Bioactive Peptides in Dairy Products

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All the research pertaining to the detection and identification of minute peptides (<4 amino acids) present in multifarious mixtures are in their early stages because of a lack of stringent peptide identification methodologies. Precise amendments like discerned censoring of ions against previously identified sequences of peptides can help overcome the aforementioned issues faced at times of optimization procedures during or after MS analysis. A state-of-the-art genesis in structure-informed
peptide identification and quantification methodologies can be guaranteed by added enrichment in the sensitivity and resolving capacity of MS, in conjunction with novel cutting edge ionization techniques. Modernization of the software for foodomics and peptidomics research and peptide identification is needed. Also, explicit and coherent structure identification in common and especially in synchronization with LC-MS requires significant attention. A continuous focus will be given to understanding of the biochemical functions of milk ingredients and their dietary implications by using a variety of powerful tools like -omics, cell models, gut microbiome research and imaging.
The introduction of innovative facilities including is an absolute requirement for the development of approaches, such as proteomics, recombinant enzymes and microbial fermentation to study and improve the metabolic and health consequences of the various roles of bioactive peptides throughout the expression of genes. Consequently, the formulation of products incorporating bioactive peptides should examine the allergenicity, toxicity and stability of the a ected metabolic functions during gastrointestinal digestion. Despite considerable progress in the isolation, purification and assessment of bioactivities of BP from various natural sources, several hurdles still remain to be overcome, particularly technological advancements to produce them on a broad scale without losing activity. In conclusion, milk-derived bioactive peptides o er substantial future prospects for product development to support health, with their multifunctional assets.

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Food-based components represent major sources of functional bioactive compounds. Milk is a rich source of multiple bioactive peptides that not only help to fulfill consumers ‘nutritional requirements but also play a significant role in preventing several health disorders. Understanding the chemical composition of milk and its products is critical for producing consistent and high-quality dairy products and functional dairy ingredients. Over the last two decades, peptides have gained significant attention by scientific evidence for its beneficial health impacts besides their established nutrient value. Increasing awareness of essential milk proteins has facilitated the development of novel milk protein products that are progressively required for nutritional benefits. The need to better understand the beneficial effects of milk-protein derived peptides has, therefore, led to the development of analytical approaches for the isolation, separation and identification of bioactive peptides in complex dairy products.

Continuous emphasis is on the biological function and nutritional characteristics of milk constituents using several powerful techniques, namely omics, model cell lines, gut microbiome analysis and imaging techniques. This review briefly describes the state-of-the-art approach of peptidomics and lipidomics profiling approaches for the identification and detection of milk-derived bioactive peptides while taking into account recent progress in their analysis and emphasizing the difficulty of analysis of these functional and endogenous peptides.

1. Introduction

Milk is a vital food that satisfies the neonate's nutritional needs and its composition is formulated to facilitate the survival of the species as a result of 200 million years of evolution [1]. Milk is a source of lactose, lipids and proteins, as well as vitamins, mineral substances, oligosaccharides, intrinsic immune factors, immune globulins, hormones, enzymes, and neonatal growing factors [2]. Such components play a significant role in different body functions like cardiovascular, immunomodulation, metabolic and neuronal growth and in the establishment and implementation of a gut microbiome [3][4][5][6]. The milk industry is the most diverse and intensively developed global dairy sector leading to the diversification of targeted dairy formulations aimed at different age groups, in order to meet particular nutritional needs. A report of the United States Department of Agriculture (USDA) Global Agricultural Information Network estimated a 38 million ton increase in liquid milk use, marking a significant increase concerning a historically low milking base, given that customers are acquiring a new taste for milk. Milk and milk products are vital to human nutrition and are known as carriers of amino acids, proteins, fat, water-soluble vitamins, calcium, essential fatty acids, and several other bioactive compounds of significant importance in diverse biochemical and physiological functions[7].

The primary sources of bioactive peptides are milk proteins. These bioactive peptides are short amino acid sequences encrypted in milk proteins and may be released by *in vivo* fermentation of milk with a proteolytic starter (lactic acid bacteria) or by enzyme hydrolysis during gastrointestinal digestion [8][9].They remain inactive within the primary structure of milk proteins and are released by casein proteolysis [10]. The bioactive peptides released are often small and range in size from 2 to 50 amino acid residues [11]. A number of these peptides found in bovine milk or synthesized *de novo* have been shown to affect the cardiovascular, neurological, digestive, endocrine and immune systems while displaying functional properties like anti-microbial, antithrombotic, anti-hypertensive, anti-atherogenic, anti-oxidant and immunomodulatory activities [12][13][14]. Since hypertension is becoming more prevalent globally, researchers have focused mainly on the evaluation of antihypertensive peptides and their intervention in cardiovascular diseases [8]. In therapeutic implementations, the potential effect of these anti-hypertensive peptides has been well documented [15][16]. Moreover, anti-hypertensive activity is one of most important functionalities of peptides but additional applications of dairy peptides can also be pinpointed, despite being less researched. Simultaneously, the increasing consumer interest in solving health issues by making conscious eating choices has led to food containing naturally occurring peptides rather than synthetically produced pharmaceuticals being preferred, especially if comparable effectiveness for targeted applications has been proven.

Analytical approaches for milk component analysis are moderately uncommon in the field of probabilistic analytical chemistry. Dietary peptide identification has been investigated mainly by subjecting food to *in vitro* digestion

simulation [17][18][19]. Table 1 summarizes the different analytical techniques used for identification and detection of milk and dairy components actively involved in human health. Over past 25 years, statistical tools for characterization and the validation of analytical method efficiency in combination with the enhancements in both instrumental and chemical approaches have achieved better testing performance. Lynch and coworkers [20] reported that methods for calculating the composition of milk in quantitative analytical chemistry are relatively uncommon since the findings determine the split of substantial sums of money between milk sellers and buyers. There is, therefore, an excellent opportunity to improve and optimize these techniques in order to achieve a degree of research rigor rarely applied to other approaches or by laboratory workers employed in analytical chemistry. Over the past 25 years, mid-infrared (MIR) milk analysis and improved performance of chemical processes for the calibration of MIR has helped the milk and dairy industry to change from weight and fat-dependent milk payment to weight-based milk assessment (e.g., fat, actual proteins, other solids) for every milk component delivered. These advances have been made possible by research in order to enhance both the chemical and instrumental analytical approaches for milk components.

Table 1. Techniques used in the identification and detection of bioactive peptides in milk and dairy products.

Analytical Techniques		Chemometric Techniques	Application	References
Electrophoretic Methods	Urea-PAGE	-	Evaluation of changes upon proteolysis in cheese	[21]
	Urea-PAGE of casein	-	Determination of concentration of peptides and age-related differences	[22]
Chromatographic and Spectrophotometric Techniques				
Mass Spectrometry	2-DE; MALDI-TOF; ESI-IT	In-gel digestion	Polymorphism of goatαs1-casein	[23]

RP-HPLC < 1000 Da	Analysis of volatile compounds	Evaluation of changes upon proteolysis in cheese	[21]
	Sensory analysis	Differences among caseins peptides and sensory attributes	[22]
NanoESI-QTOF	In-capillary tryptic hydrolysis	Characterization of elephant milk proteins	[24]
MALDI-TOF (reflectron),HPLC-ESI-IT	Tryptic digestion	Identification of truncated goat	[25]
MALDI-TOF(reflectron),HPLC-ESI-IT	Tryptic digestion	Identification of truncated forms of goat αs2-CN A and E	[26]
ESI-QqQ	Offline RP-HPLC	Degree of glycosylation and phosphorylation of ovine and caprine CMP	[27][28]

2-DE, immunoblotting, MALDI-TOF, ESI-QqQ	In-gel digestion immunoblotting	Phosphorylation and glycosylation of ovine caseins	[29]
1-DE and 2-DE,ESI-IT	Enzymatic digestion	Phosphorylation, glycosylation,and genetic variants of κ-casein	[30]
2-D LC-nanoESI-IT	Shotgun proteomics	Identification of minor human milk proteins	[31]
2-DE, HPLC-QTOF	In-gel digestion	Characterization of minor whey proteins	[32]
HPLC-QTOF	In-gel digestion	Lactosylation of β-Lg, α-La, and αs2-CN in infant formula	[33]
HPLC-ESI-QqQ	Tryptic digest in solution	Lactosylation of β-Lg	[34]
MALDI-TOF	-	Degradation of αs1-CN f(1–23) by bacterial amino and endopeptidases	[35]

	MALDI-TOF (reflectron)		Specificity of peptidases from *Lactobacillus helveticus*	[36]
Polymerase Chain reaction(PCR)		Bovine DNA in cheese	Ovine and caprine cheese samples	[37]
	PCR-RFLP	Bovine DNA in cheese	Commercial mozzarella and feta cheese samples	[38]
		Bovine DNA in cheese	Experimental binary mixtures of bovine milk with ovine, caprine, and buffalo milk	[39]
	Duplex PCR	Simultaneous detection of bovine and buffalo DNA in cheese and milk	Experimental mixtures of bovine and buffalo milk and commercial buffalo Mozzarella samples	[40]
		Commercial cheese	Simultaneous detection of	[41]

		samples	bovine and caprine DNA in cheese	
	RT-PCR	Bovine DNA in cheese	Experimental and commercial mozzarella cheese	[42]
	ELISA	Identification of immunogens	Evaluation the fraction of goat′s milk for quantification and the existence of standard immunoglobulins (IgG)	[43]

Nevertheless, the initiative, leadership and financing for most of the developments in the testing of milk in the USA during the last 25 years came from the USDA Test Procedures Committee, Dairy Programs, and the Federal Milk Market orders.

The consolidation and increased production of milk products have led to the introduction of new, fast, reliable and cost-effective methods for quality assurance and composition analysis. The sensory and instrumental evaluation of dairy food characteristics could be used for quality assurance determinations prior to the transition of the product to the customers, who perform the final sensory assessment, although it is always important to detect, identify and correct product defects. Modern instrumental and sensory strategies in dairy food production facilitate the optimization of a product′s desired attributes and the creation of new products that suit the product′s future market segment attribute profile. The sensory evaluation of dairy products has developed from both defect-oriented recognition system and intensity-development approaches that explain several more dimensions of the characteristics of a product. The data from sensory processes like quantitative descriptive analysis (QDA) can be used for defining and optimizing the characteristics of a dairy food product by combining them with the aim of measurements from a chemical (e.g., gas chromatography) or physical (e.g., rheology measurements, such as dynamic mechanical analyses) method. For the analytical and instrumental data processing, more sophisticated statistical methods such as principal component analysis are frequently used for the data analysis. Comprehending the underlying presence of milk and dairy bioactive peptides, may, therefore, reveal possibilities of custom-made processing to enhance identification of milk peptides and produce enriched milk products that improve health aspects. This review focuses on the methodologies used for the analysis of milk, and dairy products will enhance our understanding of dairy products′ bioactive properties and direct the future identification of new functional

products. This article also presents state-of-the-art approaches for complementing and profiling of the bioactive detection, regardless of the small proportion of amino acid units.

2. Milk Fat Globule Membranes (MFGM): A Value-Added Product in Milk and Dairy Industry

Due to its complex structure and health-efficient properties, milk fat globule membranes (MFGM) has received considerable attention in recent years [44]. The composition and function of MFGM proteins are essential indicators of dairy′s nutritional origin and can be integrated into a range of health benefits [45][46][47]. The use of MFGM as a nutraceutical relies on its chemical components, modifications in manufacturing and different processed food products. MFGM may be isolated from milk or dairy products, including buttermilk and butter strengthened in MFGM components. The separation of milk into cream and skimmed milk, churning of butter, flavor and milk products texture can be related to milk fat globule surface properties. Isolation and characterization procedures determine the composition of proteins. Hence, appropriate use of MFGM as well as its components could therefore greatly enhance the value added of dairy products [48].

The proteins of the MFGM are 1–4% of the overall milk proteins. The complex peripheral and integral protein system represents the MFGM [49]. They play a significant role in infant metabolism and act as a defense mechanism [50]. The milk fat globule membrane protein characterization with polyacrylamide gel electrophoresis exposed approximately nine polypeptide chains. To date, important functional bioactivities related to the fat membrane protein of milk globules include immune stimulation, antimicrobial and antiviral characteristics. The specific components of bovine dairy fat membrane protein like lactadherin show less bioactivity than the human analogous [51]. Two MFGM glycoproteins, namely, lactadherin and mucin display resistance to pepsin and sustain their biological function even at low gastric pH. The involvement of carbohydrate moieties, which form a glycocalyx around milk fats globules, may be correlated with the resistance of the glycoproteins. Glycocalyx provides a barrier against lipolysis as well as a steric barrier against aggregation and recrystallization [52]. In addition to the antibacterial and anti-inflammatory activities of the MFGM [53] proteins, MFGM phospholipids, particularly, sphingomyelin also have many potential health benefits, like gut protection and colon cancer prevention.

The concentration of neutral lipid content of MFGM varies widely. The most common components of total membrane lipid are glycerides. In comparison with bulk milk triglycerides, triglycerides constitute a greater proportion of long chain fatty acids, generally referred to as high melting triglycerides. In MFGM, free fatty acids like β-carotene, hydrocarbons, and squalene represents lipid fractions. MFGM also consists of membrane phospholipids fragment which constitutes phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin. Thus, this cross-examination offers further bit of knowledge of the complex structure of MFGM proteins, strengthening our understanding of the functional importance of MFGM proteins.

3. Factors Affecting Milk Bioactive Peptides Composition and the Variations among Animal Genetics

Biologically-active peptides can be generated from milk proteins by various mechanisms that include the activity of microbial enzymes, proteolytic enzymes, and indigenous enzymes from starter and non-starter cultures that function during milk secretion, storage, processing, and finally digestion. The key indigenous digestive enzymes were identified in caprine and ovine milk [54][55][56][57]

The composition of milk protein varies between the major dairy animals. For eg., sheep milk usually contains more casein, α-lactalbumin, serum albumin, lactoferrin, and β-lactoglobulin, than goats, buffaloes and cows [58]. There are several factors that may influence the composition of milk bioactive peptides, such as, protein composition, ruminants diet, animal genetics, environmental conditions, lactation stage, physiological state of the animal, which ultimately leads to milk yield and composition [59][60][61][62]. The components of milk protein are defined well in the literature, nonetheless, knowledge on how animal genetics, animal nutrition and the distribution of these components are scarcely found in the literature. Diverse technical approaches are being used by the dairy industry to adjust the concentration of different bioactive peptides in milk (addition of protein precursors, modified milk proteins, or processed enzymes) [63]. The preferences of customers are increasingly moving towards fresh food products and less processed packaged foods [64]. Animal genetics and nutrition might be significant assets to collect natural products enriched in bioactive peptides. The most efficient and useful way of increasing the production of the beneficial milk bioactive peptides for the consumption of humans is potentially through animal genetics. The profile and concentration of milk protein distinguished between animal species has been strongly influenced by animal nutrition and animal genetics . Unfortunately, only a few studies expand these relationships to allow the modifications in milk bioactive peptides.

4. Potential Roles and Applications for Milk and Dairy Industry Infant Formula Products

Technological advances in recent decades have greatly affected living conditions and the dairy industry. Production of food has transformed from small-scale farming to mechanical processing, transforming food supply, storage, distribution and consumption on a large scale. Infant's formula is generally used as a nutrient supplements in order to meet the needs of infant and children. The industrial production of baby formulas requires essential nutrients at a degree higher than normal in breast milk, including Cu and Mn. The only way to nutritionize many infants from the first four to six months of their life is to deliver infant formulas. These are crucial for infant's health during periods of severe shortages and inadequate nutrition, as they will adequately sustain growth and development. These formulations are consumed by many of the infants and marketed in powdered, liquid and liquid ready-to-eat forms. Notwithstanding advancements in the development of formula preparations, there are a few compounds present in human milk, including anti-infection agents (human milk proteins are well-known to promote cell growth and proliferation), enzymes and trophical factors [65].

Milk proteins are widely used in baby food. The composition difference between human and cow milk is now well known and they do not have the same composition, especially regarding proteins and carbohydrates. Moreover, lactoferrin and α-lactalbumin are the major whey proteins in human milk, while β-lactoglobulin does not exist. In addition, caseins predominate in cow milk, while human milk is rich in whey (soluble) proteins [66]. Thus, αs1- and

αs2-casein account for more than half of the total caseins in cow′s milk while bovine caseins are even more highly phosphorylated than human milk casein [67]. The hydrolysates in cow milk proteins are perhaps the most utilized protein in infant formulas due to their superior nutritional value [68]. A variety of cow′s milk products (e.g., non-fat milk, casein, casein and whey protein combinations or whey protein concentrate partially hydrolyzed) focus on providing protein for these formulas.

Milk basic formulas are divided into two categories; whey-predominant and casein-predominant formulas. Casein-predominant formulas are essentially diluted bovine skim milk, which contains fat and other nutrients but does not change the casein: whey ratio. New rules and guidelines for infant foods indicate that it is not compulsory to have both the ratios. The minimal and maximum protein must always be adhered to in baby formulas, although it varies on the source of the protein. The difference between these two formulations is their protein source. Infants fed with whey contents showed enhanced plasma threonine concentrations [69].

5. Knowledge Summary and Future Directions

All the research pertaining to the detection and identification of minute peptides (<4 amino acids) present in multifarious mixtures are in their early stages because of a lack of stringent peptide identification methodologies. Precise amendments like discerned censoring of ions against previously identified sequences of peptides can help overcome the aforementioned issues faced at times of optimization procedures during or after MS analysis. A state-of-the-art genesis in structure-informed peptide identification and quantification methodologies can be guaranteed by added enrichment in the sensitivity and resolving capacity of MS, in conjunction with novel cutting edge ionization techniques. A remarkable challenge is the invention of new technologies that will secure high production with augmented purity in the domains of chromatographic and non-chromatographic separation procedures, respectively. There is a compelling demand to revise the accessories along with the techniques themselves. Modernization of the software for foodomics and peptidomics research and peptide identification is needed. Also, explicit and coherent structure identification in common and especially in synchronization with LC-MS requires significant attention. However, due to the inadequacy of technological advancements, enriched food items and molecular approaches, there is still limited work in this area.

Over the past 100 years, the different components in milk and the primary forms of milk proteins were established with tremendous advances. Moreover, very little or no knowledge of the stability, bioavailability and efficiency of these bioactive peptides leads to a major knowledge gap that hinders a better understanding of their role in human health. At this point, further prerequisites are the establishment of resources to protect/expand the operation of bioactive peptides and encourage their maximum use in food production systems. Analytical development has contributed to the fractionation and characterization of dairy components. Throughout the past 50 years, knowledge of milk factor biosynthesis has progressed rapidly. Milk testing has also been transformed from slow laboratory procedures into quick tests of multiple components which may be carried out on the farm. Improved understanding of the different forms of milk protein has encouraged the commercialization, in dietary uses, of new milk protein ingredients. A continuous focus will be given to understanding of the biochemical functions of milk ingredients and their dietary implications by using a variety of powerful tools like -omics, cell models, gut

microbiome research and imaging. Milk monitoring has grown from gross compositional monitoring for regulatory reasons or farm remuneration to a range of assessments for uses such as agricultural management and animal welfare. Traditionally, tests were conducted in large centralized laboratories and gradually switched to the field itself. Approaches like ELISA are already being used to show the possible occurrence of Johne′s disease in cow′s milk, and these approaches also provide farmers with evidence to take steps to reduce the incidence of this disease. PCR, which is currently in use, is another example of a diagnostic testing aid used to recognize mastitis-causative bacteria. These data have helped organizations identify chronic pathogen shedders and contribute to making management decisions for removing these particular animals and reducing SCC levels. More than 30 tests (composition and indicator) can currently be performed on milk samples, and the number of valuable tests is expected to continue to grow. Increasing technology will also include robotic milking systems, making it easier to collect and test milk samples. In the future, further field milk studies will occur as this provides farmers with timely data for management decisions and the production of milk. Milk is easier to process than blood samples or other biological materials.

The introduction of innovative facilities including is an absolute requirement for the development of approaches, such as proteomics, recombinant enzymes and microbial fermentation to study and improve the metabolic and health consequences of the various roles of bioactive peptides throughout the expression of genes. Consequently, the formulation of products incorporating bioactive peptides should examine the allergenicity, toxicity and stability of the affected metabolic functions during gastrointestinal digestion. The implementation of integrated research platforms is still necessary for interdisciplinary research to clarify the role and mechanism of milk-derived bioactive peptides. In addition, before formulations are used as chemotherapeutic agents or tested directly for viable conditions, the preliminary positive effects of milk derivative products on target diseases must be considered carefully. Despite considerable progress in the isolation, purification and assessment of bioactivities of BP from various natural sources, several hurdles still remain to be overcome, particularly technological advancements to produce them on a broad scale without losing activity. In conclusion, milk-derived bioactive peptides offer substantial future prospects for product development to support health, with their multifunctional assets.

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