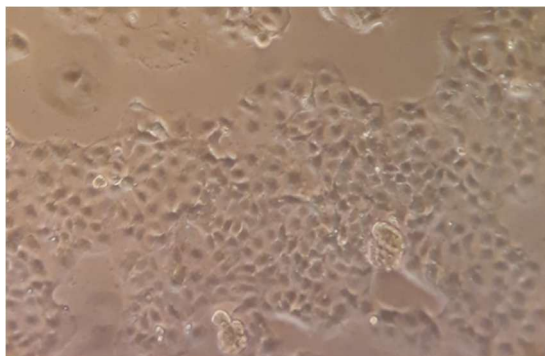


Figure 5. Caco-2 cells

3.5.2 Cell viability assay (MTT test)

To evaluate the influence of peptides on cell viability, Caco-2 cells (1×10^4), (Figure 6), were seeded in 96-well plates. After 48 hours, cells were treated with 0.05 mg/mL of peptides isolated through SPE and SAMNs or 0.01 mg/mL of SAMNs@peptides for 24 hours. Then, medium was removed and 0.5 mg/mL MTT solution was added to cells. After the incubation at 37°C for 3 hours in the dark, the MTT solution was replaced with isopropanol/ dimethyl sulfoxide solution (90:10) to stop the reaction for 15 min. The absorbance at 595 nm was evaluated using a plate reader (Tecan Infinite® M200 PRO). In order to induce oxidative stress and inflammation 250 μ M of TbOOH and 0.01 mg/mL LPS or 4 ng/mL TNF- α were used respectively.

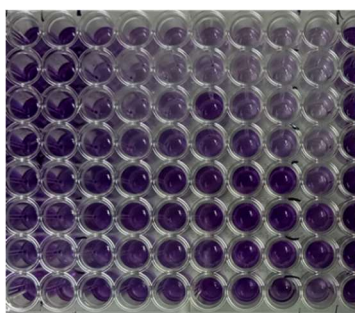


Figure 6. Example of MTT test.

4. Results and discussion

Two distinct methods were employed for the extraction and enrichment of peptides from bovine milk in order to understand which the best protocol is to isolate bioactive peptides. Moreover, this research aims to highlight the specific characteristic of the amino acidic sequence of the peptides isolated with the two different methods. At the end, the antioxidant and anti-inflammatory properties of the isolated bioactive peptides were analyzed.

4.1 Extraction and enrichment of bioactive peptides

The first method involved the utilization of superparamagnetic iron oxide nanoparticles (SAMNs). In this approach both milk samples from healthy (H) and mastitis-affected (M) cows were subjected to the binding with SAMNs. SAMNs are selective for specific sequences of the peptides and this study aimed to understand which the common features of these peptides are.

The second method was SPE (solid phase extraction). To extract the peptides starting from milk whey, we employed increasing concentrations (5%, 30%, and 50%) of acetonitrile. Notably, while the 5% acetonitrile solution had minimal impact on eluting low molecular weight peptides, the most effective elution was achieved with the 30% acetonitrile solution, which completely released the peptides from the column.

Noteworthy, the total amount of isolated peptides obtained from 100 mL of ultracentrifuged whey were 14 mg and 23 mg through SAMNs and SPE, respectively. Evidently, the SPE technique yielded a greater quantity of peptides compared to the amount obtained through the utilization of SAMNs. In the case of the SPE method, the whey was employed in its entirety, including all available peptides and the extraction is aspecific. Conversely, the nanotech approach involved the isolation of specific peptides from a mixture containing molecules with a molecular weight below 10 kDa, resulting in a lower amount of isolated peptides. A summary of both procedures was reported in Figure 7.

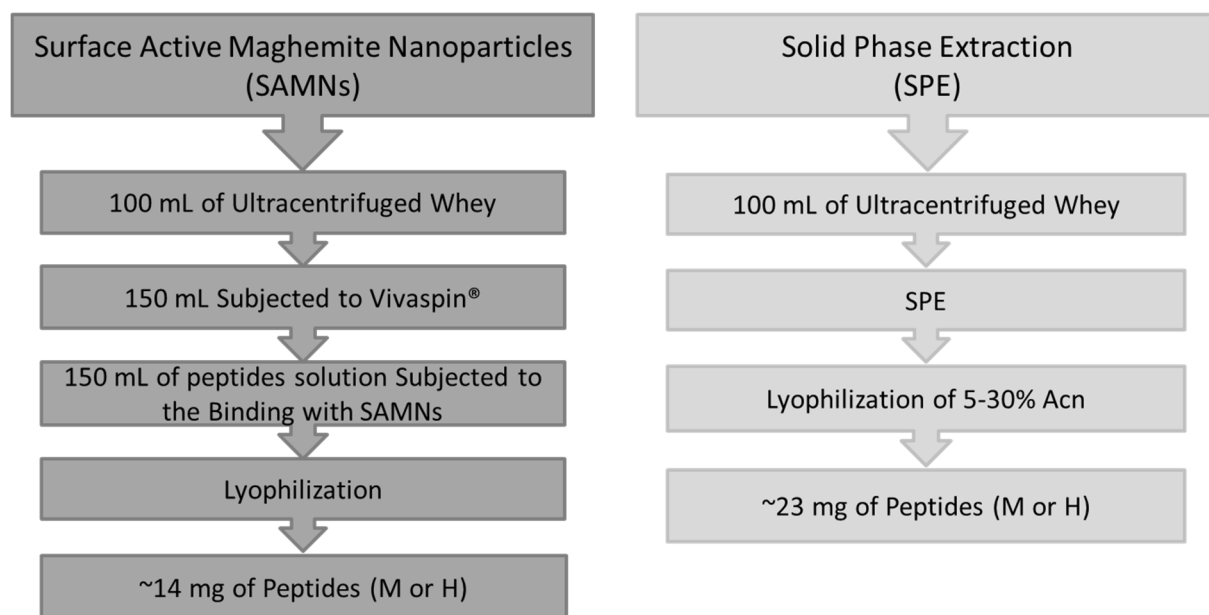


Figure 7. Summary of the Peptides Extraction with SAMNs and SPE Techniques

4.2 Evaluation of peptide content in the SAMNs and SPE fractions

As a result, in order to estimate the amount and the properties of extracted peptides, the fractions obtained through both SAMNs and SPE techniques underwent analysis using high-performance liquid chromatography (HPLC) in 220 nm abs. The absorption spectra depicting the peptides for each method are presented in Figure 9a and 9b, respectively. The profiles of chromatographic absorption spectra derived from the peptides extracted using the two distinct techniques display noticeable differences. As reported above, SAMNs possessing distinctive active surfaces tailored for the binding of specific peptides facilitated the isolation of peptides with specific sequences (Figure 8a), differing from those obtained through the SPE method (Figure 8b). Moreover, also with this technique is evident that using SPE the amount of the peptides is higher than what obtained with SAMNs. Of note, the profile of the peptides obtained with the two extraction techniques, in particular with SAMNs, is very different from M (red curve) and H samples (black curve). As evident from Figure 8, there are diverse main peaks that suggest a different peptide content that will be further confirmed through MS analysis.

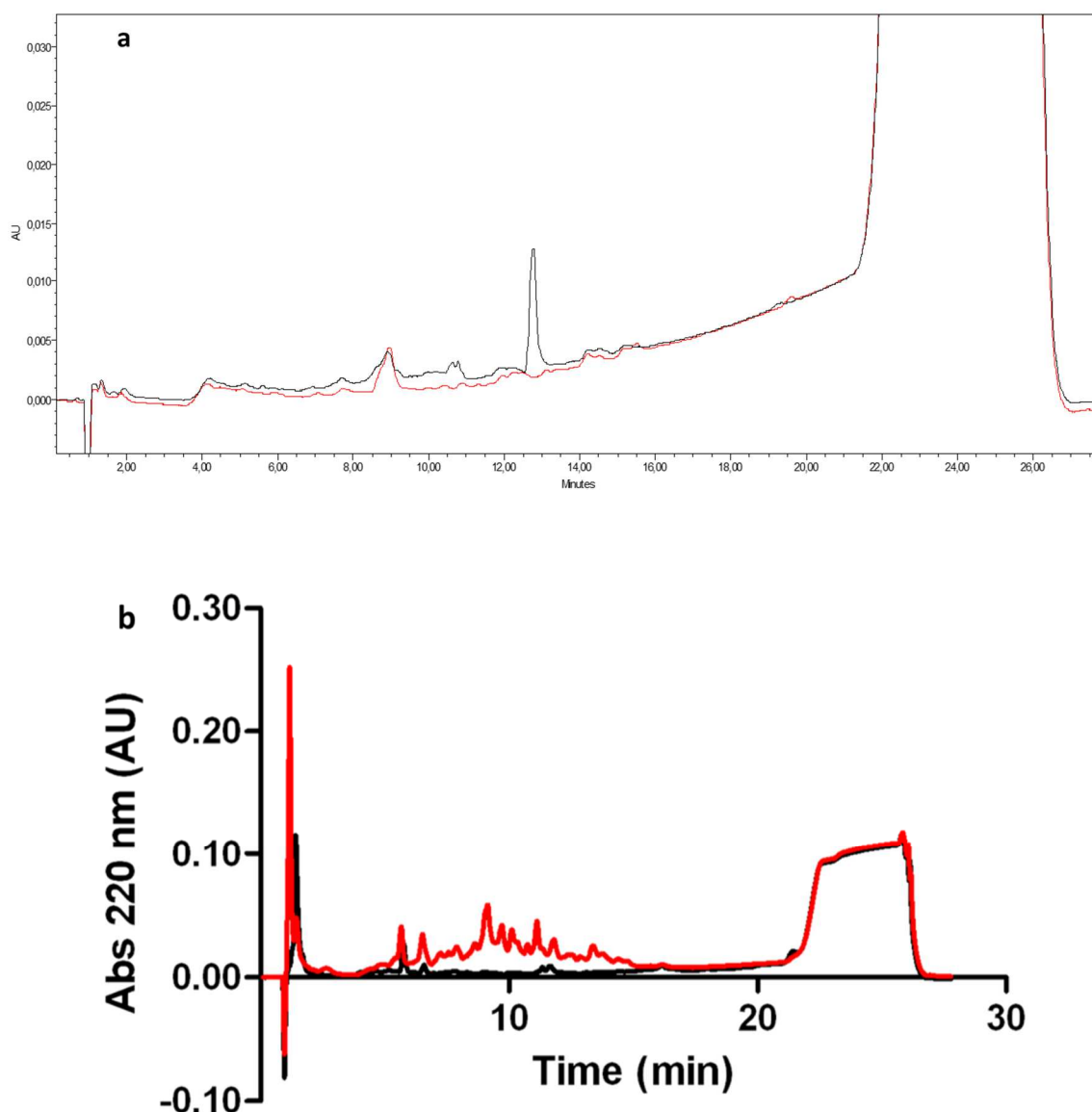


Figure 8. Chromatograms of the extracted peptides with **a)** SAMNs and **b)** SPE. Peptides from **M** (Bovine Mastitis Milk) are depicted in red, Peptides from H (Healthy Cow Milk) are reported in black.

4.3 Protection effects of extracted peptides

To evaluate the effects of peptides on cell viability, Caco-2 cells (1×10^4) were cultivated in the presence of 0.05 mg/mL of peptide fractions or 0.01 mg/mL of SAMNs@peptides for 24 hours. The outcomes are graphically represented in Figure 9. Notably, the peptides obtained through both SAMNs and SAMNs@peptides approaches displayed no harmful effects on cell viability. More interestingly, they exhibited a tendency to enhance cell viability. This aspect will be further analyzed because could be due to the activation of specific signaling pathways. Conversely, SPE isolated peptides, decrease cell viability. This results could be attributed to the hydrophobic nature of the peptides extracted with the SPE method, due to the use of acetonitrile that is a hydrophobic solvent.

These hydrophobic peptides might interact with cell membranes, potentially leading to the formation of pores that can cause cell lysis. Consequently, this provides a plausible explanation for the observed cytotoxic effects associated with these peptides.

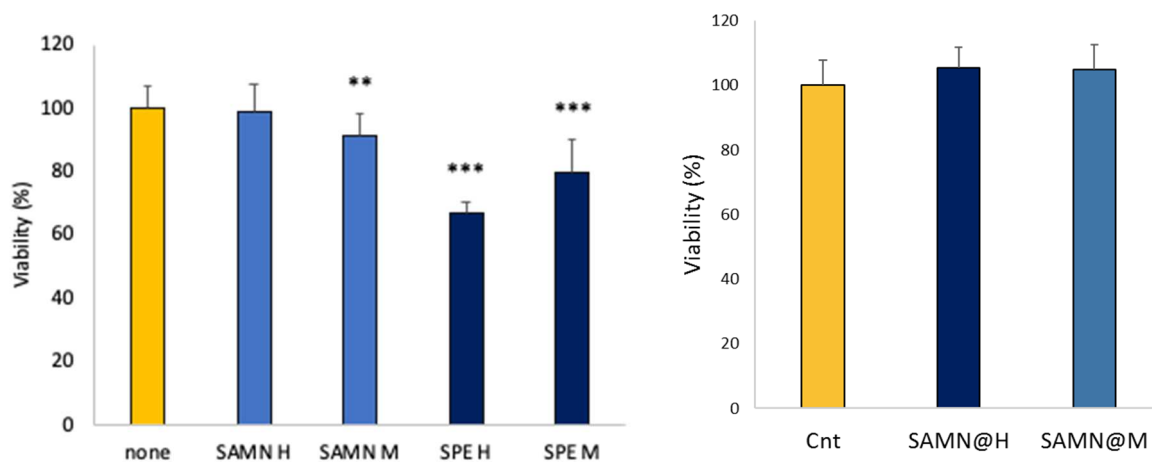


Figure 9. Effect of the peptides on cell viability

In order to evaluate possible antioxidant and anti-inflammatory properties of the isolated peptide fractions, cells (1×10^4) were pre-treated with the peptides (0.05 mg/mL) or SAMNs@peptides (0.01 mg/mL), oxidative stress or inflammation was induced by 250 μ M TbOOH, 0.01 mg/mL LPS or 4 ng/mL TNF- α for 18 hours the MTT assay was performed.

Notably, when oxidative stress and inflammation were induced in the cells without peptide treatments, cell viability decrease by 70% and 20%, respectively. On the other hand, when cells were pre-treated with the peptides isolated with SAMNs, or SPE and peptides binded to SAMNs (SAMNs@peptides), and the oxidative stress and inflammation were boosted using TbOOH, LPS and TNF- α , distinctive impacts of each peptide fraction were observed. In particular, peptides extracted from bovine affected by mastitis (M) showed more antioxidant effects than peptides obtained from the milk of healthy cows (H). Additionally, peptides extracted through SPE exhibited higher efficacy compared to those acquired through SAMNs and SAMNs@peptides in inhibiting oxidative stress induced by TbOOH. Notably, peptides isolated using SAMNs were more effective against oxidative stress than SAMNs@peptides (Figure 10).

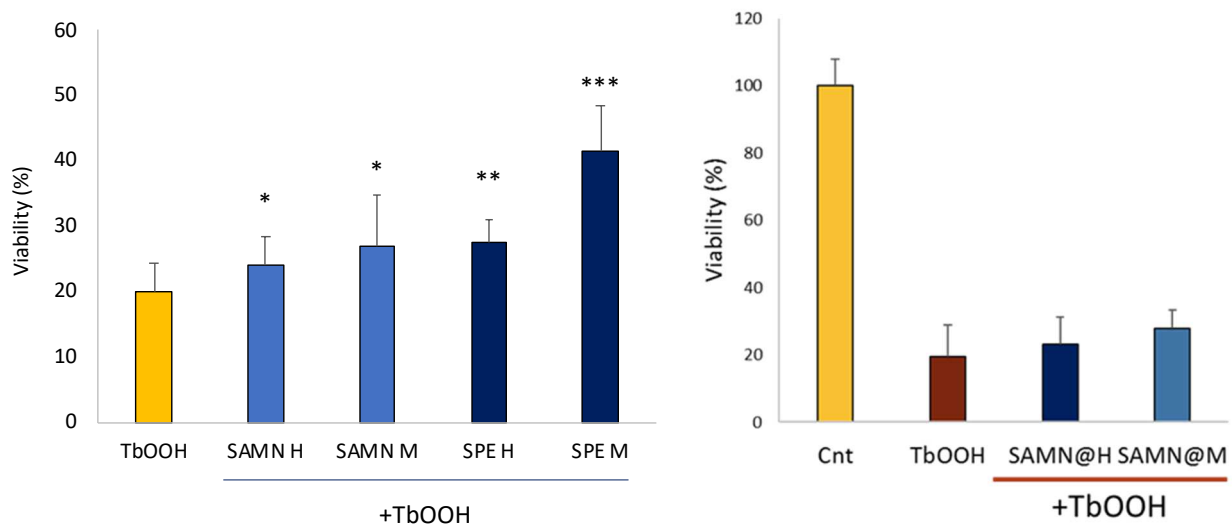


Figure 10. Influence of the Extracted Peptides with Different Techniques against Oxidative Stress (TbOOH)

In the evaluation of peptides' effects against inflammatory factors, pre-treated cells were exposed to LPS or TNF- α . After 18 hours, the findings confirmed the anticipated trend, with peptides from bovine affected by mastitis (M) displaying greater efficacy compared to peptides from the milk of healthy cows (H). This aligns with expectations due to the release of diverse bioactive peptides into milk during mastitis-induced can modulate immune responses. In direct comparison, peptides derived from both healthy and mastitis-affected bovine milk, when obtained through SAMNs, demonstrated greater anti-inflammatory capacity then those acquired through SPE (Figure 11).

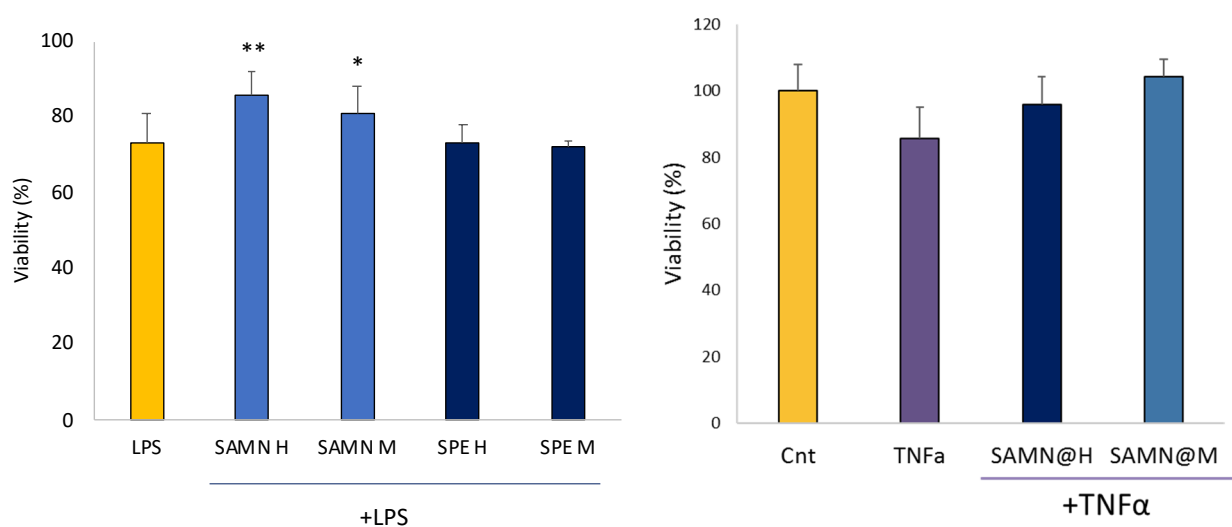


Figure 11. Influence of the Extracted Peptides with Different Techniques against Inflammatory Factor 0.01 mg/mL LPS and 4 ng/mL TNF α .

4.4 Peptidomic analysis of the peptide profile present in SPE fractions

The analysis carried on using LC-MS produced very interesting results. When examining the peptide fractions isolated from healthy (H) and mastitis-affected (M) cows using the SPE technique, we observed that peptides ranging in length from 9 to 30 amino acids. Notably, these peptides were enriched in Alanine (Ala), Proline (Pro), Glutamic acid (Glu), Glutamine (Gln), and Lysine (Lys).

Upon careful examination of the extracted peptides, a fascinating pattern emerges. The majority of these bioactive peptides find their origins in caseins, specifically the α - and β -casein varieties. Furthermore, we have detected certain peptides derived from Glycoprotein 2 in both fractions under investigation. Nevertheless, it's worth noting that we should not overlook the potential role of other proteins, such as the Polymeric immunoglobulin receptor.

A noteworthy observation is that some peptides were unique to either the H fraction or the M fraction, indicating distinct peptide profiles in these two fractions. For instance, peptides from proteins like Fibroblast growth factor, Protein canopy homolog 3, Perilipin, SYT4 protein, and Lactoperoxidase were exclusively present in the H fraction. On the other hand, Cysteine and serine-rich nuclear protein 2 exhibited bioactive peptides exclusively in the M fraction.

An interesting finding arises when shared proteins in both fractions yield different bioactive peptides, both in terms of quantity and sequence. Notably, the M fraction contained a greater number of peptides from the same proteins compared to the H fraction.

Furthermore, some peptides were common to both M and H fractions, exemplified by sequences like AAPAGAAIQSRAGEIQN and KNTmEHVSSSEESIISQETKY. Several other peptides were also shared across these two fractions, including AKPEKAKETEKDDTGTPITKIELVPSH, TKVIPYVRYL, HIQKEDVPSEK, KYKVPQLEIVPNsAEERLHSm, EPVLGPVRGPFK, EDGSDPPSGDFLTEGGGVK, VEDHIAEGSVAVR, LLYQEPVLGPVRGPFPIIV, and SEGVAIDPARVLDLGPITR.

Nonetheless, each fraction possessed its own set of exclusive peptides. In the M fraction, specific peptides like KEGIHAQQKEPMIGV, DSLNPP, GRVSLVEDHIAEGSVAVR, and KQVKAAPAGAAIQS were identified. Meanwhile, the H fraction contained its own unique peptides, including STYDLVSSAYISRKDQYPYL, ALPIIQKLEPQIA, QPTNQVVANAKGAmTG, GAKDSVASTITGVVDRTKGAVTG, WFREKVSPAVFVSREGQEQEGEEmaEYR, IQEVKASDDGEYR, and QDENYEEAIVHLK.

In summary, this analysis reveals a complex and distinct peptide landscape in each fraction, highlighting the importance of considering the unique peptide profiles within H and M fractions for a comprehensive understanding of their bioactive potential.

Detailed information about the identified peptide sequences from both M and H milk, obtained through the SPE technique, can be found in Tables 5 and 6.

Table 5. Identified peptides in the M fractions extracted with SPE

Protein Name	Sequence of the Peptides	Location of the peptides
G5E6M1 (Polymeric immunoglobulin receptor)	<i>AAPAGAAIQSRAGEIQN</i>	<i>f (358-374)</i>
	<i>APAGAAIQSRAGEIQ</i>	<i>f (359-373)</i>
	<i>APAGAAIQSRAGEIQN</i>	<i>f (359-374)</i>
	<i>PAGAAIQSRAGEIQNK</i>	<i>f (360-375)</i>
	<i>AGEIQNKALLDPSFFA</i>	<i>f (369-384)</i>
	<i>KESVKDAAGGPGAPADPGRPTGYSGSSKA</i>	<i>f (385-413)</i>
	<i>AKESVKDAAGGPGAPADPGRPTGYSGSSKA</i>	<i>f (384-413)</i>
	<i>ALLDPSFFAKESVKDAAGGPGAPADPGRPTGYSGSSKA</i>	<i>f (376-413)</i>
	<i>FFAKESVKDAAGGPGAPADPGRPTGYSGSSKA</i>	<i>f (382-413)</i>
F1N6D4 (Solute carrier family 34 member 2, from Bos taurus)	<i>AKPEKAKETEKDDTGTPI TKIELVPSH</i>	<i>f (27-53)</i>
P02663 (α-S2-casein)	<i>IIsQETYKQEK</i>	<i>f (29-39)</i>
	<i>KNTmEHVsssEESIISQETYK</i>	<i>f (16-36)</i>
	<i>KNTMEHVSSsEEsIIsQETYK</i>	<i>f (16-36)</i>
	<i>KNTmEHVsssEESIISQETYKQEK</i>	<i>f (16-39)</i>
	<i>KNTmEHVSSSEESIISQETYKQEK</i>	<i>f (16-39)</i>
	<i>KNTmEHVSSSEESIISQETYKQEK</i>	<i>f (16-39)</i>
	<i>KNTMEHVSSsEEsIISQETYKQEK</i>	<i>f (16-39)</i>
	<i>KNTMEHVSSSEESIISQETYKQEK</i>	<i>f (16-39)</i>
	<i>KITVDDKHY</i>	<i>f (85-93)</i>
	<i>GPIVLNPWDQVK</i>	<i>f (117-128)</i>
	<i>IVLNPWDQVK</i>	<i>f (119-128)</i>
	<i>LYQGPIVLNPWDQVKRNAVPITPT</i>	<i>f (114-137)</i>
	<i>QGPIVLNPWDQVK</i>	<i>f (116-128)</i>

	<i>QYLYQGPIVLNPWDQVKRNAVPITPT</i>	<i>f (112-137)</i>
	<i>YLYQGPIVLNPWDQVK</i>	<i>f (113-128)</i>
	<i>YQGPIVLNPWDQVK</i>	<i>f (115-128)</i>
	<i>YQGPIVLNPWDQVKRNAVPITPT</i>	<i>f (115-137)</i>
	<i>LNREQLSTsEENS</i>	<i>f (138-150)</i>
	<i>LNREQLSTSEENS</i>	<i>f (138-150)</i>
	<i>NREQLSTSEENS</i>	<i>f (139-150)</i>
	<i>KTVDmEsTEVFTK</i>	<i>f (152-164)</i>
	<i>KTVDMEsTEVFTK</i>	<i>f (152-164)</i>
	<i>TVDmEsTEVFTK</i>	<i>f (153-164)</i>
	<i>TVDmESTEVFTK</i>	<i>f (153-164)</i>
	<i>KTVDmEsTEVFTKK</i>	<i>f (152-165)</i>
	<i>KTCLTEEEKNRL</i>	<i>f (165-176)</i>
	<i>KTCLTEEEKNRLNFL</i>	<i>f (165-179)</i>
	<i>TKLTEEEKNRLNFL</i>	<i>f (166-179)</i>
	<i>TKLTEEEKNRLN</i>	<i>f (166-177)</i>
	<i>TKLTEEEKNRL</i>	<i>f (166-176)</i>
	<i>KTCLTEEEKNRLNFLK</i>	<i>f (165-180)</i>
	<i>LNREQLSTsEENSKKTVDMESTEVFTK</i>	<i>f (138-164)</i>
	<i>NREQLSTSEENSKKTV</i>	<i>f (139-154)</i>
	<i>NREQLSTsEENSK</i>	<i>f (139-151)</i>
	<i>LNREQLstSEENSK</i>	<i>f (138-151)</i>
	<i>LNREQLStSEENSKKTV</i>	<i>f (138-154)</i>
	<i>LNREQLSTSEENSKKTV</i>	<i>f (138-154)</i>
	<i>KTVDmESTEVFTKK</i>	<i>f (152-165)</i>
	<i>KTVDMEsTEVFTKK</i>	<i>f (152-165)</i>
	<i>KTVDMESTEVEFTKK</i>	<i>f (152-165)</i>
	<i>TKLTEEEKNRLNF</i>	<i>f (166-178)</i>
	<i>TKLTEEEKNRLNFLK</i>	<i>f (166-180)</i>
	<i>KTCLTEEEKNRLNFLKKISQRYQ</i>	<i>f (165-187)</i>
	<i>TKLTEEEKNRLNFLKKISQ</i>	<i>f (166-184)</i>
	<i>TKLTEEEKNRLNFLKKISQR</i>	<i>f (166-185)</i>

	<i>QKFALPQYLKTVYQHQQ</i>	<i>f (187-202)</i>
	<i>FALPQYLKTVYQHQQ</i>	<i>f (189-202)</i>
	<i>FALPQYLKTVYQHQQK</i>	<i>f (189-203)</i>
	<i>AmKPWIQPKT</i>	<i>f (204-213)</i>
	<i>YQHQQAmKPWIQPK</i>	<i>f (199-212)</i>
	<i>AMKPWIQPKT</i>	<i>f (204-212)</i>
	<i>AMKPWIQPKTKVIP</i>	<i>f (204-217)</i>
	<i>AmKPWIQPKTKVIPYVR</i>	<i>f (204-220)</i>
	<i>AmKPWIQPKTKVIPYVRYL</i>	<i>f (204-222)</i>
	<i>AMKPWIQPKTKVIPYVRYL</i>	<i>f (204-222)</i>
	<i>TKVIPYVRYL</i>	<i>f (213-222)</i>
	<i>TKVIPYVRY</i>	<i>f (213-221)</i>
	<i>KVIPYVRYL</i>	<i>f (214-222)</i>
P02662 (α-S1-casein)	<i>RPKHPIKHQGLPQE</i>	<i>f (16-29)</i>
	<i>RPKHPIKHQGLPQEV</i>	<i>f (16-30)</i>
	<i>RPKHPIKHQGLPQEVLN</i>	<i>f (16-32)</i>
	<i>RPKHPIKHQGLPQEVLNEN</i>	<i>f (16-34)</i>
	<i>RPKHPIKHQGLPQEVLNENL</i>	<i>f (16-35)</i>
	<i>RPKHPIKHQGLPQEVLNENLL</i>	<i>f (16-36)</i>
	<i>RPKHPIKHQGLPQEVLNENLLR</i>	<i>f (16-37)</i>
	<i>RPKHPIKHQGLPQEVLNENLLRF</i>	<i>f (16-38)</i>
	<i>RPKHPIKHQGLPQEVLNENLLRFF</i>	<i>f (16-39)</i>
	<i>GLPQEVLNENLL</i>	<i>f (25-36)</i>
	<i>GLPQEVLNENLLR</i>	<i>f (25-37)</i>
	<i>GLPQEVLNENLLRF</i>	<i>f (25-38)</i>
	<i>HQGLPQEVLNENLL</i>	<i>f (23-36)</i>
	<i>PQEVLNENLLR</i>	<i>f (27-37)</i>
	<i>VLNENLLR</i>	<i>f (30-37)</i>
	<i>LNENLLRF</i>	<i>f (31-38)</i>
	<i>LNENLLRFFVAPFPEVFGKEKV</i>	<i>f (31-52)</i>
	<i>LPQEVLNENLLR</i>	<i>f (26-37)</i>
	<i>LPQEVLNENLLRF</i>	<i>f (26-38)</i>

	<i>NENLLRFF</i>	<i>f (32-39)</i>
	<i>NENLLRFFVAPFPFVFGKEKV</i>	<i>f (32-52)</i>
	<i>RFFVAPFPFVFG</i>	<i>f (37-48)</i>
	<i>RFFVAPFPFVFGK</i>	<i>f (37-49)</i>
	<i>RFFVAPFPFVFGKEK</i>	<i>f (37-51)</i>
	<i>RFFVAPFPFVFGKEKV</i>	<i>f (37-52)</i>
	<i>RFFVAPFPFVFGKEKVNELS</i>	<i>f (37-56)</i>
	<i>VAPFPFVFG</i>	<i>f (40-48)</i>
	<i>VAPFPFVFGKEKV</i>	<i>f (40-52)</i>
	<i>APFPFVFGKEK</i>	<i>f (41-51)</i>
	<i>APFPFVFGKEKV</i>	<i>f (41-52)</i>
	<i>APFPFVFGKEKVNEL</i>	<i>f (41-55)</i>
	<i>FFVAPFPFVFG</i>	<i>f (38-48)</i>
	<i>PEVFGKEKV</i>	<i>f (44-52)</i>
	<i>FFVAPFPFVFGK</i>	<i>f (38-49)</i>
	<i>FFVAPFPFVFGKEK</i>	<i>f (38-51)</i>
	<i>FFVAPFPFVFGKEKV</i>	<i>f (38-52)</i>
	<i>PFPEVFGKEK</i>	<i>f (42-51)</i>
	<i>FPEVFGKEKV</i>	<i>f (43-52)</i>
	<i>FVAPFPFVF</i>	<i>f (39-47)</i>
	<i>FVAPFPFVFGK</i>	<i>f (39-49)</i>
	<i>FVAPFPFVFGKEKV</i>	<i>f (39-52)</i>
	<i>PFPEVFGKEKV</i>	<i>f (42-52)</i>
	<i>NELSKDIGsES TEDQA</i>	<i>f (54-68)</i>
	<i>NELSKDIGSEsTEDQA</i>	<i>f (54-68)</i>
	<i>NELSKDIGSESTEDQA</i>	<i>f (54-68)</i>
	<i>HIQKEDVP SER</i>	<i>f (95-105)</i>
	<i>HIQKEDVP SERY</i>	<i>f (95-106)</i>
	<i>HIQKEDVP SERYL</i>	<i>f (95-107)</i>
	<i>HIQKEDVP SERYLG</i>	<i>f (95-108)</i>
	<i>HIQKEDVP SERYLGYLEQLLR</i>	<i>f (95-115)</i>
	<i>HIQKEDVP SERYLGYLEQLRL</i>	<i>f (95-116)</i>

	<i>HIQKEDVPSERYLGYLEQLRLK</i>	<i>f (95-117)</i>
	<i>IQKEDVPSE</i>	<i>f (96-105)</i>
	<i>IQKEDVPSE</i>	<i>f (96-106)</i>
	<i>IQKEDVPSE</i>	<i>f (96-107)</i>
	<i>GYLEQLRLK</i>	<i>f (108-117)</i>
	<i>GYLEQLRLK</i>	<i>f (108-119)</i>
	<i>YLEQLRLK</i>	<i>f (109-117)</i>
	<i>YLGYLEQLRLK</i>	<i>f (107-117)</i>
	<i>KYKVPQLEIVPNsAEERLHSm</i>	<i>f (118-138)</i>
	<i>KYKVPQLEIVPNsAEERLHSM</i>	<i>f (118-138)</i>
	<i>YKVPQLEIVPNsAEERLHSM</i>	<i>f (119-138)</i>
	<i>YKVPQLEIVPNsAEERLHsm</i>	<i>f (119-138)</i>
	<i>YKVPQLEIVPNsAEERLHSMK</i>	<i>f (119-139)</i>
	<i>YKVPQLEIVPNsAEERLHsmK</i>	<i>f (119-139)</i>
	<i>VPQLEIVPNsAEER</i>	<i>f (121-134)</i>
	<i>VPNsAEERLHSm</i>	<i>f (127-138)</i>
	<i>VPQLEIVPNsAEERLHSm</i>	<i>f (121-138)</i>
	<i>VPQLEIVPNsAEERLHSM</i>	<i>f (121-138)</i>
	<i>VPQLEIVPNsAEERLHsm</i>	<i>f (121-138)</i>
	<i>VPQLEIVPNsAEERLHSMK</i>	<i>f (121-139)</i>
	<i>KEGIHAQQKEPMIGV</i>	<i>f (139-153)</i>
	<i>VPLGTQYTDAPSF</i>	<i>f (182-194)</i>
	<i>QYTDAPSFSDIPNPIGSENSEKT</i>	<i>f (187-209)</i>
	<i>QYTDAPSFSDIPNPIGSENSEKTTmP</i>	<i>f (187-212)</i>
	<i>QYTDAPSFSDIPNPIGSENSEKTTmPLW</i>	<i>f (187-214)</i>
	<i>QYTDAPSFSDIPNPIGSENSEKTTMPLW</i>	<i>f (187-214)</i>
	<i>TDAPSFSDIPNPIGSENSEKTTmP</i>	<i>f (189-212)</i>
	<i>TDAPSFSDIPNPIGSENSEKTTMPLW</i>	<i>f (189-214)</i>
	<i>SDIPNPIGSENSEK</i>	<i>f (195-208)</i>
	<i>SDIPNPIGSENSEKT</i>	<i>f (195-209)</i>
	<i>SDIPNPIGSENSEKTTmP</i>	<i>f (195-212)</i>
	<i>SDIPNPIGSENSEKTTMP</i>	<i>f (195-212)</i>

	<i>SDIPNPIGSENSEKTTmPLW</i>	<i>f (195-214)</i>
	<i>SDIPNPIGSENSEKTTMPLW</i>	<i>f (195-214)</i>
	<i>DIPNPIGSENSEKT</i>	<i>f (196-209)</i>
	<i>DIPNPIGSENSEKTTmP</i>	<i>f (196-212)</i>
	<i>DIPNPIGSENSEKTTMP</i>	<i>f (196-212)</i>
	<i>DIPNPIGSENSEKTTmPLW</i>	<i>f (196-214)</i>
	<i>DIPNPIGSENSEKTTMPLW</i>	<i>f (196-214)</i>
	<i>FSDIPNPIGSENSEKTTmP</i>	<i>f (194-212)</i>
	<i>FSDIPNPIGSENSEKTTmPLW</i>	<i>f (194-214)</i>
	<i>IGSENSEKTTmP</i>	<i>f (201-212)</i>
	<i>IPNPIGSENSEKT</i>	<i>f (197-209)</i>
	<i>IPNPIGSENSEKTT</i>	<i>f (197-210)</i>
	<i>IPNPIGSENSEKTTmP</i>	<i>f (197-212)</i>
	<i>IPNPIGSENSEKTTMP</i>	<i>f (197-212)</i>
	<i>IPNPIGSENSEKTTmPL</i>	<i>f (197-213)</i>
	<i>IPNPIGSENSEKTTmPLW</i>	<i>f (197-214)</i>
	<i>IPNPIGSENSEKTTMPLW</i>	<i>f (197-214)</i>
	<i>NPIGSENSEKTT</i>	<i>f (199-210)</i>
	<i>NPIGSENSEKTTmP</i>	<i>f (199-212)</i>
	<i>NPIGSENSEKTTmPLW</i>	<i>f (199-214)</i>
	<i>PIGSENSEKTTmP</i>	<i>f (200-212)</i>
P02666 (β-casein)	<i>RELEELNVPGEIVE</i>	<i>f (16-29)</i>
	<i>RELEELNVPGEIVESLSSSEESITRIN</i>	<i>f (16-42)</i>
	<i>RELEELNVPGEIVESLsssEESITRINK</i>	<i>f (16-43)</i>
	<i>RELEELNVPGEIVESLsssEESITRINK</i>	<i>f (16-43)</i>
	<i>RELEELNVPGEIVESLSSSEESITRINK</i>	<i>f (16-43)</i>
	<i>RELEELNVPGEIVESLsssEESITRINKK</i>	<i>f (16-44)</i>
	<i>INKKIEKFQsEEQQQTEDELQDK</i>	<i>f (41-63)</i>
	<i>FQsEEQQQTEDELQDK</i>	<i>f (48-63)</i>
	<i>FQsEEQQQTEDELQDKIHPF</i>	<i>f (48-67)</i>
	<i>IEKFQsEEQQQTE</i>	<i>f (45-57)</i>
	<i>IEKFQsEEQQQTEDELQDK</i>	<i>f (45-63)</i>

	<i>IEKFQSEEQQTEDELQDK</i>	<i>f (45-63)</i>
	<i>IEKFQsEEQQQTEDELQDKIHPF</i>	<i>f (45-67)</i>
	<i>IEKFQsEEQQQTEDELQDKIHPFA</i>	<i>f (45-68)</i>
	<i>KFQsEEQQQTEDELQDK</i>	<i>f (47-63)</i>
	<i>KFQsEEQQQTEDELQDKIHPF</i>	<i>f (47-67)</i>
	<i>KIEKFQsEEQQQTE</i>	<i>f (44-57)</i>
	<i>KIEKFQsEEQQQTEDEL</i>	<i>f (44-60)</i>
	<i>KIEKFQsEEQQQTEDELQ</i>	<i>f (44-61)</i>
	<i>KIEKFQsEEQQQTEDELQD</i>	<i>f (44-62)</i>
	<i>KIEKFQsEEQQQTEDELQDK</i>	<i>f (44-63)</i>
	<i>KIEKFQSEEQQTEDELQDK</i>	<i>f (44-63)</i>
	<i>KIEKFQsEEQQQTEDELQDKIHPF</i>	<i>f (44-67)</i>
	<i>KIEKFQsEEQQQTEDELQDKIHPFA</i>	<i>f (44-68)</i>
	<i>KKIEKFQsEEQQQTEDELQD</i>	<i>f (43-62)</i>
	<i>KKIEKFQsEEQQQTEDELQDK</i>	<i>f (43-63)</i>
	<i>DELQDKIHP</i>	<i>f (58-66)</i>
	<i>LTQTPVVVPPFLQPEVmGVSKVKEAmAPK</i>	<i>f (92-120)</i>
	<i>LTQTPVVVPPFLQPEVMGVSKVKEAmAPK</i>	<i>f (92-120)</i>
	<i>FLQPEVmGVSKVKEAmAPK</i>	<i>f (102-120)</i>
	<i>FLQPEVMGVSKVKEAmAPK</i>	<i>f (102-120)</i>
	<i>VPPFLQPEVmGVSKV</i>	<i>f (99-113)</i>
	<i>VVPPFLQPEVMGVSKV</i>	<i>f (98-113)</i>
	<i>VPPFLQPEVMGVSKV</i>	<i>f (99-113)</i>
	<i>VPPFLQPEVmGVSKVKEA</i>	<i>f (99-116)</i>
	<i>VPPFLQPEVMGVSKVKEA</i>	<i>f (99-116)</i>
	<i>VPPFLQPEVmGV</i>	<i>f (99-110)</i>
	<i>VPPFLQPEVMGV</i>	<i>f (99-110)</i>
	<i>VVPPFLQPEVmGVSKV</i>	<i>f (98-113)</i>
	<i>PEVmGVSKVKEAmAPK</i>	<i>f (105-120)</i>
	<i>PPFLQPEVmGVSKVKEA</i>	<i>f (100-116)</i>
	<i>PPFLQPEVmGVSKVKEAmAPK</i>	<i>f (100-120)</i>
	<i>PPFLQPEVMGVSKVKEAmAPK</i>	<i>f (100-120)</i>

	<i>PPFLQPEVMGVSKVKEAMAPK</i>	<i>f (100-120)</i>
	<i>KEAmAPKHKEmPFPK</i>	<i>f (114-128)</i>
	<i>SKVKEAMAPKHKEmPFPK</i>	<i>f (111-128)</i>
	<i>SKVKEAMAPKHKEmpFPK</i>	<i>f (111-128)</i>
	<i>VKEAmAPKHKEmpFPK</i>	<i>f (113-128)</i>
	<i>VKEAMAPKHKEmpFPK</i>	<i>f (113-128)</i>
	<i>EmPFPKYpVEpFTESQS</i>	<i>f (123-139)</i>
	<i>EmPFPKYpVEpFTESQSL</i>	<i>f (123-140)</i>
	<i>EMPFPKYpVEpFTESQSL</i>	<i>f (123-140)</i>
	<i>EmPFPKYpVEpFTESQSLTLTDVENL</i>	<i>f (123-148)</i>
	<i>FPKYpVEpFTESQSL</i>	<i>f (126-140)</i>
	<i>HKEmpFPKYpVEpFTESQSL</i>	<i>f (121-140)</i>
	<i>mPFPKYpVEpFTESQSL</i>	<i>f (124-140)</i>
	<i>MPFPKYpVEpFTESQSL</i>	<i>f (124-140)</i>
	<i>HKEMPFPKYpVEpFTESQSL</i>	<i>f (121-140)</i>
	<i>HKEmpFPKYpVEpFTESQSLTLTDVENL</i>	<i>f (121-148)</i>
	<i>HKEMPFPKYpVEpFTESQSLTLTDVENL</i>	<i>f (121-148)</i>
	<i>KEmpFPKYpVEpFTESQSL</i>	<i>f (122-140)</i>
	<i>KEMPFPKYpVEpFTESQSL</i>	<i>f (122-140)</i>
	<i>YPVEpFTESQSL</i>	<i>f (129-140)</i>
	<i>TLTDVENLHLPLP</i>	<i>f (141-153)</i>
	<i>TLTDVENLHLPLPLLQS</i>	<i>f (141-157)</i>
	<i>LLQSWMHQPHQPLPPTVmFPPQSV</i>	<i>f (154-177)</i>
	<i>TVmFPPQSVLSLSQS</i>	<i>f (169-183)</i>
	<i>TVmFPPQSVLSLSQSK</i>	<i>f (169-184)</i>
	<i>LSLSQSKVLPVPQKAVPYPQRDmPIQA</i>	<i>f (178-204)</i>
	<i>LSLSQSKVLPVPQKAVPYPQRDmPIQAFL</i>	<i>f (178-206)</i>
	<i>LSLSQSKVLPVPQKAVPYPQRDMPIQAFL</i>	<i>f (178-206)</i>
	<i>LSLSQSKVLPVPQK</i>	<i>f (178-191)</i>
	<i>LSQSKVLPVPQK</i>	<i>f (180-191)</i>
	<i>SLSQSKVLPVPQK</i>	<i>f (179-191)</i>
	<i>SLSQSKVLPVPQKA</i>	<i>f (179-192)</i>

	<i>SLSQSKVLPVPQKAVPYPQRD</i> <i>SLSQSKVLPVPQKAVPYPQRDmPIQA</i> <i>SLSQSKVLPVPQKAVPYPQRDmPIQAF</i> <i>SLSQSKVLPVPQKAVPYPQRDMPIQAF</i> <i>SLSQSKVLPVPQKAVPYPQRDMPIQAFL</i> <i>VLSLSQSKVLPVPQKAVPYPQRD</i> <i>VLSLSQSKVLPVPQKAVPYPQRDmPIQA</i> <i>KVLPVPQKAVPYPQRD</i> <i>KVLPVPQKAVPYPQRDmPIQA</i> <i>KVLPVPQKAVPYPQRDMPIQA</i> <i>KVLPVPQKAVPYPQRDmPIQAF</i> <i>VPYPQRDmPIQ</i> <i>VPYPQRDMPIQA</i> <i>VPYPQRDMPIQAF</i> <i>LYQEPVLGPVRGPFPIIV</i> <i>QEPVLGPVRGPFPIIV</i> <i>YQEPVLGPVRGPFPIIV</i> <i>EPVLGPVRGPF</i> <i>EPVLGPVRGPFPI</i> <i>EPVLGPVRGPFPII</i> <i>EPVLGPVRGPFPIIV</i> <i>FLLYQEPVLGPV</i> <i>FLLYQEPVLGPVRGPFPIIV</i> <i>LLYQEPVLGPV</i> <i>LLYQEPVLGPVRGPFPIIV</i>	<i>f (179-199)</i> <i>f (179-204)</i> <i>f (179-205)</i> <i>f (179-205)</i> <i>f (179-206)</i> <i>f (177-199)</i> <i>f (177-204)</i> <i>f (184-199)</i> <i>f (184-204)</i> <i>f (184-204)</i> <i>f (184-205)</i> <i>f (193-203)</i> <i>f (193-204)</i> <i>f (193-205)</i> <i>f (207-224)</i> <i>f (209-224)</i> <i>f (208-224)</i> <i>f (210-221)</i> <i>f (210-222)</i> <i>f (210-223)</i> <i>f (210-224)</i> <i>f (205-216)</i> <i>f (205-224)</i> <i>f (206-216)</i> <i>f (206-224)</i>
A0A3Q1M5U9 (κ-casein)	<i>ALINNQFLPYPPYAKPA</i> <i>ALINNQFLPYPPYAKPAAV</i> <i>EINTVQVTSTAV</i> <i>SPPEINTVQVTSTAV</i>	<i>f (59-75)</i> <i>f (59-77)</i> <i>f (168-180)</i> <i>f (165-180)</i>
A0A452DHW7 (β-casein)	<i>AQTQSLVYFPFGPIHNSLPQNIPPLTQTPV</i> <i>HNSLPQNIPPLTQTPV</i> <i>IHPFAQTQSLVYFPFGPIHNSLPQNIPPLTQTPV</i>	<i>f (103-132)</i> <i>f (117-132)</i> <i>f (99-132)</i>

	<i>LVYFPFGPIHNSLPQNIPPLTQTPV</i> <i>QTQSLVYFPFGPIHNSLPQNIPPLTQTPV</i> <i>VYFPFGPIHNSLPQNIPPLTQTPV</i>	<i>f (108-132)</i> <i>f (104-132)</i> <i>f (109-132)</i>
E1BM76 (Cysteine and serine rich nuclear protein 2)	<i>DSL NPP</i>	<i>f (39-44)</i>
P02672 (Fibrinogen α chain)	<i>EDGSDPPSGDFLTEGGGVR</i>	<i>f (20-38)</i>
E1BH17 (Butyrophilin subfamily 1 member A1)	<i>GRVSLVEDHIAEGSVAVR</i> <i>VEDHIAEGSVAVR</i> <i>THRGEEFPSmSES RNPDEEGLFTVR</i>	<i>f (93-110)</i> <i>f (98-110)</i> <i>f (179-203)</i>
P80195 (Glycosylation-dependent cell adhesion molecule 1)	<i>ILNKPEDETHLE</i> <i>ILNKPEDETHLEAQPTDASAQF</i> <i>ILNKPEDETHLEAQPTDASAQFI</i> <i>ILNKPEDETHLEAQPTDASAQFIR</i> <i>SSRQPQSQNPKLPLS</i> <i>SSRQPQSQNPKLPLSILK</i> <i>SSRQPQSQNPKLPLSILKEK</i> <i>SSRQPQSQNPKLPLSILKEKHL</i>	<i>f (19-30)</i> <i>f (19-40)</i> <i>f (19-41)</i> <i>f (19-42)</i> <i>f (72-86)</i> <i>f (72-89)</i> <i>f (72-91)</i> <i>f (72-93)</i>
G5E6M1 (Polymeric immunoglobulin receptor)	<i>KQVKAAPAGAAIQS</i> <i>KQVKAAPAGAAIQSR</i> <i>KQVKAAPAGAAIQSRAGEIQNK</i>	<i>f (354-367)</i> <i>f (354-368)</i> <i>f (354-375)</i>
A0A3Q1M193 (Glycoprotein 2)	<i>SEGVAIDPARVLDLGPITR</i>	<i>f (481-499)</i>

Table 6: Identified peptides in the H fraction extracted with SPE

Protein Name	Sequence of the Peptides	Location of the peptides
G5E6M1 (Polymeric immunoglobulin receptor)	<i>AAPAGAAIQSRAGEIQ</i> <i>AAPAGAAIQSRAGEIQN</i> <i>AAPAGAAIQSRAGEIQNK</i> <i>APAGAAIQSRAGEIQ</i> <i>APAGAAIQSRAGEIQN</i> <i>PAGAAIQSRAGEIQNK</i> <i>FFAKESVKDAAGGPGAPADPGRPTGYSGSSKA</i>	<i>f (358-373)</i> <i>f (358-374)</i> <i>f (358-375)</i> <i>f (359-373)</i> <i>f (359-374)</i> <i>f (360-375)</i> <i>f (382-413)</i>
A0A3Q1M193 (Glycoprotein 2 from Bos taurus)	<i>AIDPARVLDLGPITR</i> <i>EGVAIDPARVLDLGPIT</i> <i>EGVAIDPARVLDLGPITR</i> <i>GVAIDPARVLDLGPIT</i> <i>GVAIDPARVLDLGPITR</i> <i>IDPARVLDLGPITR</i> <i>PARVLDLGPITR</i> <i>SEGVAIDPARVLDLGPIT</i> <i>SEGVAIDPARVLDLGPITR</i>	<i>f (459-473)</i> <i>f (456-472)</i> <i>f (456-473)</i> <i>f (457-472)</i> <i>f (457-473)</i> <i>f (460-473)</i> <i>f (462-473)</i> <i>f (455-472)</i> <i>f (455-473)</i>
F1N6D4 (Solute carrier family 34 member 2, from Bos taurus)	<i>PWPELENSQPTSEKYTVKADGEQS</i> <i>WPELENSQPTSEK</i> <i>WPELENSQPTSEKYTVKADGEQ</i> <i>WPELENSQPTSEKYTVKADGEQS</i> <i>EKYTVKADGEQS</i> <i>AKPEKAKETEKDDTGTPTKIELVPSH</i> <i>AKETEKDDTGTPTKIELVPSH</i> <i>TPITKIELVPSH</i> <i>TKIVSSVTAL</i>	<i>f (3-26)</i> <i>f (4-16)</i> <i>f (4-25)</i> <i>f (4-26)</i> <i>f (15-26)</i> <i>f (27-53)</i> <i>f (32-53)</i> <i>f (42-53)</i> <i>f (684-693)</i>
F1MHI1 (Perilipin)	<i>STYDLVSSAYISRKDQYPYL</i> <i>ALPIIQKLEPQIA</i> <i>LPIIQKLEPQIA</i> <i>QPTNQVVANAKGAmTG</i>	<i>f (24-43)</i> <i>f (64-77)</i> <i>f (65-77)</i> <i>f (99-114)</i>

	GAKDSVASTITGVVDRTKGAVTG	<i>f</i> (125-147)
	VVDRTKGAVTGSVKTKSVVSG	<i>f</i> (137-158)
	GVENALTKSELLVDQY	<i>f</i> (175-190)
	TKSELLVDQY	<i>f</i> (181-190)
	LPLTKDELEK	<i>f</i> (191-200)
P02662 (α-S1-casein)	RPKHPIKHQGLPQEVLNENLL	<i>f</i> (16-36)
	RPKHPIKHQGLPQEVLNENLLR	<i>f</i> (16-37)
	RPKHPIKHQGLPQEVLNENLLRF	<i>f</i> (16-38)
	HPIKHQGLPQEVLNENLLR	<i>f</i> (19-37)
	GLPQEVLNENLLR	<i>f</i> (25-37)
	LPQEVLNENLL	<i>f</i> (24-36)
	LPQEVLNENLLR	<i>f</i> (24-37)
	LPQEVLNENLLRF	<i>f</i> (24-38)
	FFVAPFPEVFGKEK	<i>f</i> (38-51)
	FFVAPFPEVFGKEKVNELS	<i>f</i> (38-56)
	FVAPFPEVFGK	<i>f</i> (39-49)
	FVAPFPEVFGKEK	<i>f</i> (39-51)
	APFPEVFGK	<i>f</i> (41-49)
	APFPEVFGKEK	<i>f</i> (41-51)
	HIQKEDVPSER	<i>f</i> (95-105)
	HIQKEDVPSERYLG	<i>f</i> (95-108)
	KYKVPQLEIVPNsAEER	<i>f</i> (118-134)
	KYKVPQLEIVPNsAEERLHSm	<i>f</i> (118-138)
	KYKVPQLEIVPNsAEERLHSM	<i>f</i> (118-138)
	LKKYKVPQLEIVPNsAEERLHSm	<i>f</i> (116-138)
	YKVPQLEIVPNsAEER	<i>f</i> (119-134)
	YKVPQLEIVPNsAEER	<i>f</i> (119-134)
	YKVPQLEIVPNsAEERLHSm	<i>f</i> (119-138)
	YKVPQLEIVPNsAEERLHSM	<i>f</i> (119-138)
	YKVPQLEIVPNsAEERLHSm	<i>f</i> (119-138)
	VPQLEIVPNsAEERLHSM	<i>f</i> (121-138)
	VPQLEIVPNsAEER	<i>f</i> (121-134)

	<i>VPQLEIVPNSAEER</i> <i>VPQLEIVPNsAEERLHSM</i> <i>VPQLEIVPNsAEERLHSMK</i> <i>VPQLEIVPNSAEERLHsmK</i> <i>APSFSDIPNPIGSENSEKTTmPLW</i> <i>IPNPIGSENSEKTTmP</i> <i>IPNPIGSENSEKTTmPLW</i> <i>IPNPIGSENSEKTTMPLW</i> <i>NPIGSENSEKTTmP</i> <i>PIGSENSEKTTmP</i> <i>PIGSENSEKTTmPLW</i> <i>SDIPNPIGSENSEKTTMPLW</i>	<i>f (121-134)</i> <i>f (121-138)</i> <i>f (121-139)</i> <i>f (121-139)</i> <i>f (191-214)</i> <i>f (197-212)</i> <i>f (197-214)</i> <i>f (197-214)</i> <i>f (199-212)</i> <i>f (200-212)</i> <i>f (200-214)</i> <i>f (195-214)</i>
A5D7F2 (SYT4 protein)	<i>APISTSREEFDEIPTVVGIFSA</i>	<i>f (2-23)</i>
A0A3Q1M193 (Glycoprotein 2)	<i>DPARVLDLGPITR</i>	<i>f (487-499)</i>
P80025 (Lactoperoxidase)	<i>DTIAQAASTTTISDAVSK</i> <i>DTIAQAASTTTISDAVSKVK</i> <i>LKTTLSsEAPTTQQLSEYFKHAKGRT</i> <i>TLSSEAPTTQQLSEYFK</i> <i>TTLSSEAPTTQQLSEYFKHAKG</i> <i>TTLSsEAPTTQQLSEYFKHAKGRT</i> <i>TAIRNGQVWEESLK</i> <i>NGQVWEESLKRL</i> <i>GQVWEESLKRL</i> <i>VWEESLKRL</i>	<i>f (121-139)</i> <i>f (121-139)</i> <i>f (191-214)</i> <i>f (197-212)</i> <i>f (197-214)</i> <i>f (197-214)</i> <i>f (199-212)</i> <i>f (200-212)</i> <i>f (200-214)</i> <i>f (195-214)</i>
P02672 (Fibrinogen α-chain)	<i>EDGSDPPSGDFLTEGGGVR</i>	<i>f (20-38)</i>
E1BHI7 (Butyrophilin subfamily 1 member A1)	<i>WFREKVSPAVFVSREGQEQEGEEmaEYR</i> <i>EDHIAEGSVAVR</i> <i>LVEDHIAEGSVAVR</i> <i>RVSLVEDHIAEGSVAVR</i>	<i>f (65-92)</i> <i>f (99-110)</i> <i>f (97-110)</i> <i>f (94-110)</i>

	SLVEDHIAEGSVAVR VEDHIAEGSVAVR VSLVEDHIAEGSVAVR IQEVKASDDGEYR QDENYEEAIVHLK THRGEFFPSmSES RNPDEEGLFTVR THRGEFFPSMSES RNPDEEGLFTVR NPDEEGLFTVR IPLSPmGEDSASGDIETLH IPLSPMGEDSASGDIETLH SPmGEDSASGDIETLH GEDSASGDIETLH	<i>f</i> (96-110) <i>f</i> (98-110) <i>f</i> (95-110) <i>f</i> (111-123) <i>f</i> (128-140) <i>f</i> (179-203) <i>f</i> (179-203) <i>f</i> (193-203) <i>f</i> (495-513) <i>f</i> (495-513) <i>f</i> (498-513) <i>f</i> (501-513)
P02666 (β-casein)	FQsEEQQQTEDELQDK FQsEEQQQTEDELQDK IEKFQsEEQQQTEDELQDK IEKFQsEEQQQTEDELQDK KFQsEEQQQTEDELQDK KIEKFQsEEQQQTEDELQDK KIEKFQsEEQQQTEDELQDK KIEKFQsEEQQQTEDELQDKIHPFA LLYQEPVLGPVRGPFPIIV LYQEPVLGPVRGPFPIIV YQEPVLGPVRGPFPIIV EPVLGPVRGPF EPVLGPVRGPFPII EPVLGPVRGPFPIIV	<i>f</i> (48-63) <i>f</i> (48-63) <i>f</i> (45-63) <i>f</i> (45-63) <i>f</i> (47-63) <i>f</i> (44-63) <i>f</i> (44-63) <i>f</i> (44-68) <i>f</i> (206-224) <i>f</i> (207-224) <i>f</i> (208-224) <i>f</i> (210-221) <i>f</i> (210-223) <i>f</i> (210-224)
P80195 (Glycosylation-dependent cell adhesion molecule 1)	ILNKPEDETHLEAQPTDASAQ ILNKPEDETHLEAQPTDASAQF ILNKPEDETHLEAQPTDASAQFI ILNKPEDETHLEAQPTDASAQFIR ILNKPEDETHLEAQPTDASAQFIRN SSRQPQSQNPKLPL	<i>f</i> (19-39) <i>f</i> (19-40) <i>f</i> (19-41) <i>f</i> (19-42) <i>f</i> (19-43) <i>f</i> (72-85)

	SSRQPQSQNP ~~K~~ LPLSILKE SSRQPQSQNP ~~K~~ LPLSILKEK SSRQPQSQNP ~~K~~ LPLSILKEKH SSRQPQSQNP ~~K~~ LPLSILKEKHL SRQPQSQNP ~~K~~ LPLS QPQSQNP ~~K~~ LPLSILKEK SQNP ~~K~~ LPLSILKEKHL NP ~~K~~ LPLSILKEKHL LPLSILKEKHL PLSILKEKHL	<i>f</i> (72-90) <i>f</i> (72-91) <i>f</i> (72-92) <i>f</i> (72-93) <i>f</i> (73-86) <i>f</i> (75-91) <i>f</i> (78-93) <i>f</i> (80-93) <i>f</i> (83-93) <i>f</i> (84-93)
P02663 (α-S2-casein)	KNTmEHVSSSEESIISQETY KNTmEHVSSsEEsIISQETYK KNTmEHVSSsEEsIISQETYK KNTmEHVSSSEESIIsQETYK KNTmEHVSSSEESIISQETYK KNTMEHVsssEEsIISQETYK KNTMEHVSSsEEsIISQETYK KNTMEHVSSSEESIIsQETYK KNTMEHVSSSEESIISQETYK KNTmEHVSSSEESIIsQETYKQE KNTmEHVSSSEESIISQETYKQE KNTmEHVSSsEEsIISQETYKQEK KNTmEHVSSSEESIISQETYKQEK KNTMEHVSSSEESIIsQETYKQEK KNTMEHVSSSEESIISQETYKQEK KTVDMEsTEVFTK KTVDmEStEVFTKK KTVDMEsTEVFTKK TVDmESTEVFTK TVDMEStEVFTK VDmEsTEVFTK KTKLTEEEKNRLNFL	<i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-34) <i>f</i> (16-36) <i>f</i> (16-36) <i>f</i> (16-37) <i>f</i> (16-37) <i>f</i> (16-37) <i>f</i> (16-37) <i>f</i> (152-164) <i>f</i> (152-165) <i>f</i> (152-165) <i>f</i> (153-164) <i>f</i> (153-164) <i>f</i> (154-164) <i>f</i> (165-179)

	<i>KTKLTEEEKNRLNFLK</i> <i>TKLTEEEKNRLNFL</i> <i>TKLTEEEKNRLNFLK</i> <i>TKVIPYVRY</i> <i>TKVIPYVRYL</i>	<i>f (165-180)</i> <i>f (166-179)</i> <i>f (166-180)</i> <i>f (213-221)</i> <i>f (213-222)</i>
Q9MZ06 (Fibroblast growth factor-binding protein 1)	<i>GSKASADESLALGKPGKEPR</i> <i>KASADESLALGKPGKEPR</i> <i>RGSKASADESLALGKPGKEPR</i>	<i>f (32-51)</i> <i>f (34-51)</i> <i>f (31-51)</i>
Q0P5N1 (Protein canopy homolog 3)	<i>LPAPELGPRQA</i>	<i>f (31-41)</i>
A0A3Q1MJE5 (α-S1-casein)	<i>YLGYLEIVPNsAEERLHSm</i>	<i>f (106-124)</i>

5. Conclusion and future perspectives

This thesis represents a significant advancement in our understanding of the intricate relationship between peptides and nanoparticles. Within this study, we identified specific peptide sequences that reveal a discernible linear pattern, characterizing the interaction dynamics between peptides and iron oxide nanoparticles. This breakthrough has the potential to greatly enhance our comprehension and predictive abilities regarding the protein corona formation selectivity. Importantly, this insight extends beyond iron oxide nanoparticles to encompass a wide range of diverse nanomaterials.

Furthermore, our findings emphasize the inherent value of utilizing specialized colloidal bare iron oxide nanoparticles. These nanoparticles offer a sustainable means to isolate bioactive peptides distinguished by their unique amino acid attributes and distinctive physicochemical characteristics from complex biological matrices. Of particular note is the discovery that milk affected by mastitis contains an abundance of peptides, making it a promising reservoir for bioactive peptides in future investigations. Intriguingly, peptides refined through SAMN nanoparticles have demonstrated noteworthy antioxidant and anti-inflammatory properties, suggesting the need for further exploration.

It is noteworthy to highlight that peptides obtained through solid-phase extraction have also demonstrated commendable antioxidant and anti-inflammatory properties. An intriguing aspect worth considering is the effectiveness of iron nanoparticles in selectively adsorbing and segregating peptides characterized by specific structural traits.

In future research endeavors, the synthesis of peptides extracted from milk, in conjunction with peptide sequences identified through mass spectrometry and precise structural elucidation, opens up opportunities for strategic manipulation. This could involve the introduction of functional groups to peptides with significant protective potential, thereby enhancing their efficacy. One particularly promising avenue involves the synthesis of bio-derived peptides with anti-cancer properties, representing a crucial step towards transformative biomedical applications.

6. References

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APPENDIX

Amino Acid	Three letter symbol	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V