

REVIEW

Advances in separation and identification of biologically important milk proteins and peptides

Miroslava Stastna 

Institute of Analytical Chemistry of the Czech Academy of Sciences, Brno, Czech Republic

Correspondence

Miroslava Stastna, Institute of Analytical Chemistry of the Czech Academy of Sciences, Veveri 97, 602 00 Brno, Czech Republic.

Email: stastna@iach.cz

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Abstract

Milk is a rich source of biologically important proteins and peptides. In addition, milk contains a variety of extracellular vesicles (EVs), including exosomes, that carry their own proteome cargo. EVs are essential for cell–cell communication and modulation of biological processes. They act as nature carriers of bioactive proteins/peptides in targeted delivery during various physiological and pathological conditions. Identification of the proteins and protein-derived peptides in milk and EVs and recognition of their biological activities and functions had a tremendous impact on food industry, medicine research, and clinical applications. Advanced separation methods, mass spectrometry (MS)-based proteomic approaches and innovative biostatistical procedures allowed for characterization of milk protein isoforms, genetic/splice variants, posttranslational modifications and their key roles, and contributed to novel discoveries. This review article discusses recently published developments in separation and identification of bioactive proteins/peptides from milk and milk EVs, including MS-based proteomic approaches.

KEYWORDS

bioactive proteins/peptides, extracellular vesicles, milk, MS-based proteomics, separation methods

1 | INTRODUCTION

Milk is a nutritious food that is required for healthy growth and development. In addition to essential nutrients, milk is a rich source of biologically important proteins and

peptides [1] that are important for physiological functions, have therapeutic effects, and can prevent from pathological conditions [2–7]. On the other hand, bioactive peptides, for example, β -casomorphin 7 (BCM-7), enzymatically released from casein variants during gastrointestinal digestion, can cause milk intolerance and allergy [8, 9]. The proteins in milk can be separated into major components, which are casein micelles (α -, β -, and κ -casein [CN] and their variants) [10–12], soluble milk whey (β -lactoglobulin [β -LG], lactoferrin [LF], α -lactalbumin [α -LA], BSA, immunoglobulins [Igs], and glycomacropeptide) [13, 14], and fat globule membrane

Abbreviations: ACE, angiotensin converting enzyme; CPPA, α -cyano-5-phenyl-2,4-pentadienic acid; DDA, data dependent acquisition; DIA, data independent acquisition; DPP-IV, dipeptidyl peptidase IV; EVs, extracellular vesicles; LF, lactoferrin; MFGM, fat globule membrane; MGONPs, methacryloyl graphene oxide nanoparticles; PLL, poly-L-lysine; α -CN, α -casein; α -LA, α -lactalbumin; β -CN, β -casein; β -LG, β -lactoglobulin; κ -CN, κ -casein.

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(MFGM) proteins [15]. In addition, milk proteins are present in various genetic/splicing variants [16] and are abundantly posttranslationally modified [17, 18]. As well, milk contains extracellular vesicles (EVs) of various sizes which carry, in addition to other bioactive components, their own specific proteome cargo [19–21]. EVs have distinct roles in biological processes and they are involved in activities crucial for cell–cell communication.

Detection of milk proteins/peptides and recognition of their bioactivities and various functions led to the production of specific diet additives and products that can be used in medicine and in a treatment of a variety of health disorders. However, milk proteins/peptides of interest need to be first isolated and identified, and various separation methods have been implemented on both laboratory and industrial scale. Concurrently, more efficient and advanced methods were developed, which can overcome the challenges related to bioactive protein/peptides purification and can target and characterize novel biocomponents present in milk. Milk is a complex and heterogeneous fluid with many proteins and protein-derived peptides which still need to be discovered and their functions confirmed, and thus, powerful separation technologies that include HPLC, CE, and especially mass spectrometry (MS) complemented with advanced up-to-date biostatistical methods [22–24] are the tools to contribute to this goal.

Separation methods carried out for milk proteome/peptidome characterization have extensive applications in food industry, for example, for the guarantee of authenticity and quality of final products [25]. Dairy products from animal species, such as goat, sheep, mare, and buffalo, are often subjected to adulteration by improper addition of cheaper cow milk, which decreases their quality [26]. However, milk of each species exhibits its own unique profile of casein variants and whey proteins that can be used as protein fingerprints for milk assessment. For example, the potential of LC–MS/MS as a reference method has been evaluated in processing of fresh cheese from cow milk or buffalo milk to monitor the replacement of raw milk with whey [27]. In [27], the tryptic peptide of β -LG, TPEVDDEALEK, was used as a marker of adulteration, and LC–MS/MS method was efficient to detect fresh cheeses adulterated with whey content above 10%. Another study [28] has aimed to analyze mare milk adulteration by cow milk using MS-based proteomics and metabolomics. The proteins and metabolites of cow milk in mare milk were detected at the levels as low as 1%. Identification of cow milk in concentration as low as 1% in goat milk samples by use of LC–MS/MS has been reported in another study [29]. In [29], bovine species-specific peptides of β -LG and α -casein (α -CN) were used as peptide markers for the evaluation

of goat milk authenticity. In the past, the authenticity assessment of dairy products has been reviewed in [30].

Related to the field of medicine, bioactive milk proteins/peptides have been shown to have many beneficial effects and possess an array of biological activities. They have antioxidant [31], antimicrobial/antiviral properties [16, 31, 32], and have been shown to have antihypertensive [16, 31], anticarcinogenic [33–35], antithrombotic [36], and immunomodulatory/anti-inflammatory [37–39] activities. The peptides from milk casein can ameliorate high-glucose-induced insulin resistance [40]. Milk bioactive peptides were associated with angiotensin converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) inhibitory activities [41, 42]. The therapeutic potential and attributes of milk proteins and milk-derived bioactive peptides have been discussed in [2, 3, 7, 33–35, 43].

In this review article, the proteins, protein-derived peptides, and EVs that are present in milk were briefly overviewed. Further, recently published advances in the separation, purification, and characterization of bioactive proteins and protein-derived peptides from milk and EVs were discussed, including their identification using MS-based proteomic approaches. The databases of milk proteins, bioactive peptides, and EV-derived proteins from milk and other sources, which have been reported up to now and can support the research on milk proteome and milk peptidome, were included.

2 | MILK PROTEINS/PEPTIDES

Milk is composed of water, solids, fat, proteins, lactose, minerals, and vitamins [44]. The bovine milk contains about 3.4% of proteins (30–35 g/L) which consists of soluble whey proteins (α -LA, β -LG, Igs, BSA, and LF), about 80% of caseins (genetic variants α_{S1} -, α_{S2} -, β -, κ -, and γ -CN), mostly in form of casein micelles, and around 1%–4% of MFGM proteins [14, 15, 45]. However, the composition of milk and protein content varies in species, among animal breeds as well as over lifetime (age, lactation, diet, environment, diseases, and so on) [1, 44–49]. In addition to genetic variants, milk proteins are present as splice variants and are posttranslationally modified by various PTMs (mostly phosphorylation and glycosylation) [17, 18], which are the regulators of protein functions, are related to the changes in protein activities, and can be associated to various pathologies.

Moreover, various enzymes and endogenous proteases are present in milk. For example, most predominant plasmin (secreted in milk as plasminogen) causes proteolysis of α_{S1} -CN, α_{S2} -CN, and β -casein (β -CN) into γ -CN [50], and it can have beneficial or detrimental results depending on the extent of hydrolysis [51]. Further, hydrolysis of

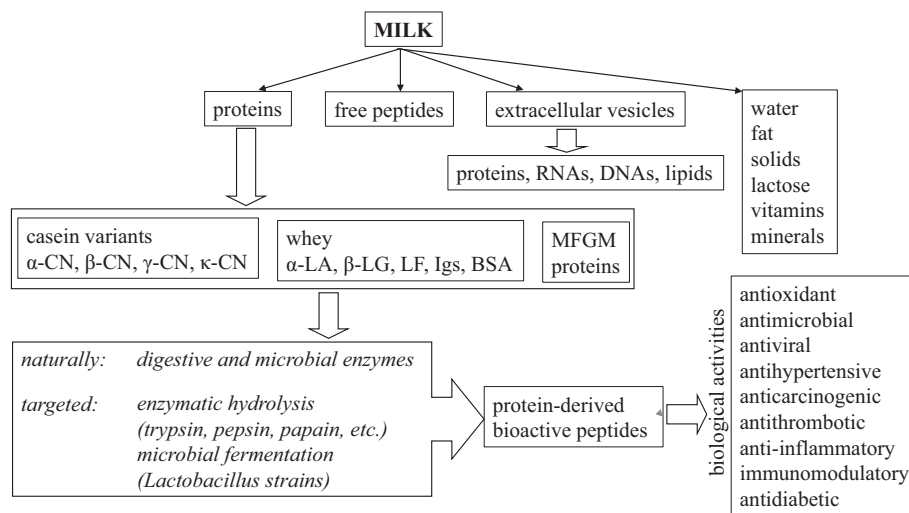


FIGURE 1 Schema of milk composition, production of milk protein-derived bioactive peptides, and the examples of their bioactivities.

milk proteins and release of bioactive peptides can occur via various processes, for example, naturally, by digestive enzymes and/or microbial enzymes, by enzymatic hydrolysis in laboratories [3, 52], and by fermentation of milk [32]. After protein hydrolysis, the bioactive peptides are isolated and validated for their activities. In food industry, bioactive peptides can be produced using hydrolysis at strong conditions, that is, heat and pressure treatments, or by hydrolysis at mild conditions, that is, enzymatic protein cleavage by pepsin, trypsin, or papain, by microbial fermentation (lactobacillus strains) and their combination [53].

For rapid identification of potential bioactive peptides, the analysis *in silico* (by means of computer simulation and modeling) has been exploited to evaluate and predict the possibilities for the release of bioactive peptides from milk proteins [54, 55]. The milk protein hydrolysates and milk protein-derived peptides have been shown to have various bioactive functions connected to health-enhancing properties and were components of diets preventing development of diseases [2–6]. Figure 1 shows schematically milk composition, production of milk protein-derived bioactive peptides, and the examples of their bioactivities.

3 | MILK EVS

Milk contains high amount of EVs [19, 56–59], which are the nanoparticles enclosed by phospholipids bilayers. EVs are naturally secreted by all cell types into extracellular space and in addition to milk, they can be found in cell cultures and various body fluids, such as plasma, saliva, spinal fluid, and urea. Various classifications of EV subtypes have been used based on their sizes, membrane components, biogenesis processes, cargo, and functions (e.g., exosomes, ectosomes, and apoptotic bodies). The exosomes belong to

small EVs derived from multivesicular bodies with diameters about 30 to 150 nm and density between 1.1 and 1.18 g/mL. They were first isolated from human milk in 2007 [60]. Although the exosomes have been mostly studied, other EV subsets were isolated from milk [61–63]. EVs contain and carry biologically active molecules, such as RNAs, DNAs, proteins, and lipids. EVs are essential for intercellular communication and signaling, modulate a variety of biological processes [19, 59, 61, 64, 65], and play crucial roles in immunity transfer and neonate development [60, 63]. Milk EVs can deliver RNA and protein cargo to recipient cells, which can be taken up by various cell types and affect their functions [61, 66]. Moreover, milk-derived EVs possess key properties which make them suitable for therapeutic applications. In addition to their abundant presence in milk and capacity to carry bioactive cargo, they exhibited no tumorigenesis in the body, had long half-life and good biocompatibility, caused low immune response, and were able to contact the cells via biomarkers at their surface [65]. It was demonstrated that milk EVs protect the cargo from enzymatic activity and degradation *in vitro* under conditions of simulated digestion in the gastrointestinal tract [67]. Thus, EVs can act as biocompatible carriers and transporting vehicles for the delivery of various contents for health prevention and during the treatment of various diseases [68]. The clinical applications and therapeutic potential of milk EVs including exosomes have been discussed in [65, 68–71].

4 | ISOLATION/PURIFICATION OF EVS FROM MILK

Protein content of milk EVs has been investigated using various methods including LC–MS/MS. However, prior to

analysis, the EVs of interest need to be effectively isolated from milk. The separation of EVs is challenging, and EVs were purified using various techniques, usually, by the series of centrifugation and ultracentrifugation steps. The major problem was a poor efficiency in the separation of various EV subtypes due to their similarities in morphology and chemistry. In addition, EVs co-precipitated with milk proteins/peptides, and thus, for example, the proteins/peptides which were not truly of exosomal origin were identified, as they co-purified with exosomes. As well, using ultracentrifugation, EVs can be trapped in non-resuspendable casein fraction which co-sediment with EV fraction, and thus, the EV yield can be affected and purity compromised. Even low-speed centrifugation for casein removal caused the loss of EVs [72] and acid precipitation prior to EVs isolation [73, 74] was often applied for this purpose because of casein low pI at 4.5 [75]. It was shown that acetic acid precipitation yielded higher counts of EVs with higher purity as compared to ultracentrifugation [74, 76] and acidification by acetic acid was more efficient than acidification by HCl as higher quantities of EVs were obtained [73].

Gel filtration, density gradient centrifugation, SEC, immunoaffinity techniques, and their combination are other methods applied for EVs purification. Several methods have been proposed recently for the isolation of EVs, for example, salting out method [77], in which bovine milk EVs were precipitated by ammonium sulfate with saturation solution between 30% and 40%. Isolated EVs showed spherical shapes with sizes of 60–150 nm. In another work, multiple EV subsets from bovine milk, including exosomes and membrane vesicles, have been isolated using sodium citrate in combination with differential centrifugation [78]. In [78], biocompatible 1% sodium citrate was able to disintegrate casein micelles (70–200 nm in sizes) into 40 nm monomers, allowing for efficient purification of high quantities of both non-exosome and exosome EV subsets by subsequent differential ultracentrifugation. In other study [79], ultracentrifugation was first applied followed by gel filtration that allowed to isolate purified exosomes from co-precipitating proteins and their complexes. The application of affinity chromatography, using Sepharose resin with antibodies against exosome surface protein markers CD9 and CD63, resulted in additional exosome purification [79]. Recently, two protocols have been reported [80] that used the incorporation of solubilization steps, optimized timing, temperature, and divalent cation chelation into either centrifugation workflow or tangential flow filtration workflow. Divalent cation chelation using 30 mM EDTA for 1 h at 37°C promoted solubilization of casein micelles and resulted in efficient isolation of EVs from casein aggregates. Both workflows yielded the large amounts of small EVs in ultradense concen-

tration and they were free of contamination from milk proteins and peptides. The aim of the next study was to compare the efficacies of several commercially available kits for the isolation of EVs from milk [76]. In [76], three different methods have been used first for casein removal (ultracentrifugation, acetic acid precipitation, and EDTA precipitation), followed by isolation of EVs from resulted whey fractions by commercial kits. The kits were based on different separation principles: ultracentrifugation, SEC (EVSecond L70 and qEV), membrane affinity isolation (exoEasy Maxi Kit), immune-affinity isolation (MagCapture Exosome Isolation Kit PS), and polymer-based isolation (ExoQuick-TC and Total Exosome Isolation Reagent). The best method for casein removal from defatted milk was acetic acid precipitation as compared to ultracentrifugation alone and EDTA precipitation (EDTA disturbs casein micelles in milk by attacking their Ca^{+} cores and disrupts protein–protein aggregates by changing electrostatic bonds [81]). Subsequent SEC (qEV column) and immuno-affinity isolation (MagCapture kit) exhibited the highest purity of isolated EVs (about 58×10^{11} and 65×10^{11} EV counts/mg of contaminating protein, respectively) with sizes of approximately 166 nm for both methods. Although other isolation methods exhibited the preparations with either high EV quantities or high EV purity or high concentration of RNA, SEC-based qEV column kit allowed to collect EVs of high purity and high quantity (about 11×10^{11} particles/mL of raw milk) with shortest time required for isolation (15 min) [76].

It has been reported that different isolation processes can affect the bioactivity of milk EVs and the proteins that they carry [72]. For example, the effect of acidification (HCl or acetic acid) for acceleration of casein removal during the isolation of pure EVs from bovine milk has been studied [73] in terms of EVs morphology, sizes, and concentration. Compared to ultracentrifugation used for casein removal (three-step process with 12 000 x g for 1 h, 35 000 x g for 1 h, and 75 000 x g for 3 h at 4°C), the concentration of EVs was significantly higher by acid treatment; however, with acid treatment, some of the EVs showed rough surfaces and EV-surface marker proteins were partially degraded (transmembrane 4 superfamily proteins (tetraspanins); CD9 and CD81) [73].

The EVs and exosome isolation/purification methods have been reviewed in Refs. [56, 57, 59, 82, 83].

5 | RECENT ADVANCES IN SEPARATION OF MILK BIOACTIVE PROTEINS/PEPTIDES

Various methods and their combination have been used for the separation/isolation of milk proteins and the

peptides derived from them either in laboratory setup or on industrial scale. Among them, membrane separation, solvent extraction, and centrifugation have been applied abundantly [84, 85] as well as various affinity enrichment techniques have been utilized [86–88]. As well, to simplify the complex protein mixture in milk, casein fraction is often separated from whey proteins, for example, by isoelectric precipitation of casein at pH 4.5–4.6 using various acids followed by centrifugation and filtration steps. Whey proteins remain soluble after CN precipitation [89].

Further, the separation of milk proteins/peptides has been based on PAGE [90] and on chromatographic separations [84], such as SEC [13], IEC [13, 91], and affinity chromatography [92]. However, analytical separation methods, such as capillary HPLC (especially RP-HPLC) and CE, are very efficient and sensitive for milk proteins/peptides purification, and various techniques have been developed over years [84]. For example, CZE has been used for milk protein quantitation [93], estimation of their phosphorylation and genetic polymorphism [94], quantitation of casein genetic variants [10], and analysis of genetic variants of milk proteins [45]. HPLC has been applied for whey protein separation in milk of different species [95], and RP-HPLC has been validated as a method for the separation of milk protein variants [96]. However, CE and HPLC are not able, in many cases, to identify and differentiate the protein variants and PTMs, and thus, they are combined with MS techniques [25, 97]. In next sections, the examples of advances applied for the separation of proteins and peptides from milk and EVs using capillary chromatography and capillary electrophoresis are overviewed including MS-based proteomic approaches that were published from year 2019 until now.

5.1 | Electrophoretic and chromatographic separation methods

5.1.1 | Milk proteins

One of the methods used for milk protein characterization at the intact level is capillary electrophoresis. Recently, novel approach has been used for separation of intact proteins in cow milk by capillary electrophoresis [98]. In the workflow, PDMS capillary (internally coated with neutral dimethyl polysiloxane) was used, and a combination of capillary dynamic coatings (using either CTAB or SDS) with BGE of different pHs was tested in order to reach efficient separation selectivity. The CTAB-PDMS capillaries worked better for BGE with low pH, and SDS-PDMS capillaries were best working for BGE with high pH. The separation selectivity was dependent on protein *pI* value.

Using CTAB-PDMS capillary and BGE with pH 3.2, β -CN (including isoforms β -CN A1 and β -CN A2), α -CN, BSA, and α -LA were separated with resolution at baseline level. However, for isoforms of β -LG (β -LG A and β -LG B) which have higher values of *pIs* (5.1 and 5.2), another BGE with pH 3.8 has to be used for their full resolution.

Another method has been used for the separation of whey proteins BSA, α -LA, and two isoforms β -LG A and β -LG B, in which electrophoresis and LC were combined [99]. Here, novel features were used, that is, (i) online connection of transient isotachopheresis/micellar electrokinetic chromatography with capillary IEF, (ii) fused silica capillary (100 μ m id and 360 μ m od; 500 mm length), in which a part of its inner surface was roughened using supercritical water, and (iii) dynamic adsorption of milk proteins into layer of PEG 4000 polymer formed on the capillary roughened surface. By using this approach, the milk proteins were separated and concentrated. The *pI* values for β -LG A and β -LG B variants were confirmed as 5.1 and 5.2, respectively, with detection limits as low as 1 pg/mL.

The accurate, efficient, and robust method has been reported for the detection and quantification of LF in bovine and human milk during lactation, using the technology of monolithic cation exchange HPLC [100]. Herein, due to the positively charged surface of LF at N-terminus domain (peptide lactoferricin), LF strongly bound to the chromatographic column and eluted as last peak during analysis. The presence of human LF in eluted fractions was confirmed by SDS-PAGE and identified by MS with concentrations in range from 2.03 to 5.79 mg/mL.

An open tubular nano-LC has been developed for sensitive separation of casein variants in milk with a novel column coating procedure [101]. It included fused silica capillary with 20 μ m id (20 cm length), in which the inner surface was coated with a stationary phase consisting of monolayer of methacryloyl graphene oxide nanoparticles (MGONPs) modified by poly-L-lysine (PLL). It was found that PLL modification was necessary to obtain sharp separated peaks of analytes, as compared to analysis performed in the MGONPs grafted open tubular column without PLL modification. The amino groups of PLL were positively charged during analysis which resulted in electrostatic interactions between CN variants and the column surface. Thus, the CN variants present in milk were separated as sharp peaks with elution order of κ -casein (κ -CN), α_{s2} -CN, α_{s1} -CN, and β -CN. In addition, α_{s1} -CN, a heavily phosphorylated variant (highly charged with eight phosphoserine groups) known to contribute to milk allergy [102], was separated as a sharp peak, which demonstrated, that this strategy can be suitable for both separation of phosphorylated CN isoforms and for allergy studies.

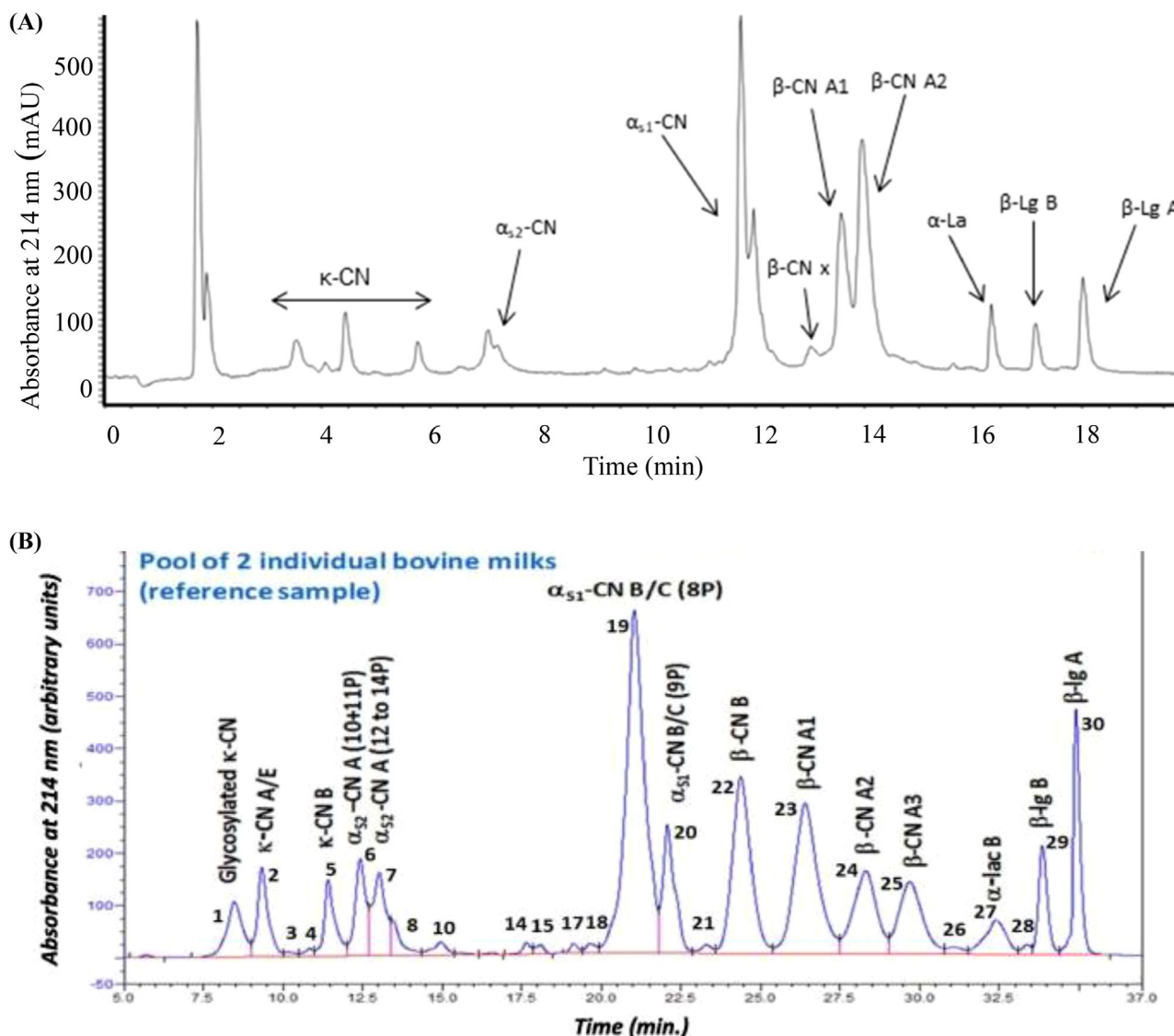


FIGURE 2 Separation of the proteins, their genetic variants, and PTM isoforms in milk samples by RP-HPLC and RP-HPLC-ESI-mass spectrometry (MS). (A) Chromatographic profile of rapid separation of the proteins and their variants in bovine milk (purchased from market) as obtained by RP-HPLC; (B) chromatographic profile of separated proteins, their variants, and PTM isoforms in a pool of two reference bovine milk samples with known genotypes as identified by RP-HPLC-ESI-MS; major peaks: 1—glycosylation isoform of κ -CN variant, 2—co-elution of κ -CN A and κ -CN E variants, 5— κ -CN B variant, 6—co-elution of phosphorylation isoforms of α_{s2} -CN A variant with 10 and 11 phosphate residues, 7—co-elution of multiple phosphorylation isoforms of α_{s2} -CN A variant with 12–14 phosphate residues, 19—co-elution of phosphorylation isoforms of α_{s1} -CN B and α_{s1} -CN C variants with 8 phosphate residues, 20—co-elution of phosphorylation isoforms of α_{s1} -CN B and α_{s1} -CN C variants with 9 phosphate residues, 22–25— β -CN B, β -CN A1, β -CN A2, and β -CN A3 variant, respectively, 27— α -LA B variant, 29 and 30— β -Lg B and β -Lg A variant, respectively. More description can be found in the text: *Source:* (A) adapted with permission from Ref. [11], copyright (2022) Elsevier; (B) adapted from Ref. [120], open access article under the Creative Commons Attribution (CC BY-NC-ND) license.

Further, rapid and reliable RP-HPLC method has been developed for the separation and quantitation of bovine milk proteins and their genetic variants [11]. The proteins were separated simultaneously in a single run within 20 min and they eluted in order of κ -CN, α -CN, β -CN, α -LA, and β -LG. In the chromatogram of Figure 2A, the peaks of κ -CN were observed which corresponded to different degrees of (de)glycosylated

κ -CN A and κ -CN B variants. The peak of α_{s2} -CN was followed by double peak of α_{s1} -CN, which was related to different phosphorylated groups of α_{s1} -CN. Further, β -CN variants A1 and A2 (heterozygous genetic variants) were preceded by the peak of additional β -CN variant which was not identified (β -CNx). Then, the peaks of separated α -LA, β -LG B, and β -LG A followed. Figure 2A shows chromatographic profile of separated proteins in

usual bovine milk purchased from market as obtained by RP-HPLC.

5.1.2 | Milk protein-derived peptides and hydrolysates

Usually, degree of hydrolysis of milk proteins is kept at low values of 3%–5% to maximize their functional properties and as well, to minimize hydrophobic peptides that cause bitter taste of final dairy products. The bitterness of protein hydrolysates is dependent on amino acid composition, molecular weight, hydrophobicity, and structure of resulting peptides [103]. In the past, the studies have been carried out to reduce bitter taste of casein hydrolysates, which are important nutrition additives, by applying various exo- and endopeptidases and processing methods [104]. For example, specific enzymatic techniques have been applied [105, 106], and exopeptidases have been used that cleaved the protein at the site adjacent to hydrophobic amino acids or cut off hydrophobic amino acids from protein terminus [107, 108]. Recently, the combination of two separation techniques has been used in 2D-LC arrangement [109], that is, SEC and RPLC for the characterization of bitter peptides in casein hydrolysates. Herein, 2D-LC method was applied to correlate both hydrophobicity and size of peptides to their bitterness and predict their sensory properties. Various endo- and exopeptidases and their combination were tested for hydrolysis of casein concentrate powder dissolved in water. The casein solution was kept at 55°C during 90 or 210 min to ensure that various degrees of hydrolysis and peptide sizes were achieved. The application of SEC followed by RPLC allowed for the determination of both peptide sizes and hydrophobicities in the sample using peptide standards with known properties. It was confirmed that the increasing bitterness of casein hydrolysate was related to higher concentration of peptides that possessed high hydrophobicities and molecular weights smaller than 6.5 kDa. This method demonstrated that based on sample elution pattern, the bitter taste of hydrolysate can be predicted [109].

By using RP-HPLC, the peptide fractions obtained from dried fermented cow milk products have been evaluated in terms of their different biological properties that resulted from various types of processing [110]. The processing of salted and unsalted cow milk by use of sun-drying or freeze-drying method showed the differences in peptides biological activities toward ACE and α -amylase. It was concluded that dried fermented milk products are the sources of bioactive peptides with various antioxidant capacities, ACE inhibitory activities, and anti- α -amylase functions and these functions can be adjusted by different salt content and drying conditions.

5.2 | MS-based proteomic approaches

5.2.1 | Milk proteins, protein-derived peptides, and hydrolysates

MS-based proteomics is an efficient tool for the characterization of biological samples. MS-based analysis of human milk can identify differentially expressed proteins, point out to protein dysregulation, and provide biomarkers for the early detection of disease [111]. It can provide detection of milk adulteration and efficient screening of protein allergens in milk [112]. For milk proteome and peptidome characterization, effective sample preparation techniques were developed as well as various proteomic techniques, such as 2DE, electrophoretic, and chromatographic techniques performed in capillaries and followed by MS identification [22–24, 113]. For example, the qualitative/quantitative efficacy of three protein extraction methods has been evaluated for allergenomic analysis of goat milk [114] using (i) urea/thiourea-based extraction; (ii) methanol/chloroform triphasic extraction, and (iii) sodium sulfite-based extraction. Based on 2DE maps and analysis by MALDI-TOF/TOF MS, the highest protein recovery, number of protein spots, and number of proteins yielded the method with urea/thiourea buffer extraction. Another study has evaluated peptide isolation/purification strategy for analysis of endogenous peptides that naturally occurred in donkey milk [115]. It included precipitation and removal of milk proteins either by cold acetone or acetic acid (pH 4.6) to enrich the fraction of endogenous peptides in resulted supernatants prior their identification by LC-MS/MS. These peptides have not been analyzed in typically applied proteomic workflows. In [115], the strategy considered the fact that endogenous peptides in milk did not effectively precipitate and thus, they were present in the supernatant, not in the pellet. In addition, even if the part of endogenous peptides precipitated, they could be lost during MS identification and database search since their cleavage specificities were different from the conditions usually used in proteomic experiments (e.g., cleavages after lysine and arginine in the case of trypsin digestion). It was confirmed that both precipitation protocols were efficient for endogenous peptide isolation and characterization. Moreover, they were complementary, as 758 peptides identified in supernatant samples were common to both protocols, and additional 346 and 226 peptides were unique to acetone purification and acetic acid purification, respectively [115]. In another example, the transient capillary isotachopheresis has been used, first for concentration of tryptic peptides from bovine β -LG A [116] and then followed by their separation by CZE using acidic BGE. Online coupling of CZE to TOF-MS via triple-tube coaxial sheath flow interface with ESI allowed to

obtain peptide mass fingerprint and identification [116]. As well, MS-based proteomics has been used for quantitative the characterization of differentially expressed MFGM proteins in bovine and donkey colostrum [49].

The milk protein profiling using MALDI TOF/TOF MS has been reported recently [117]. MALDI MS has been included regularly in proteomic strategies for food analysis, especially for the identification of milk and milk product authentication and adulteration. In [117], the matrix α -cyano-5-phenyl-2,4-pentadienyl acid, synthesized for MALDI TOF/TOF MS analysis proved to significantly increase the protein signals, enhance the spot homogeneity, and decrease the spot-to-spot variability as compared to conventional matrices. In [118], MALDI TOF MS has been used for screening of three characteristic peptides of casein in cow milk (m/z of 830, 1195, and 1759) to detect adulteration of goat milk. Herein, the adulteration was detected when the proportion of cow milk in goat milk appeared as low as 1%. The MALDI 2,5-dihydroxybenzoic acid matrix was more effective for detection of peptides of α -CN and β -CN (LODs about 0.1 mg/L) than α -cyano-4-hydroxycinnamic acid matrix [118].

As β -CN exists in A1 and A2 variants, cows with A1A1 gene will produce milk with only A1 variant (A1A1 milk), cows with A2A2 gene will produce milk with only A2 variant (A2A2 milk), and cows with A1A2 gene will produce milk that has both A1 and A2 variants (A1A2 milk). Recently, comparative label-free proteomic profiling of bovine milk containing various β -CN genetic variants (A1A1, A2A2, and A1A2 milk) has been performed using LC-MS/MS [8]. The data obtained by MS were confirmed and validated by multiple reaction monitoring (MRM) analysis for selected proteins. Differentially expressed proteins that associated with each of this particular β -casein variant present in milk have been identified: (i) in milk containing A1A1 variant, cathelicidin-2, and ceruloplasmin were the most abundant proteins; (ii) in milk containing variant A2A2, CD5 molecule-like protein, and LF showed the most abundance; and (iii) in milk containing heterozygote A1A2, osteopontin, selenoprotein P, and β -glucuronidase were mostly present. The study showed that the variability in milk protein composition was dependent on the presence of particular β -CN variant in milk.

In another work, the separation and identification of intact whey and casein proteins in bovine milk has been performed using CE coupled to MS in less than 15 min [89]. It was applied as an alternative method to routinely used CE with UV detection for bovine milk protein profiling which lacks molecular mass confirmation. The results obtained by CE-MS were compared to the results achieved by use of MALDI TOF/TOF MS. Although res-

olution and mass accuracy of applied MALDI TOF/TOF MS were not able to resolve β -CN variants, by application of CE-MS/MS hyphenation, the variants β -CN A1 and β -CN A2, which differ in one amino acid (His67 compared to Prp67; $\Delta M_r = 40$), were resolved. This is important for the identification of milk with specific β -CN content for people with health issues. In most widely available milk type A1A2, bioactive peptides produced from β CN-A1 and β -CN-B by digestive enzymes are responsible for milk allergy and intolerance, whereas milk type A2A2 contains only β -CN A2 which does not cause these issues. Thus, infallible and precise identification of β -CN variants in milk is of a high interest [89]. In addition, another study has been carried out to identify/quantify β CN A1 or β CN A2 in bovine milk [119]. In [119], automated online immobilized digestion system utilizing trypsin was applied (online digestion was completed in 4 min compared to classical in-solution digestion taking from 2 to 24 h), followed by separation and identification of tryptic peptides by LC-MS/MS (total 18.5 min for analysis process completion). Trypsin was used to fragment one peptide from β -CN A1 and one peptide from β -CN A2, both with amino acid sequence from 49 to 97 (His67 vs. Prp67 difference) and they were used as marker peptides. MRM analysis with three most intense transitions of the marker peptides was applied to determine the quantification limit of β CN A1 and β CN A2 as 0.8 and 2.4 μ g/g, respectively.

The study has been carried out that used reversed RP-HPLC-ESI-MS approach to qualify/quantify six intact milk proteins (four caseins, α -LA, and β -LG) with their genetic and splicing variants and their posttranslationally modified phosphorylation and glycosylation isoforms [120]. In addition, the method was able to identify the degradation products of milk proteins by endogenous milk protease plasmin, that is, γ -CNs and their complements. To be able to make automatic protein identification, the authors built a library of about 3000 of theoretical masses corresponding to six milk proteins, their genetic/splicing variants, PTMs, and plasmin proteolysis products from literature data and genomic/proteomic sequence databases. The method was developed and optimized for bovine milk; however, it could be adopted for all mammalian species with minimum optimization in RP-HPLC conditions and a creation of mass library for a specific species [120]. Figure 2B demonstrates the chromatographic profile with major peaks of bovine milk proteins, their genetic variants, and PTM isoforms separated and identified from a pool of two reference milk samples by RP-HPLC-ESI-MS. This research study was targeted on identification of PTMs of various milk protein variants, and thus, milk samples with specific content of known protein genotypes were used and analyzed. In addition to the major peaks that are marked, other milk protein variants and their glycosylation and

phosphorylation isoforms were identified and quantified as well [120].

The temporary changes in whey proteome of goat milk have been evaluated during lactation period from 1 to 240 days after delivery by quantitative proteomics [48]. The proteins were identified using LC-MS/MS, first in data independent acquisition (DIA) mode, and the results with data dependent acquisition (DDA) mode served as to verify the accuracy of the DIA results. As a result, significant variances in the expression of 238 proteins out of total 344 proteins were detected during the lactation period. For example, significant upregulation of lactoperoxidase, calgranulin-A, calgranulin-B, and polymeric immunoglobulin receptor was detected from day 1 to 240 as well as significant downregulation of vitamin D-binding protein, lipopolysaccharide-binding protein, IgG, and fibronectin. Interestingly, LF levels were downregulated from day 1 to 30 and then upregulated till day 240. Most importantly, the expression changes of most whey proteins obtained by DIA analysis corresponded to the results acquired by DDA analysis. Further, gene ontology analysis (GO; compendium that includes data about protein/gene known cellular compartments, molecular functions, and biological processes) of differentially expressed whey proteins during lactation period revealed that most common molecular function was protein binding, receptor binding, and molecular function regulation. Biological processes in which the differentially expressed whey proteins were involved were associated with immune function, complement and coagulation cascades, protease activity, and in transport.

The fast stop-flow 2DLC-MS in-house constructed instrumentation has been used for peptide separation, identification, and search for bioactive peptides in various food-derived protein hydrolysates including casein protein hydrolysates [121]. The combination of several types of chromatographic columns for coupling of SEC with RPLC was tested. Separation efficiency, sample injection volume, and transfer volume between SEC and RPLC were evaluated. The instrumentation was designed in such a way, that the fractions from SEC (1st dimension) were transferred interruptedly to RPLC (2nd dimension), hence, designation as stop-flow 2DLC; and with short stop-flow time, hence, indication as a fast stop-flow 2DLC. Both features were optimized to ensure low levels of band broadening. The peptides identified by MS were compared to the database of bioactive peptides BIOPEP-UWM [122]; however, only six peptides matched peptides of known bioactivities from this database, probably due to limited number of bioactive peptides included and insufficient tools available for short peptide identification. Nevertheless, the authors identified the large amounts of peptides in food-derived protein hydrolysates that possessed bioactive amino acid fragments, for example, the peptides including

the fragments that are known to have ACE inhibitory, DPP-IV inhibitory, stimulative, and antioxidative activities.

Peptidomic-based analyses have been carried out to search for the peptides with potential antimicrobial activities. The goat milk was fermented by *Lactobacillus rhamnosus* C25 (2%) at 37°C for 48 h [32] to generate the peptides from milk proteins. The peptides were subjected to ultrafiltration with the MWCO membranes of 3, 5, and 10 kDa, and each resulting peptide fraction was evaluated for microbial activities against gram-positive/negative bacteria. Both gram-positive and gram-negative strains were inhibited to certain levels by peptides in all three fractions; however, the peptides in MWCO < 5 kDa fraction exhibited the highest antimicrobial activity compared to the peptides in MWCO < 3 and <10 kDa fractions. The isolated peptide fractions were analyzed by RP-HPLC, and microbial peptides in the fraction with MWCO < 5 kDa were further identified by LC-MS/MS. More than 560 peptides were detected by LC-MS/MS, and *in silico* analysis (using computer modeling) of LC-MS/MS data predicted 36 peptides to be antimicrobial (with pIs in range from 4.8 to 11). Out of 36 peptides, 21 antimicrobial peptides were characterized as cationic (+1 to +3 charge; important feature for their interaction with bacteria negative charged membranes), and 6 antimicrobial peptides were predicted as positively charged and helical in shape. Thus, LC-MS/MS and the *in silico* approach in this study confirmed the ability of goat milk proteins to release bioactive peptides that also could be synthesized and act as potential antimicrobial agents [32].

The antioxidant and antimicrobial peptides in hydrolysates released from fermented bovine milk by lactic acid bacteria have been characterized using LC-MS/MS [123, 124]. Their potential bioactivity functions were searched against BIOPEP-UWM database [125, 126]. In [123], three *Lactobacillus* strains were investigated, and the antioxidant activities of hydrolysates were measured (based on the scavenging rates) by four different assays (DPPH, ABTS, hydroxyl free, and superoxide anion radical scavenging activity). The potential antioxidant activities of 43 bioactive peptides were detected in the hydrolysate of casein and whey proteins derived from fermentation of milk by *Lactobacillus reuteri* WQ-Y1. For example, bioactive peptides from α_{S1} -CN (YLGYLEQLLR), β -CN (VKEAMAPK), and κ -CN (YIPIQYVLSR) exhibited antioxidant functions. In [124], sheep milk was fermented with *Lactobacillus plantarum* (KGL3A) with incubation period optimized to 48 h for attainment of maximum peptide production with the highest proteolysis activities. The antioxidant activities of the peptides were determined by three radical scavenging assays, and their antimicrobial activities were detected against *Escherichia coli*, *Salmonella typhimurium*, *Enterococcus faecalis*, and

Bacillus cereus. Six bioactive peptides were identified and confirmed against BIOPEP-UWM database. These studies confirmed that bovine and sheep milk are the sources of antioxidant/antimicrobial peptides after their fermentation by various *Lactobacillus* strains [123, 124].

Other approaches have been used for screening the peptide profiles and peptide bioactivities in hydrolysates of milk protein concentrates generated by four different proteases treatment [52, 127, 128]. For example, in [52], one-step enzymatic hydrolysis of milk was used, and LC-MS/MS was applied for the identification of peptide composition. The peptide profiles differed among hydrolysates generated by different proteases as well as number of peptides identified (307, 260, 373, and 472 peptides). It was confirmed that small peptides (MW < 3 kDa) were the main components of the hydrolysates (termed as “extensive” hydrolysates), they were responsible for preventing/reducing oxidative stress and had specific biological activities, that is, enzyme inhibition and antioxidant activity.

In addition to the proteins, there are free low molecular weight peptides (LMWPs) present in milk. In [129], LMWPs in human milk have been extracted, isolated, and identified by using techniques based on cation-exchange SPE, and ultrahigh-performance LC (UPLC) coupled to QTOF MS [129]. Herein, the extraction method with hexane was applied first to precipitate the proteins from milk and remove fat. The subsequent ultrafiltration with 5 kDa cut off was applied for isolation of the LMWPs from the extract, and it was followed by cation-exchange SPE for additional LMWPs purification. By using UPLC-QTOF MS analysis, total 56 LMWPs were detected and quantified, predominantly from the β -CN amino acid regions 16–40, 85–110, and 205–226, with several peptide fragments from κ -CN.

Over last years, glycosylation of milk proteins (PTM resulted from attachment of oligosaccharides [glycans] to proteins) from different species has been profusely investigated, as well as the changes in protein glycosylation profiles over time [130–133]. According to the attached glycans, glycoprotein can be present in many different glycoforms. Most common is covalent attachment of *N*-linked glycans to the protein via its nitrogen in asparagine or arginine side chains in protein *N*-glycosylation, and attachment of *O*-linked glycans to the protein through oxygen of serine, threonine, or tyrosine side chains in protein *O*-glycosylation. For example, the proteins in colostrum possessed unique *N*-glycosylation profiles compared to mature milk and *N*-linked glycan composition affected their specific biological functionalities [132]. In addition, various *N*-glycan types and their heterogeneity in milk glycoproteins resulted in species-specific milk *N*-glycomes (a set of all *N*-linked glycans) [133–135].

Recently, *N*-glycoprofiling of IgG and LF from goat milk has been performed using LC-MS/MS approach [131], and the site-specific glycan diversity and individual glycosylated sites of both proteins were identified. In other study, the dynamic changes of LF glycosylation isolated from colostrum and mature milk have been reported during lactation cycle of cows from diverse genetic backgrounds [132]. Herein, the major alterations in *N*-glycan distribution were determined over the first 72 h, and the glycosylation profiles remained stable behind 1-month lactation after delivery. In another study, dynamic changes in human milk β -CN phosphorylation and *O*-glycosylation have been analyzed during lactation period (16 weeks) using LC-MS/MS [130]. In [130], the main task was to define PTM changes (phosphorylation and glycosylation) in the β -CN protein and β -CN endogenous peptides. For identification of β -CN proteome, in-solution digestion of milk was performed prior to LC-MS/MS analysis. For isolation of β -CN endogenous peptides, milk proteins were precipitated by 10% (v/v) TCA, supernatant was collected and analyzed by LC-MS/MS. In this study, *O*-glycosylation of β -CN at its C-terminus (threonine sites Thr207 and Thr214) was reported for the first time. In addition, the study detected gradual changes in phosphorylation and *O*-glycosylation of both β -CN and its endogenous peptides over lactation period. As well, β -CN endogenous peptidome contained more phosphopeptides and *O*-glycopeptides compared to β -CN proteome [130].

5.2.2 | Milk EV-derived proteins

The quality and quantity of proteome contents in both milk and EVs have been shown to depend on physiological and pathological conditions [39, 136–138]. In addition, the proteomic profiles of EVs differ between EVs derived from milk and other body fluids [139] as well as among EVs originated in milk of various species [20]. For example, the exosomes isolated from milk and plasma samples which have been collected from the same cow at the same time point showed differential protein profiles (LC-MS/MS) associated with different molecular functions and biological processes [139]. In another study, the proteomes of human and bovine exosomes characterized by LC-MS/MS have exhibited similarities as well as differences [20]. In [20], out of 229 proteins and 239 proteins identified in the human and milk exosomes, respectively, 53 proteins were common to both species. However, 176 and 186 proteins were unique to human and bovine exosomes, respectively. Among common proteins, lactadherin, perilipin-2, butyrophilin, and xanthine dehydrogenase/oxidase were present.

The proteomic and functional analysis of exosomes from porcine milk over lactation stages have been performed [138]. Comparison of the proteins in exosomes from colostrum to the proteins in exosomes of mature milk revealed different expression patterns. The differentially expressed proteins possessed the functions associated with the regulation of cellular processes, cellular development, and development of immune response. In another work, proteomic analysis has identified a large number of newly identified proteins in the small EVs from milk of late-stage lactating cows [21]. Herein, compared to previous reported proteomic data on bovine milk-derived small EVs, 429 proteins newly identified expanded the number of proteins already reported for small EVs during early- and mid-stage lactation.

LC-MS/MS has been applied for the identification and quantitation of the protein markers specific to EV subsets in bovine milk [61]. The proteins of EV subset that sedimented at 35 000g (35 K) during differential ultracentrifugation of milk were compared to the proteins of EV subset that pelleted at 100 000g (100 K). Out of 1838 proteins common to both EV subsets, 20 proteins were unique to 35 K pellet and 41 proteins specific to 100 K pellet. These unique proteins were suggested as markers for EV subsets identified in 35 and 100 K pellets, respectively. The prediction analysis of cellular origin (GO) of EV subset proteins determined that the exosomes were predominantly found in 100 K pellet, whereas cytoplasmic membrane-derived vesicles were in 35 K pellet. Further, reactome pathway analysis predicted that 35 K pellet proteins were related to cell migration, proliferation, survival, and regulation of translation, whereas the proteins present in 100 K pellet were associated with metabolic and extracellular matrix turnover and immunity modulation. Previously, protein markers have been accepted and proposed for different EV subsets from milk and biological fluids (light EVs [ADAM10, EHD4, SDCBP, and TSG101], dense EVs [FN1, C2, C6, and C7], large EVs [ACTN1, MVP, and EEF2]), and exosomes (tetraspanins, Rab family, and ESCRT complex proteins) as well as markers for EV biological activities (integrins ITGA1, ITGAM, ITGA6, ITFG1, and ITGB2) [60, 61]. Surprisingly, using these usually reported EV markers, the authors in [61] were not able to clearly distinguish between milk EV subsets that sedimented at 35 000g and 10 000g, as for example, 100 K milk pellet contained EVs similar to exosomes; however, the protein markers for exosomes, ESCRT, and Rab family, were identified in 35 K milk pellet as well.

The application of consecutive ultracentrifugation and gel filtration followed by affinity chromatography containing Sepharose resins with immobilized antibodies against EVs surface markers CD9 and CD63 [79] has resulted in the preparation of highly purified EVs that corresponded

to vesicles of exosomal nature (confirmed by transmission electron microscopy and flow cytometry analysis). The exosomal proteins/peptides were analyzed by MALDI-TOF-MS/MS, either using solutions of purified vesicles directly or after vesicle destruction by TFA. By direct analysis of vesicles, major proteins were detected, such as CD9, CD63, CD81, actin, LF, lactadherin, butyrophilin, and xanthine dehydrogenase in addition to various molecules within 2.5–8.5 kDa range. To identify those small molecules (<10 kDa) more specifically, the purified exosomal vesicles were destructed with TFA, and resulted extracts were subjected to filter centrifugation, consecutively with 10 and 3 kDa cut-offs. MALDI-TOF MS/MS analysis of resulting filtrates identified peptides with molecular masses of 2.0–7.6 and 0.9–4.4 kDa, respectively. As the peaks of these peptides were present in the MS spectra of direct MALDI-TOF-MS/MS analysis as well, the contribution of acid hydrolysis by TFA to peptide generation was excluded. As well, simultaneous treatment of exosomal vesicle extracts (<10 kDa) with chymotrypsin, trypsin, and proteinase K resulted in hydrolysates containing different peptides than the peptides detected in MS spectra of direct MALDI-TOF-MS/MS analysis. This study confirmed that exosomes contained the peptides with molecular masses in range from 0.8 to 8.5 kDa.

Characterization and distribution of protein glycosylation in bovine milk-derived exosomes and whey have been reported [140] using MS-based proteomics. The analysis identified 114 glycoproteins, most of them were found in exosomes. Further, 95 glycopeptides and 5 specific glycosites were confirmed in exosomes. It was concluded that bovine milk-derived exosomes have specific glycoproteome that can be distinguished from glycoproteome detected in bovine whey. The determination of site-specific protein glycosylation of bovine milk-derived exosomes is important for clarification of the role of glycosylation during, for example, exosome drug delivery.

MS-based proteomics has been used for profiling of differentially expressed proteins in small EVs from milk of cattle infected by bovine leukemia virus (BLV) and uninfected cattle [141]. Out of total number of identified proteins in milk EVs, 1212 proteins overlapped between both groups. However, 118 proteins were unique to BLV-infected cattle. Compared to EV proteins from milk of uninfected cattle, 26 proteins significantly differed in expression with over twofold up/down regulation. For example, ciliary neurotrophic factor receptor, histamin H1 receptor, and endothelial differentiation-related factor 1 were upregulated and cytochrome b reductase 1, semaphoring 7A, and integrin subunit alpha 6 (ITGA60 and myoferlin MYOF) were downregulated. These results confirmed that BLV infection altered encapsulated proteins in milk EVs and these proteins that newly

appeared can be used as identifiers for this pathology in cattle.

The reviews have been published about protein content of milk EVs [19] as well as other information about methodological approaches used in human small EVs proteomics [142].

6 | DATABASES OF PROTEINS, PEPTIDES, AND EV-DERIVED PROTEINS FROM MILK AND OTHER SOURCES

Bovine milk proteins databases BoMiProt (from 2020) [143] and upgraded BoMiProt 2.0 (from 2022) [144] are manually curated databases with over 10 642 proteins from whey, MFGM, and exosomes, including 1287 proteins with PTMs (<http://www.bomiprot.org>). Another protein atlas reporting of 4654 proteins from healthy cows found in 20 publications of milk proteomes has been compiled, in which the proteins are categorized based on milk fractions (skimmed milk, whey, MFGM, and exosomes), and according to five lactation stages [137].

The databases of bioactive peptides from various sources have been published over years. Although many of them did not target the peptides of milk in particular, they were valuable tools for prediction and identification of bioactive peptides originated from milk. They included milk bioactive peptide database (MBPDB; <http://mbpdb.nws.oregonstate.edu>) [125], BIOPEP-UWM (*in silico* database of bioactive peptides from food) [126], PeptideDB (bioactive peptides from food proteins collected from literature, public bioactive peptide databases and reference datasets [53], PepBank (the peptides identified during sequence text mining and from public peptide data sources) [145]), BioPepDB (an integrated data platform for bioactive peptides from food) [146], APD3 (antimicrobial peptide database from bacteria, archaea, protists, fungi, plants, and animals; initially online in 2003 (ADP); version from 2009 (ADP2) is continually updated and it expanded into ADP3; <https://aps.unmc.edu>) [147], and CAMP_{R4} (an updated CAMP database [148]; Collection of AntiMicrobial Peptides from various organisms; <http://camp.bicnirrh.res.in>) [149, 150].

The databases of proteins identified in exosomes and other EVs of various origins have been reported. A manually curated web-based compendium of exosomal cargo has been published in 2016, which contains 9769 proteins, 3408 mRNAs, 2838 miRNAs, and 1116 lipid entries from about 286 exosomal studies in multiple organisms (<http://exocarta.org>) [151]. Another web-based compendium of RNA, proteins, lipids, and metabolites that were identified in various EVs has been published as Vesiclepedia (<http://microvesicles.org>) and it contains 349 988 protein

entries, 27 646 mRNA entries, 10 520 miRNA entries, and 639 lipid entries for ectosomes or shedding microvesicles, exosomes, and apoptotic bodies [152].

The datasets/databases of milk proteins and bioactive peptides and EV-derived proteins from milk and other sources are summarized in Table 1 with additional details.

7 | CONCLUDING REMARKS AND CONSIDERATION ABOUT FUTURE DIRECTIONS

Bioactive milk proteins and milk protein-derived peptides attracted tremendous attention over the last years. The advances in separation methods allowed for their isolation from complex milk matrix enabled the identification of protein biologically important variants, isoforms, and PTMs and contributed to the characterization of their unique functionalities and bioactivities. The highly sensitive instrumentation applied in MS-based proteomic approaches and progress in biostatistical methods for accurate interpretation of the gathered data resulted in novel findings. Moreover, these experimental approaches can highly benefit from evolving *in silico* methods that conduct computer simulation and modeling for prediction of the potential milk proteins as the sources of specific bioactive peptides. Thus, combination of advanced experimental methods and *in silico* techniques is efficient tool for the future exploration.

Bioactive peptides are released from milk proteins during gastrointestinal digestion. However, tailored hydrolysis of the milk proteins can be induced by various enzymes and microbial fermentation to obtain the peptides with specific physiological functions and targeted therapeutic effects. These peptides consist of natural amino acids and when used in therapies, possible side effects of regular drugs are eliminated. The biological activities of milk protein hydrolysates and derived peptides are dependent on a degree of hydrolysis (a number of peptide bonds cleaved), class of enzyme, and other factors applied during hydrolysis (pH, temperature, time, and so on). Hence, the separation methods and MS-based proteomic approaches can be used for optimization of milk protein hydrolysis and the identification of resulted peptides.

Although many studies have been published dealing with the separation and identification of milk proteins and peptides, far fewer examples have been reported on usage of MS-based proteomics for the characterization of the protein content in milk EVs. As various types of EVs, including exosomes, have been identified as crucial for cell-to-cell signaling and communications, the identification of the

TABLE 1 The datasets/databases of milk proteins, bioactive peptides, and extracellular vesicle (EV)-derived proteins from milk and other sources.

Dataset/Database	Description		Ref.
BoMiProt 2.0	Manually curated database of bovine milk proteins from whey, MFGM, and exosomes that provides 10 642 proteins including 1287 proteins with PTMs. https://bomiprot.org	Milk proteins	[144]
Bovine milk protein dataset	The atlas of 4654 proteins identified in milk of healthy cows that was compiled from 20 publications; the proteins are categorized according to milk fractions and lactation stages	Milk proteins	[137]
MBPDB	Comprehensive database of milk bioactive peptides from mammalian sources that was compiled from literature search; bioactive peptides were mapped to the amino acid sequences of parent proteins to show the sites with highest presence of bioactive peptides. http://mbpdb.nws.oregonstate.edu	Milk bioactive peptides	[125]
BIOPEP-UWM	Database of bioactive peptides derived from food; the bioactive fragments are marked to protein sequences; database allows to find enzymes that can release particular peptides. It has the option allowing users to send new bioactive peptides for uploading into database	Bioactive peptides from food	[126]
PeptideDB	Web application of bioactive peptides from food proteins that was collected from published research articles, public bioactive peptide databases, and reference datasets. It includes tool for generation of possible peptides from input proteins using cleavage with various enzymes	Bioactive peptides from food	[53]
PepBank	Database of peptide sequences and associated biological data obtained by text mining of MEDLINE abstracts and by search of data from public sources (ASPD and UniProt). It enables prediction of binding partners of bioactive peptides, as well as their molecular targets or binding specificities	Peptides from various organisms	[145]
BioPepDB	A searchable database of food-derived bioactive peptides that includes more than 4000 entries; it can be used for prediction of novel peptides by simulated hydrolysis and as a reference database for evaluation of peptide functions. http://bis.zju.edu.cn/biopepdb/	Bioactive peptides from food	[146]
APD3	Antimicrobial peptide (AMP) data analysis system and database with data collected from literature over 20 years; it contains 3559 AMPs with 3282 AMPs from natural sources of 6 organisms (3067 with activity data), 187 predicted peptides from genomes, and 90 synthetic peptides (derivatives of natural AMPs). https://aps.unmc.edu	Bioactive peptides from various organisms	[147]
CAMP _{R4}	Collection of AntiMicrobial Peptides from various organisms; it provides curated information on synthetic and natural AMPs; it contains AMPs sequences, structures, family-specific peptide signatures, N and C terminal modifications, and it links to other databases. It has been created to accelerate AMP oriented studies. http://camp.bicnirrh.res.in	Bioactive peptides from various organisms	[149, 150]
ExoCarta	Manually curated web-based compendium of exosomal proteins, mRNAs, miRNAs, and lipids from 286 studies targeted on exosomes that were isolated from various tissue/cell types; it includes biological pathways and protein–protein interaction networks of exosomal proteins. http://exocarta.org	Proteins of exosomes	[151]
Vesiclepedia	Compendium of proteins, RNAs, lipids, and metabolites identified in all classes of EVs (exosomes, ectosomes, or shedding microvesicles and apoptotic bodies) isolated from 41 organisms; it was collated from literature and it includes experimental details; it provides top 100 proteins that were abundantly detected in EVs. http://microvesicles.org	Proteins of various EVs	[152]

Abbreviation: MFGM, fat globule membrane.

qualitative/quantitative changes in their proteome cargos carried to the recipient cells during pathological conditions should have a top priority in the future experiments. The obtained results will bring the knowledge how to manage the diagnosis, prevention, and treatments of various diseases.

Further, the identification of bioactive proteins/peptides using high tech separation instruments/techniques as well as recognition of their particular biological functions and activities are essential; however, they are only first steps in a broad picture. Major challenge is to use this knowledge and apply it for health improvement and therapy. The stability and biofunctionality of proteins/peptides can decrease in an *in vivo* environment. Thus, their encapsulation in certain types of nanoparticles appears as a good solution to enhance and preserve proteins/peptides stability, bioactivity, and delivery, as well as to increase the proportion of proteins/peptides that enter the circulation system in the body and have an active effect (bioavailability). This is a promising area and various nanoparticle delivery systems for therapeutic agents have been evaluated and approved for clinical application, such as polymeric nanoparticles and liposomes, as they increase agent circulation half-life, sustained release, and targeted deliveries [64]. However, these synthesized polymeric systems have to meet important requirements, such as biocompatibility, stability, reproducibility in preparation, permeability for efficient agent release, and the ability to load a variety of hydrophobic/hydrophilic compounds. On the other hand, exosomes and other small EVs from milk of various mammals are nature carriers which deliver bioactive proteins from donor to recipient cells and they possess the features needed to perform this task. They have abundant availability, noninvasiveness, resistance to stomach acid, and they are able to cross cellular membranes. Hence, various encapsulation methods for loading of drugs and therapeutic agents into exosomes have been tested and discussed [64]. Exosomes and liposomes are other nanocarriers have been reported as delivery systems for milk bioactive proteins/peptides as well [153, 154]. Moreover, the fluorophore probes can be covalently attached to milk exosomes [64, 155] to localize them, for example, during inflammatory processes [155] or to track a specific cargo delivery. It can be expected, that more studies will appear in the future, dealing with the delivery of specific bioactive milk proteins/peptides while fully preserving and retaining their biological functionalities, using this approach.

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CONFLICT OF INTEREST STATEMENT

The author has declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Miroslava Stastna  <https://orcid.org/0000-0003-1448-1901>

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