REVIEW

Dairy Milk Protein–Derived Bioactive Peptides: Avengers Against Metabolic Syndrome

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Abstract

Purpose of Review Metabolic syndrome is continuously increasing among the world's populations. Metabolic syndrome is a medical condition in which individuals suffer from high blood pressure, high blood glucose levels, and obesity. The in vitro and in vivo bioactivities of dairy milk protein–derived peptides (MPDP) have proven their potential as an excellent natural alternative to the current medical treatment for metabolic syndrome. In this context, the review discussed the major protein source of dairy milk and provides current knowledge on the novel and integrated approach to MPDP production. A detailed comprehensive discussion is provided on the current state of knowledge regarding the in vitro and in vivo bioactivities of MPDP against metabolic syndrome. In addition, the most important aspect of digestive stability, allergenicity, and further directions for MPDP application is provided.

Recent Findings The major proteins found in milk are casein and whey, while a minor portion of serum albumin and transferrin are reported. Upon gastrointestinal digestion or enzymatic hydrolysis, these proteins produce peptides with various biological activities including antioxidative, antiinflammatory, antihypertensive, antidiabetic, and antihypercholesterolemic, which could help in ameliorating metabolic syndrome.

Summary Bioactive MPDP has the potential to curtail metabolic syndrome and potentially act as a safe replacement for chemical drugs with fewer side effects.

Keywords Dairy milk · Protein · Peptide · Bioactivities · Metabolic syndrome · Health benefits

Abbreviations

AALI	Acute alcoholic liver-injured
ABTS•+	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin-converting enzyme
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase IV
GLP	Glucagon-like peptide
HUA	Hyperuricemia
IFN-γ	Interferon-gamma
IL	Interleukin
MDA	Malondialdehyde
MPDP	Milk protein–derived peptides
NF-κB	Nuclear factor-κB
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2–related factor 2
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SGD	Simulated gastric digestion
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α
XO	Xanthine oxidase

Statement of Significance This review summarizes our current state of knowledge regarding dairy MPDP and their bioactivities against metabolic syndrome. Literature suggests that various MPDPs and their bioactivities can be produced from milk proteins. These identified MPDPs have the potential to reduce metabolic syndrome symptomology via various enzymatic and biochemical pathways. Designing an effective peptide therapy is of prime importance for the application of MPDPs in metabolic syndrome.

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Introduction

Bioactive peptides have been gaining recognition on both nutraceutical and pharmacological fronts due to their high therapeutic potential and compatibility without adverse interactions with their drug counterparts. The bioactive peptides are characteristically short peptide chains of 2 to 20 amino acids having less than 6000 Da molar mass [1, 2]. They are obtained from the proteolytic clipping of specially sequenced amino acid chains catalyzed by endogenous and exogenous enzymes from plant, animal, and/or microbial sources during food processing [3]. In vitro, the desired peptides may be fractionated and tracked during complex proteolytic processes by using peptidome tools [4]. These peptides can be derived from both animal and plant proteins, whose functionality and potency are determined by C- and N-terminal amino acids, sequence, and affinity after proteolysis from their derivative proteins [1]. For their pharmacological and therapeutic application, the most potent peptide sequence is synthesized in a pure form and subjected to confirmation tests for their specific biological activities. These activities include anticancer, antioxidant, antiinflammatory, antimicrobial, antihypertensive, hypoglycemic, and immunoregulatory actions [1]. There are extensive ongoing investigations into the effects of bioactive peptides on metabolic syndrome, particularly because the modern sedentary lifestyle is a prime cause of metabolic syndrome, which increases the chance of developing cardiovascular disease fivefold. Therefore, bioactive peptides are rapidly gaining recognition for their diverse biological properties [5]. Grundy [6] explains that metabolic syndrome is a proinflammatory and prothrombotic condition characterized by hypertension, hyperglycemia, atherogenic dyslipidemia, and insulin resistance that can be treated by adopting a healthy lifestyle. Bioactive peptides play a vital role in modulating cholecystokinin (a hormone-stimulating pancreatic enzyme secretion) receptor expression, glucose uptake, insulin signaling regulation, adipocyte differentiation, and even mimicking the insulin hormone itself. Bioactive peptides have a potential future in modern medicine because of these functional bioactivities [7•].

Major proteins in milk are casein (α_{S1}- and α_{S2}-caseins, β-casein, and κ-casein) and whey (α-lactalbumin, β-lactoglobulin, lactoferrin, and glycol-macropeptide), while serum albumin and transferrin are minor proteins. Milk proteins subject to systematic hydrolysis form amino acid sequences having activities analogous to hormones [8] and are thus an excellent source of bioactive peptides. Milk proteins may either be enzymatically hydrolyzed or microbially fermented in in vitro conditions followed by the application of favorable separation and purification techniques. Various chromatography techniques are used for the fractionation, purification, and identification of the bioactive peptides in the hydrolysate [9••].

Bovine and buffalo milk are commercially abundant kinds of dairy milk, and their peptides obtained by hydrolysis have promising health benefits, including antioxidative, antiinflammation, antihypertensive, antidiabetic, antihypercholesterolemic, and anticarcinogenic [10, 11]. These peptides are often categorized as excellent natural substances that could be used as alternatives to current treatments for metabolic syndrome with fewer side effects. In vivo research on milk-derived peptides has been conducted for two decades, which has reported their potential effectiveness to treat metabolic syndrome by their effects on multiple body systems such as digestive, endocrine, immune, circulatory, and cardiovascular systems [11]. Thus, dairy MPDPs, solely or in combination, possess multifunctional properties that can be used to control or treat metabolic syndrome as medicinal alternatives.

Even though numerous studies have emphasized the production and identification of bioactive MPDPs, very few have evaluated their in vivo conditions. This has led to a huge gap in the demonstrated efficacy of in in vitro conditions and in vivo assessment and applications. This review provides a comprehensive overview of the potential effects of dairy (bovine and/or buffalo) milk–derived peptides on metabolic syndrome as well as their mechanisms of action. The search for scientific literature published since 2010 was conducted using Scopus, PubMed, and Google Scholar search engines. Relevant articles examining the effects of bovine and/or buffalo milk–derived peptides on antioxidants, hypertension, diabetes, inflammation, cardiovascular disease, and lipidemia are included in the review. The following sections review the composition of milk proteins; peptide production methods; the mechanism of action of bovine and/or buffalo peptides against various diseases associated with metabolic syndrome; peptide digestibility; and allergenicity.

Dairy Milk Proteins

The major milk proteins are casein micelle and whey, while there is a certain residual protein in the milk fat globule membrane. Milk proteins constitute around 4% of the total milk components, and casein and whey comprise 80% and 20% of total protein, respectively, where caseins are categorized into α-casein, β-casein, and κ-casein and whey into β-lactoglobulin and α-lactalbumin mainly [12] (Fig. 1). Proteins such as lactoferrin, fatty acid binding protein, lactoferrin, prolactin, and folate binding proteins are also found in milk and are considered minor proteins. Positive effects of casein and whey have been found in the digestive, nervous, hormonal, circulatory, immune, and cardiovascular systems, along with other body functions [13, 14]. Additionally, milk proteins form the basis for a wide range of peptides with outstanding functional and immunological activities [8, 15].

Fig. 1 Overall constituent of dairy milk protein and peptide with its potential health protective activity
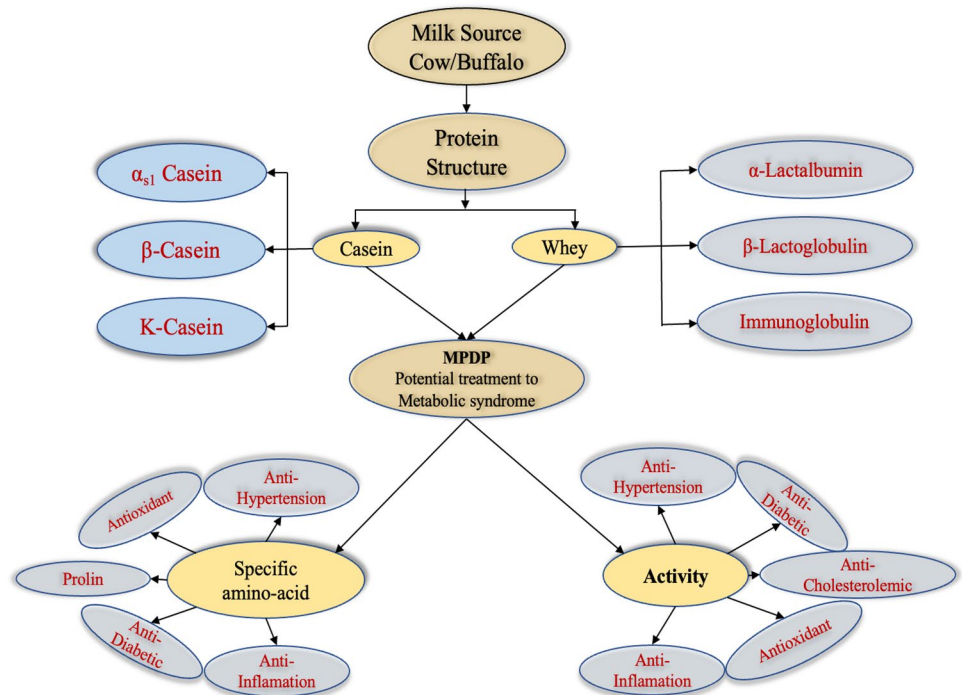
Casein

Casein protein contains all nine essential amino acids, with an unusually high leucine fraction. Cow and buffalo milks contain 108 mg/g and 90 mg/g of casein respectively [16]. Due to the presence of proline and cystine fractions, casein lacks the disulfide bridge with α-helix structures. The casein protein is also known as casein phosphoproteins because it contains 0.7–0.9% phosphorous, is hydrophobic, and has a strong affinity for calcium binding, which makes them water-insoluble [14]. The α_{s1}-casein, α_{s2}-casein, β-casein, γ-casein, and κ-casein fractions in the buffalo milk are 14.4–18, 2.2–2.8, 12.5–15.8, 1.6, and 4.3–5.4 g/kg, respectively. These casein fractions in bovine milk are 12–15, 3–4, 9–11, 1–2, and 3–4 g/kg, respectively. Compared to bovine milk, buffalo milk has a 40% higher concentration of α_{s1}-casein and a 35% higher concentration of β-casein fractions. In contrast, buffalo milk has lower fractions of α_{s2}-casein and κ-casein than bovine milk, at just 6.3 and 12% respectively. Because casein from both species has homogeneous phosphoserine clusters, the proteolysis of buffalo milk casein (i.e., α_{s1}, α_{s2}, and β) is similar to that of bovine milk casein [12]. NAVPITPTL, YQEPVLGPVR, YFYPQL, and VLPVPQK are some buffalo milk casein–derived peptides, and HLPGRG and QNVLPLH are derived from the casein of bovine milk either through hydrolysis or some other proteolysis process [17–21].

Whey Protein

Whey proteins are regarded as beneficial proteins from a nutritional standpoint because of their abundance in branched-chain amino acids and bioactive proteins such as α-lactalbumin, β-lactoglobulin, lactoferrin, immunoglobulins, and serum albumin [22]. Whey protein consumption has a wide range of beneficial effects on metabolic health, including the reduction of metabolic syndrome, hyperlipidemia, atherosclerosis, and hypertension [23, 24]. Proteomics analysis has revealed that buffalo whey has a potent hypotensive and immune-enhancing component, and is one of the main sources of immunoreactive components, indicative of good health-promoting functions [25]. The major whey proteins in milk are alpha-lactalbumin and beta-lactoglobulin. Concentrations of α-lactalbumin and β-lactoglobulin in buffalo milk are 1.4 and 3.9 g/kg, respectively, while those of cow milk are 2–4 and 1–5 g/kg [26]. Alpha-lactalbumin, beta-lactoglobulin, and lactoferrin contain 129, 162, and 690 amino acids, respectively, and alpha-lactalbumin has a comparatively high proportion of cysteine, lysine, tryptophan, and branched-chain amino acids [14]. IQKVAGTW and SVDGKEDLIW are some examples of buffalo milk whey–derived peptides; LDQWLCEKL, ELKDLKGY, and ILDKVGINY are derived from bovine whey [27–30]. Whey proteins can thus be used to produce bioactive peptides with various health benefits [11].

Novel and Integrated Approach for the Production of Milk Protein–Derived Peptide (MPDP)

Apart from conventional methods (enzymatic hydrolysis, fermentation, and in vitro digestion) mentioned in the supplementary file for producing bioactive peptides, novel technologies, such as high hydrostatic pressure, ultrasounds, microwave-assisted extractions, ohmic heating, pulsed electric fields, and subcritical water hydrolysis (Fig. 2), are being recently explored to achieve efficient proteolysis of the parent proteins without compromising their functionality and bioactivity [31]. These novel technologies, when coupled with microbial fermentation or enzymatic hydrolysis to produce bioactive peptides, can increase their yield bioactivity and reduce production cost and time as compared to conventional approaches [32, 33]. For instance, ultrasonic waves produce cavitation bubbles and generate tremendous energy due to oscillations that enhance the production of bioactive peptides during enzymatic hydrolysis. Peptides produced from milk proteins by pretreatment with ultrasonic waves (single frequency 28 kHz, power density 20 W/L) followed by enzymatic hydrolysis showed higher angiotensin I-converting enzyme (ACE) inhibition rate than non-ultrasound treated peptides [34].

High-pressure processing, on the other hand, is the most recent approach to cleaving peptides and assisting hydrolysis by proteolytic enzymes [35, 36••]. High hydrostatic pressure involves the application of isostatic pressures ranging from 100 to 1000 MPa with or without thermal treatment. Combined high pressure and heat cause protein denaturation which exposes hidden peptide sequences and increases the number of sites for proteolytic activity. High hydrostatic pressure–assisted enzymatic hydrolysis of β-lactoglobulin from cow milk increases the yield

of bioactive peptides with antioxidant and antiinflammatory properties [31]. Likewise, evidence-based research on the application of microwave pulses of electric fields and ohmic heating has improved the release of bioactive peptides by unfolding and denaturation of peptide sequences [37]. Furthermore, previous studies have suggested an increase in the degree of hydrolysis of whey protein isolate when hydrolyzed with subcritical water [38]. However, there is still a dearth of studies relating to the application of these novel technologies to extract peptides from cow and buffalo milk proteins.

In addition to the intervention of green technology for generating bioactive peptides, in silico approaches also play a crucial role in predicting the bioactivities and binding affinity and mechanism of the peptides released from a parent protein [9••]. In silico approaches are also useful for overcoming the hurdle of gastrointestinal transit by simulating the conditions, molecular docking, protein structure, protein–ligand interaction, and quantitative structure–activity relationship models. Such an approach provides some insight into the fate of these peptides during their transit through the gastrointestinal tract. Even though the in silico approach has enormous potential for understanding virtual protein and peptide phenomena in humans, in vitro and in vivo testing remain the only valid experimental approaches for peptide identification and understandings their biological activities [39].

Milk Protein–Derived Peptide Effects on Metabolic Syndrome

In the last decade, several functional peptides derived from cow and buffalo milk have been identified and investigated for both their in vitro and in vivo biological activities.

Fig. 2 The schematic diagram showing bioactive peptide production from cow and buffalo milk
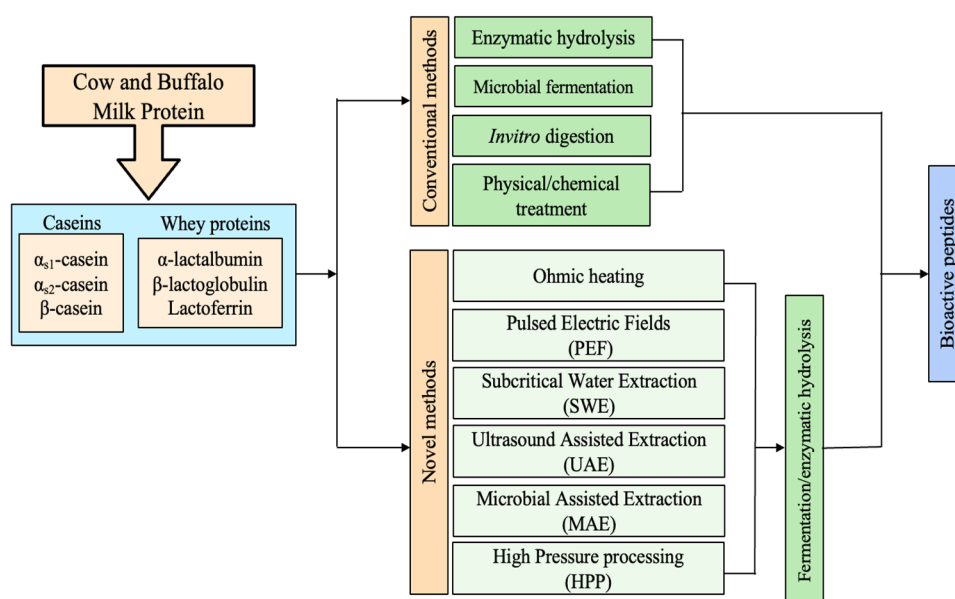
These peptides, produced during the processing of milk proteins, serve as a source of inhibition for several metabolic syndrome-related enzymes and pathways, as indicated in Tables 1 and 2.

Antioxidant Activity

In Vitro

Studies have shown that metabolic syndrome is associated with oxidative stress, a proinflammatory state, and intracellular redox imbalance as a result of an increase in reactive oxygen species (ROS) formation, which results in mitochondrial dysfunction, protein accumulation, lipid oxidation, and ROS-related impairment [40, 41]. Novel buffalo casein peptides (RELEE, TVA, MEDNKQ, EQL) and bovine peptides (PYPQ, YFYPE, EMPFPK, PQSV) were identified in milk samples hydrolyzed by the two enzymes, trypsin and alcalase. In vitro, peptides RELEE, TVA, and EQL have demonstrated ROS binding potential in 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) radical [42, 43]. Furthermore, the investigation of buffalo casein–derived peptides YQEPVLGPVR, YFPQL, and LLY displayed extraordinary free radical scavenging capacity [18–20]. According to Abdel-Hamid et al. [44], peptides with Tyr/His residues and Pro and/or Phe residues were found to have high antioxidant activity in papain hydrolyzed buffalo milk. The hydrolyzed buffalo milk fractions included a variety of peptides with antioxidant activity, some of which have been reported in previous studies, and some peptides (YPSG, KFQ, and HPFA) were anticipated to contribute to the antioxidant activity [44]. In addition, research by Srivastava et al. [45] outlined that fermented milk–derived peptides AGWNIPM and YLGYLEQLLR possess higher antioxidant activity with AGWNIPM possessing the highest ABTS$^{\bullet+}$ radical inhibition (73.45%) followed by YLGYLEQLLR (64.46%). Also, peptides YLGYLEQLLR, VKEAMAPK, and YIPIQYVLSR derived from the milk fermentation by different strains of lactobacillus (*L. brevis* CGMCC15954, *L. plantarum* A3, and *L. reuteri* WQ-Y1) displayed potent 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity [46].

Besides the in vitro antioxidant inhibitory assay, understanding the mechanism of a peptide in cell lines is crucial. The antioxidant activity in the cell medium is mediated by activation of the Keap$_1$-nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathway [18, 20, 47]. Peptides derived from milk proteins can shield Caco-2 cells from peroxide-induced oxidative stress (lipid peroxidation) by activating the Keap$_1$-Nrf2 pathway [47]. Antioxidant activities of bovine milk–derived tripeptide peptide (LLY) were explored in Caco-2 cells, which revealed peptide-induced antioxidative properties by inhibition of intracellular ROS production, mitigation of malondialdehyde (MDA), and protein carbonyls production; augmented catalase activity had little impact on glutathione peroxidase and superoxide dismutase (SOD). Also, relative expression of genes Nrf2 and Keap$_1$ and Nrf2 nuclear translocation by LLY peptide were allied with antioxidative signaling [19]. Evidence of transportation of milk casein–derived peptides YFYPQL and YQEPVLGPVR across the epithelial membrane was reported wherein their antioxidant activity was achieved by activating the Nrf-2 stress signaling pathway [18, 20]. Tonolo et al. [48••] showed that fermentation-derived milk peptides VPYPQR, ARHPHPHLSFM, and RHPHPHLSFM exhibited ABTS$^{\bullet+}$ and DPPH radical scavenging activity and an inhibition of TbOOH-stimulated lipid peroxidation in a cell line by the activation of the Keap$_1$/Nrf2 pathway. The peptide VLPVPQK (PEP), which was released from buffalo milk β-casein, increases cellular protection by the relative expression of a gene in the Nrf2 inhibitory pathway and reduces oxidative stress in fibroblast cells [49, 50]. Studies on the buffalo casein–derived peptide VLPVPQK also suggest that it can reduce lactate dehydrogenase (LDH) activity and intracellular ROS production. Even at low doses, VLPVPQK showed positive antioxidant effects, including a decrease in glutathione levels and changes in SOD and catalase activity brought on by H$_2$O$_2$ [49, 51]. Overall, recent studies have shown increasing implementation of cell line-based models for investigating the antioxidant properties of bioactive peptides.

In Vivo (Mouse/Human)

The antioxidant properties of milk-derived peptides are multifaceted and partake in multiple pathways, including damage cell recovery and oxidative enzyme modulation or inhibition. They can also play a role in chelating metal ions, controlling the production and removal of ROS by enzyme modulation glutathione peroxidase and SOD, and maintaining cellular integrity [52]. To have an effective antioxidant capacity, peptides should comprise amino acids such as tryptophan, tyrosine, histidine, and proline, and display a hydrophobic character. In addition, two hydrophobic amino acids, leucine or valine, influence peptide antioxidant and lipid peroxidation capacity. The sequence, configuration, structure, and molecular weight of amino acids also have an impact on these processes. In an in vivo study, tripeptide (LLY) unveiled remarkable antioxidative potential against ethanol-induced oxidative stressed mice by increasing glutathione and reducing the activities of MDA. In addition, tripeptide administration revealed reduced activity of glutathione peroxidase and catalase regardless of the dosage, but higher peptide doses (1 mg/kg BW/day) were only sufficient to lower the activity of SOD [19].

Table 1 Peptides from cow or buffalo milk's in vitro activities in the treatment of the metabolic syndrome and associated diseases

Species	Isolated and identified peptide	Techniques used for synthesis and isolation of MPDP	Assay methods	Biological activity	Reference
Bovine and buffalo milk	PYPQ, YFYPE, EMPFK, PQSV; RELEE$_{(f18-20\beta-CN)}$, TVA$_{(f163-165\kappa-CN)}$, MADNKQ$_{(f69-74\ \alpha s1-CN)}$, EQL	Enzymatic hydrolysis (trypsin, alcalase); Ultrafiltration (UF)/ RP-HPLC/ LC–MS/MS	Antioxidant	ABTS$^{\bullet+}$ radical scavenging	[42, 43]
Buffalo milk casein	YQEPVLGPVR YFPYQL LLY	-	Antioxidant/antiinflammatory	ORAC/ABTS$^{\bullet+}$/Caco-2 cells/ Swiss Albino mice	[18–20]
Buffalo milk	YPSG, HPFA, KFQ; FPGPIPK, IPPK, IVPN, QPPQ	Enzymatic hydrolysis: (papain, pepsin, or trypsin)	Antioxidant/antihypertensive	ACE inhibitory activity	[44]
Commercial milk	AGWNIPM, YLGYLEQLLR	Fermentation (3 different strains of *Lactobacillus*)	Antioxidant/immunomodulatory activity	ABTS$^{\bullet+}$ radical inhibition, modulation of the pro- and antiinflammatory cytokines and NO release	[45]
Commercial milk (bovine milk)	YLGYLEQLLR (α_{S1}-casein), VKEAMAPK (β-casein), YIPIQY-VLSR (κ-casein)	Fermentation (3 different strains of *Lactobacillus*) LC–MS/MS	Antioxidant	DPPH/ABTS$^{\bullet+}$ radical scavenging	[46]
Bovine milk	VPYPQR, ARHPHPHLSFM, RHPHPHLSFM	Fermentation milk (four synthetic peptides)	Antioxidant	ABTS$^{\bullet+}$/DPPH radical scavenging; lipid peroxidation inhibition; activated Keap1-Nrf2 signaling pathway	[47, 48••]
Buffalo/bovine milk β-casein	VLPVPQK (PEP)	-	Antioxidant; antiinflammatory; antihypertensive	ABTS$^{\bullet+}$ radical scavenging; Nrf2 inhibitor (Keap1); reduced LDH activity, lipid peroxidation and intracellular ROS production, oxidative stress in fibroblast cells; ACE inhibitory activity	[50, 51, 59, 63]
Casein	YQLD, FSDIPNPIGSEN, FSDIPN-PIGSE, YFYP	Hydrolysis by two microbial proteases, protein SD-NY10 and protease A "Amano" 2SD	Antioxidant	Exhibited different antioxidant activity by activating the Keap1-Nrf2 signaling pathway in oxidative damaged HepG2 cell model	[114]
Bovine/buffalo milk	IPP VPP	-	Antioxidant; antiinflammatory; antidiabetic; antihypertensive	Inactivate inflammatory signaling pathway; DPP-IV inhibition	[56–58, 72, 86]
Buffalo milk casein	YQEPVLGPVR	-	Antiinflammatory	Alteration in cytokines and macrophages	[18]
Bovine milk casein	MKP	-	Antihypertensive	ACE-inhibitory activity	[65]
Bovine milk casein hydrolysate	EKVNELSK$_{\alpha s1-casein}$, NMAINPSKENLCSTFCK$_{\alpha s2-casein}$	—	Antihypertensive	ACE-inhibition	[67, 68]
β- and α$_{s1}$-casein	YPFPGPIPN, HLPLP, AYFYPEL	Human jejunal digests and SGD	Antihypertensive	ACE-inhibition	[69]
Bovine milk whey	EVLNENLLRF	Fermentation (*Pediococcus acidilactici* SDL1414)	Antihypertensive	ACE inhibition	[73]

Table 1 (continued)

Species	Isolated and identified peptide	Techniques used for synthesis and isolation of MPDP	Assay methods	Biological activity	Reference
Bovine milk	Lys-Ala-Ala-Leu-Ser-Gly-Met; Lys-Pro-Ala-Gly-Asp-Phe; Lys-Lys-Ala-Ala-Met-Ala-Met; Leu-Asp-His-Val-Pro-Gly-Gly-Ala-Arg	Fermentation (*Lactobacillus* strains)	Antihypertensive	ACE inhibition	[74]
Bovine whey α-lactalbumin	LDQWLCEKL$_{f(115-123)}$	Enzymatic hydrolysis (trypsin)	Antidiabetic	DPP-IV inhibition	[29]
Bovine α-lactalbumin hydrolysates	ELKDLKGY, ILDKVGINY	Enzymatic hydrolysis (Alcalase)	Antidiabetic	DPP-IV inhibitory activity	[27]
β-Lactoglobulin	LKPTPEGDL; LKPTPEGDLEIL	Enzymatic hydrolysis (Pepsin)	Antidiabetic	DPP-IV inhibition	[84]
Commercial milk (αS1, αS2-CN)	-	Enzymatic hydrolysis (*Dregea sinensis* protease)	Antidiabetic	α-glucosidase inhibition	[83]
Bovine casein	HLPGRG, QNVLPLH, PLMLP, MFE; GPAHCLL, ACGP	Enzymatic hydrolysis (alcalase and pronase E)	Antidiabetic	Inhibition of DPP-IV; α-glucosidase and α-amylase	[21]
Commercial milk	YPSYGL, HPHPHLSFMAIPP, SLPQNIPPL	Fermentation (two strains of *Lactococcus*)	Antihypertensive/antidiabetic	Dual function (ACE and DPP-IV) inhibition	[88]

In a recent study, after 6 weeks of oral administration of the peptide SVDGKEDLIW derived from buffalo milk lactoferrin, there was a significant improvement in the activity of antioxidant enzymes SOD and GSH-P$_X$ in several organs and systems, including the liver, heart, brain, and blood. Additionally, it has also been reported that the levels of MDA in blood and heart tissue were also lowered by the peptide [30]. In another study, a similar observation was reported wherein acute alcoholic liver-injured (AALI) mice were treated with bovine milk–derived peptide PGPIPN via oral gavage to evaluate the role of dairy peptides in preventing and reducing AALI [53]. The study suggested that PGPIPN reduced alcoholic hepatocyte damage and oxidative stress in mouse liver tissues, including a lowering of MDA levels and increased GSH-Px and SOD activity [53]. An investigation of peptide VLPVPQK from buffalo milk casein administration in the mice model was carried out by Mada et al. [51], which indicated a suppression of excessive body weight gain and lipid peroxidation, and an enhanced antioxidative status. The in vivo studies investigating the antioxidative properties of dairy protein–derived peptides are limited, hence more emphasis on carrying out confirmative studies on the biological activities of peptides in the in vivo model should be carried out.

Antiinflammatory

In Vitro

Numerous aspects of metabolic syndrome–related oxidative stress and inflammation have been identified, including circulating inflammatory biomarkers like fibrinogen, C-reactive protein, serum amyloid A, macrophage/monocyte, neutrophil, cytokines, immune cells, and adipose tissue abnormalities [54]. Obesity-induced inflammation is characterized by an elevation of cytokines and dysregulation of adipokines like interleukin (IL)-6, IL-8, IL-1β, interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α), and transforming growth factor (TGF-β) [55]. Antihypertensive peptides such as VPP and IPP derived from bovine milk have varying inhibitory effects on inflammatory pathways and potent impacts on the migration, proliferation, and mitigation of inflammatory factors in vascular smooth muscle cells [56, 57]. To elucidate, mitogen-activated protein kinases and tyrosine kinases are the main intracellular kinases that are activated when the AT$_1$R is activated by Ang II, which results in excessive proliferation, an inflammatory response, and oxidative stress in vascular smooth muscle cells. When IPP and VPP were evaluated for their effects on AT$_1$R expression in vascular smooth muscle cells, the results showed no consistent effect on AT$_1$R protein levels, indicating that the protective effect was exerted downstream of AT$_1$R. Tripeptide VPP reduced

Table 2 In vivo/ex vivo bioactivities of a peptide derived from bovine/buffalo milk in the treatment of metabolic syndrome and related diseases

Species	Peptide	Methods and study models	Biological activity	References
Bovine milk	LLY	Antioxidant and antiinflammatory in mice-model	Reduce the activities of antioxidative enzymes; cytokines modulation	[19]
Buffalo milk lactoferrin	SVDGKEDLIW	Mice-model study	Improve antioxidant enzymes (SOD and GSH-P$_X$) activity	[30]
Dairy peptide	PGPIPN	Antioxidant/antiinflammatory/antihypercholesteremic in AALI mice-model	Lower MDA, improve GSH-Px and SOD activity; antiinflammatory markers	[53]
Buffalo milk casein	VLPVPQK	Mice-model	Suppress weight gain and lipid peroxidation; enhance antioxidation	[51]
-	VLPVPQKAV, RYPSYGLN	Lipopolysaccharide (LPS)-stimulated rodents	Pro- and antiinflammatory markers alteration	[61]
Buffalo milk casein	NAVPITPTL	Antiinflammatory activity in ovariectomized (OVX) rats	Cytokines modulation	[62]
Buffalo casein	YFYPQL	Antiinflammatory activity in cultured mice splenocytes	Pro- and antiinflammatory markers modulation	[20]
Commercial Milk	KFWGK	Antihypertensive in spontaneously hypertensive rats (SHRs)	CCK-dependent vasorelaxation	[75]
Bovine casein	YQKFPQYLQY (YQK)	Antihypertensive in Wistar rats and SHRs	Lowering SBP	[76]
Bovine casein	MKP	Antihypertensive in spontaneously hypertensive rats (SHRs)	Lowering SBP	[65]
Bovine casein	[90]RYLGY[94] [143]AYFYPEL[149]	Antihypertensive in spontaneously hypertensive rats (SHRs)	Lowering SBP	[77]
Bovine/buffalo milk	IPP; VPP	Antihypertensive in spontaneously hypertensive rats (SHRs)	Decreased CASNA following mean arterial pressure reduction	[78]
Bovine/buffalo milk	IPP; VPP	Antihypertensive in human subject	BP-lowering effects of lacto-tripeptide	[79]
Bovine milk	LIVTQTMKG	Antidiabetes in mice-model	Protective effect on β cells of alloxan-induced type-1 diabetic mice	[91]
Cow milk beta casein	VPYPQ (f 193–197)	Antidiabetes in mice	Reduced postprandial blood glucose levels in a dose-dependent manner	[86]
Milk whey	ALPM, LWM	Antihyperuricemia in Sprague–Dawley rats	xanthine oxidase inhibition	[96]

the activation of inflammatory signaling kinases such as NF-κB pathways, which can trigger inflammation and cell proliferation [58]. Furthermore, possible antiinflammatory effects were observed through cytokine (IL-10, IL-1β, and IL-8) and nitric oxide (NO) production, indicating that they may cause proinflammatory activity through IL-8 or IL-6 production only if an inflammatory stimulus was already present [56, 57].

In another study, the effects of VLPVPQK derived from buffalo casein on inflammation were examined, wherein immune cells, including monocytes, mast cells, and leucocytes, were observed at the site of peptide administration. Additionally, the proinflammatory mediator TNF-α, produced by fibroblasts and endothelial cells, was lowered on treatment with various concentrations of the peptide VLPVPQK [49, 59]. Moreover, decapeptide

YQEPVLGPVR from buffalo casein inhibited the growth of murine splenocytes, decreased levels of proinflammatory cytokines (Interferon-γ), while increasing antiinflammatory cytokines TGF-β and Interleukin-10, and increasing macrophage phagocytosis [18].

In Vivo/Ex Vivo

Currently, the exploration of the antiinflammation effects of peptides is being extensively studied in animal model systems. The amino acid type in the amino-terminus and carboxyl-terminus of peptides plays a pivotal role in cytokine regulation caused by the peptides. This finding demonstrates that the dominant molecule recognized by receptors on lymphocytes and macrophages is the amino acid from the R group in each terminus of peptides. Research

on immunomodulating effects of hexapeptide PGPIPN on acute alcoholic liver injured (AALI) mice demonstrated prevention and reduction of AALI, mitigation of alcoholic hepatocyte damage, and a dose-dependent alleviation of hepatocyte oxidative stress and endoplasmic reticulum stress by regulating IL-1β, TNF- α, and IL-6 expression [53, 60]. Furthermore, the elevation of serum alanine transaminase and aspartate aminotransferase levels reduced inflammation and hepatocyte damage [53, 60]. Research on the administration of peptides derived from milk fermented by a specific strain of *L. fermentum* on lipopolysaccharide (LPS)-stimulated mice demonstrated that peptides VLPVPQKAV and RYPSYGLN have a possible antiinflammation effect after 3 weeks of treatment. At the same time, the formation of proinflammatory cytokines IL-6 and TNF-α decreased, while production of the antiinflammatory cytokine IL-10 increased [61] upon treatment with these peptides.

Recent research has investigated changes in proinflammatory and antiinflammatory factors upon oral ingestion of peptide NAVPITPTL derived from buffalo milk casein in ovariectomized (OVX) rats. Interestingly, after administration of NAVPITPTL at 100 μg/kg for 8 weeks, there was a significant increase in serum TGF-β levels and a decrease in IL-6 and TNF-α levels [62]. Likewise, when hexapeptide YFYPQL derived from buffalo casein was incubated in cultured mice splenocytes (ex vivo), production of the proinflammatory cytokine IFN-γ, which is a crucial component of the metabolic inflammation circuit, was reduced, and an inhibition of proliferation and phagocytosis of peritoneal macrophages was observed. Moreover, activation of the antioxidative enzyme and an increment in antiinflammatory cytokine-IL-10 was detected, thus a combination of antioxidant and antiinflammatory properties was noted [20]. Tripeptide LLY has also been shown to have an antiinflammatory response in an ex vivo environment by modulating several cytokines (IL-10, IFN-γ, and TGF-β) and associated pathways with improvement in peritoneal macrophage phagocytosis [19]. Likewise, when treated with aged rat skin fibroblasts, the bovine milk β-casein–derived peptide VLPVPQK increased cell migration by decreasing IL-6 and TNF-α, nuclear transmigration of the dormant Nrf2, and cell proliferation. The peptide reverses the growth arrest in aged fibroblast cells by lessening the activities of caspase-9 and -3, retaining nuclear integrity, and downregulating NF-κB/p38MAP kinase signaling by means of decreasing phosphorylated p38MAP kinases in the cytoplasm and by Nrf2 activation [63, 64].

Antihypertensive Activity

In Vitro

In both in vivo and in vitro model systems, the mechanisms responsible for hypertension rely on blood pressure–lowering

effects via renin inhibition, nitric oxide (NO)-mediated vasodilation, ACE inhibition, and increased antioxidant response. The different MPDPs are shown to inhibit ACE through various inhibitory mechanisms such as competitive, noncompetitive, or a mixed-type of enzyme inhibition. Abdel-Hamid [44] discovered three novel peptides with active ACE inhibition activity (FPGPIPK$_{β-CN}$, QPPQ$_{β-CN}$, IVPN$_{αS1-CN}$, and IPPK$_{κ-CN}$) derived from hydrolyzed buffalo milk protein, where each has a terminal hydrophobic amino acid and IC$_{50}$ values ranging from 9 to 49 μgmL^{-1}. Other ACE inhibitory bioactive peptides (YPVEPFT, GPFPIIV, YPF-PGPIPK, YPFPGPIPN, LPVPQ, and DMPIQ) reported by Abdel-Hamid et al. [44] were also identified from bovine milk protein. Likewise, peptide Met-Lys-Pro (MKP), as a fraction of AMKPW, was isolated from bovine milk casein and reported to be a potent ACE inhibitor (IC50: 0.43 μM) [65]. The mechanism behind antihypertension and ACE inhibition can be understood to be due to the functional ability of the renin–angiotensin–aldosterone system (RAAS). Conversion of angiotensinogen by renin to form angiotensin I (Ang I) activates the RAAS system, which then proceeds to the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) by ACE (EC 3.4.15.1). Vasoconstriction is caused by Ang II binding to angiotensin II receptor type 1 in smooth muscle cells of blood vessels [66]. Thus, inhibition of ACE is the plausible therapeutic target for hypertension. Peptides EKVNELSK and NMAINPSKENLCSTFCK were two novel ACE inhibitor peptides derived from bovine casein hydrolysate (α$_{s1}$-casein and α$_{s2}$-casein, respectively), with IC$_{50}$ values of 5.998 mM and 129.07 μM, respectively [67, 68].

In addition, antihypertensive peptides derived from β- and α$_{s1}$-casein (YPFPGPIPN, HLPLP, and AYFYPEL) were found to be present in human jejunal digests after oral intake and upon simulated gastric digestion (SGD) of dairy casein and whey milk protein powders [69]. Exploration of partially hydrolyzed whey protein yielded the four potential ACE inhibitory peptides, PQVSTPTL, MPGP, PMHIR, and PPLT, with no allergenicity or toxicity and IC$_{50}$ values of 86, 179, 90, and 168 μM, respectively [70]. Simulated gastrointestinal digestion of cow and buffalo milk protein subsequently resulted in the release of an ACE inhibition peptide. The novel peptide VLPVPQK obtained from simulated gastrointestinal digestion of buffalo milk casein had the highest ACE inhibitory activity and the strongest bond with hACE [71]. IPP (Ile-Pro-Pro) and VPP (Val-Pro-Pro), on the other hand, isolated solely or in conjugation with other amino acids, exhibited antihypertensive effects via RAAS modulation, upregulated endothelial nitric oxide, proinflammatory cytokine expression, and monocyte activity, and/or reduced oxidative stress in vascular smooth muscle and endothelial dysfunction. Furthermore, VPP has been shown to suppress Ang II–induced cell proliferation, oxidative stress, and inflammation [56, 58, 72].

Besides enzymatic hydrolysis–derived milk peptides, numerous studies have been conducted on the antihypertensive peptides formed by proteolytic cleavage during the fermentation of milk. Microbe-specific proteolytic cleavage is common during fermentation, where hydrophobic peptides show higher ACE inhibitory activity. For instance, EVL-NENLLRF, a previously known ACE inhibitor, was present in bovine milk whey fermented by *Pediococcus acidilactici* SDL1414, which demonstrated strong ACE inhibitory activity (IC$_{50}$: 19.78 μg/ml) [73]. Similarly, *Lactobacillus helveticus* KLDS.31 and *Lactobacillus casei* KLDS.105 fermentation of bovine milk resulted in the release of four ACE inhibitory peptides (Lys-Ala-Ala-Leu-Ser-Gly-Met, Lys-Pro-Ala-Gly-Asp-Phe, Lys-Lys-Ala-Ala-Met-Ala-Met, and Leu-Asp-His-Val-Pro-Gly-Gly-Ala-Arg) whose IC$_{50}$ value ranged from 77.45 to 201 μM [74].

In Vivo

Several animal studies have been conducted to investigate potential metabolic modifications after ingestion of peptides (Table 2), but it is crucial to note that these studies do not replicate the anticipated benefit of oral administration of peptides and bio-active compounds in humans. The mechanisms governing physiology may vary depending on the species, e.g., human or mouse. Nevertheless, before human consumption investigations begin, animal studies of peptides are necessary to understand their potential mechanisms, bioactive modifications, speculated potency, bioavailability, and toxicity. In vivo experiments studied over the past decades provide invaluable information for the human application of bovine or buffalo milk–derived peptides as an antihypertensive agent. The milk-derived pentapeptide KFWGK, for instance, demonstrated a potent and long-lasting antihypertensive effect via cholecystokinin (CCK)-dependent vasorelaxation. KFWGK also decreased blood pressure with a minimum effective dose of 5 g/kg when administered orally to spontaneously hypertensive rats (SHR) with advanced hypertension [75]. An interesting study on Wistar rats and SHRs assessed the effects of single and repeated doses of oral administration of the antihypertensive peptide-YQKFPQYLQY (YQK), obtained by pepsin and trypsin hydrolyzed bovine casein. Intriguingly, 11-week-old male SHRs, weighing 260 g on average, who had received the three different doses of 1, 3, or 9 mg/kg body weight of the peptide YQK (IC$_{50}$: 11.1 M) displayed a significant reduction in systolic blood pressure (SBP) after 5 h of administration of all three doses. The maximum reduction in the single dose of 1, 3, or 9 mg YQK/kg body weight was 17.5, 26.7, and 40 mmHg, respectively. In a repeated dose experiment, SBP declined considerably following the first dose, which after 4 h dropped by a maximum of 36.8 mmHg. When the second dose was administered after

4 h of the first dose, SBP remained stable. At 8 h following the initial YQK dose, SBP increased once more, and SBP changed by 18.2 mmHg at 12 h, yet still lower than it had been before the first oral administration [76], thus demonstrating an effective antihypertensive effect.

To explain the antihypertensive action of bovine casein–derived peptide in vivo, peptide MKP was orally administered in single and repeated doses to SHRs. Upon oral ingestion of peptide MKP, brief SBP reduction was noted, which occurred in a dose-dependent manner. The ingestion of 1 mg/kg MKP resulted in a drop of SBP to 158.8, 152.2, 158.2, and 166.0 mmHg at 2, 4, 6, and 8 h, respectively. Daily repeated administration of MKP at 10 mg/kg, on the other hand, demonstrated that prolonged oral administration subsequently lowered blood pressure to 163.3 mmHg as compared to 171.7 mmHg in controls [65]. Similarly, the peptides ^{90}RYLGY^{94} and ^{143}AYFYPEL^{149} derived from bovine casein produced a significant reduction in the SBP of the animal model, with a maximum decrease after administration of ^{90}RYLGY^{94} at 6 h, 23.8 mmHg, and ^{143}AYFYPEL^{149} at 4 h, 21.1 mmHg [77]. Inhibition of cutaneous arterial sympathetic nerve activity (CASNA) contributes to lowering blood pressure by regulating peripheral artery and arteriole constriction, which is intrinsically related to peripheral vascular resistance. Possibly via the afferent vagus nerve, IPP and VPP gastric administration significantly decreased CASNA following mean arterial pressure reduction. Additionally, it has been demonstrated that IPP and VPP inhibit renal sympathetic nerve activity, whose stimulation promotes the release of renin and sodium reabsorption, both of which are mechanisms that can lead to hypertension [78].

In addition to animal studies, a double-blind randomized, placebo-controlled cross-over human study to evaluate the effect of milk tripeptide on blood pressure and vascular renal function in prehypertensive Japanese subjects, IPP/VPP supplementation reduced SBP to 127 mmHg in comparison to the placebo group of 130 mmHg on 7-day mean tele-monitored BP [79]. Although the detailed mechanism governing the blood pressure–lowering effects of lacto-tripeptide supplementation remains unclear, a plausible mechanism might involve the inhibition of an enzyme involved in the RAAS system and/or an increase in the release of vasodilatory peptides like bradykinins. The improvement of arterial stiffness and endothelial function by lacto-tripeptide supplementation has been reported in a study by Cicero et al. [80], while Tomiyama et al. [79] demonstrated no effect on either of these parameters in prehypertensive subjects. Overall, sufficient in vitro and significant in vivo studies on antihypertensive dairy–derived bioactive peptides have been carried out since the last decade, which have demonstrated their antihypertensive effects.

Antidiabetic

In Vitro

Diabetes mellitus, a hyperglycemic condition, is caused by insulin resistance or the inability to produce insulin, and appropriate ways of maintaining blood glucose homeostasis are critically needed. Dipeptidyl peptidase IV (DPP-IV) inhibits the activity of incretin hormones, glucagon-like peptide (GLP), and glucagon-like peptide-1 (GLP-1), which signal insulin secretion from pancreatic beta cells, resulting in hyperglycemia; DPP-IV inhibitors, on the other hand, provides an opportunity to control blood sugar by blocking DPP-IV action. Additionally, inhibition of α-glucosidase and α-amylase, two enzymes involved in complex carbohydrate digestion, is considered to be an efficient approach to lower blood sugar levels [81]. It is anticipated that α-amylase competitively interacts with peptide sequences having hydrophobic amino acids Leu, Met, and Pro at the terminal. Furthermore, α-glucosidase is inhibited by the hydrophobic amino acids Met, Pro, Phe, and Leu, while DPP-IV inhibition occurs with both hydrophobic Ala, Gly, Leu, Pro, Met, and Trp amino acids and by hydrophilic Gln, His, Arg, and Ser amino acids at peptide termini [21]. Enzymatic hydrolysis of milk and milk protein from cow and buffalo liberate peptides with diabetes marker inhibition potential [82, 83]. Several novel peptides with strong inhibition of DPP-IV, alpha-glucosidase, and alpha-amylase have been identified, such as LDQWLCEKL [29], LKPTPEGDL, and LKPTPEGDLEIL [84], ELKDLKGY, ILDKVGINY, and KILDK [27, 85•], and RNAVPITPTLNR, TKVIPYVRYL, YLGYLEQLLR, and FALPQYLK [83].

A novel DPP-IV inhibitory peptide LDQWLCEKL (IC$_{50}$: 131 µM) was isolated from bovine whey α-lactalbumin (f 115–123) upon trypsin hydrolysis, indicating that this peptide is effective as a preventive or adjuvant therapy for type 2 diabetes management [29]. The majority of peptides with proline or alanine at the N-terminus possess DPP-IV inhibition, such as milk-derived peptide VPYPQ, Diprotin A (IPI), and Diprotin B (VPL), which have either one or two proline residues and that are considered strong competitive inhibitors of DPP-IV [86, 87]. However, for LDQWLCEKL, the hydrophobic amino acid Leu was located at the penultimate position, and it is hypothesized that it interacted with the hydrophobic pocket found at the active site of DPP-IV [29]. Similarly, bovine α-lactalbumin hydrolysate–derived peptides, ELKDLKGY and ILDKVGINY, were obtained by alcalase hydrolysis and demonstrated the DPP-IV inhibitory activity [27]. Interestingly, when TNF-stimulated 3T3-L1 adipocytes interacted with peptide KILDK, it sufficiently minimized insulin resistance in the adipocytes by suppressing JNK phosphorylation (Thr183/Tyr185) and it inhibited proinflammatory gene expression by impeding NF-κB

signaling [85•]. Previously, pepsin digested β-lactoglobulin were reported to be enriched with the most potent uncompetitive DPP-IV inhibiting fragments, LKPTPEGDL and LKPTPEGDLEIL, with IC$_{50}$ values of the fractions of 45 µM and 57 µM, respectively [84].

Apart from DPP-IV inhibitory peptides, potent alpha-glucosidase inhibitory peptides have been identified from different fractions of milk hydrolyzed by *Dregea sinensis* protease. Four novel peptides from α$_{S1}$, α$_{S2}$-casein exhibited promising α-glucosidase inhibition. These peptides are anticipated to occupy the active sites of α-glucosidase—potentially Arg428, Arg387, Arg801, Arg727, Arg799, and Trp710—by the formation of hydrogen bonds, thereby circumventing the complexation formation and glycosylation of α-glucosidase with the substrate [83]. Likewise, upon hydrolysis of bovine caseins with alcalase and pronase E at different times led to the production of prospective antidiabetic peptides (HLPGRG, QNVLPLH, PLMLP, MFE, GPAHCLL, and ACGP) capable of inhibiting three diabetic-related enzymes (DPP-IV, α-glucosidase, and α-amylase) [21]. Milk fermentation with the two strains of *Lactococcus* released YPSYGL, HPHPHLSFMAIPP, and SLPQNIPPL peptides with the dual function of ACE and DPP-IV inhibition [88].

Lastly, two well-known DPP-IV inhibitors, IPP and VPP, demonstrated effective involvement through enhancement of insulin signals, antiinflammation via NF-κB pathway under TNF stimulation, and prospective contribution to insulin resistance prevention. Furthermore, IPP and VPP possess insulin-sensitizing effects that are independent of insulin receptors present in adipocytes. Upon the administration of VPP, glucose transporter-4 (GLUT-4) expression was enhanced in adipocytes, which restored the absorption of glucose in TNF-treated adipocytes [72, 89].

In Vivo

Diabetes is a common complication of metabolic syndrome, which is characterized by hyperinsulinemia, hyperglycemia, insulin resistance, and multisystem inflammation as a result of impaired glucose-insulin metabolism. With the hypothesis that milk peptides are protective against GLP-1 degradation and attenuate DPP-IV activity in vivo, data indicated that whey protein is taken orally elevated plasma GLP-1 levels, which was effective without influencing the DPP-IV activity [90].

Studies in animal models demonstrated ingestion of bovine and buffalo milk–derived peptides ameliorates pancreatic cell damage, improves oxidative stress, and decreases blood glucose. Lacto-ghrestatin (LGP9), a bovine milk–derived peptide with the sequence LIVTQT-MKG, has been shown to protect β cells of alloxan-induced type-1 diabetic mice. On treatment with LGP9, alloxan-induced type-1 diabetic mice with hyperglycemia showed

a reduction in blood glucose levels and glycated serum proteins (GSP). At the same time, LGP9 treatment increased glucose transporter-2 expression, protected injured β cells by suppressing apoptosis, rescued Ki67 immunoreactivity through IRS2/PI3K/Akt signaling, increased the phosphorylation of FOXO1, and upregulated PDX-1 expression, which resulted in increased insulin secretion [91]. Another peptide, VPYPQ, derived from cow milk beta-casein fraction (fraction number 193–197) was administered orally to 6-week-old mice and found to decrease the postprandial blood glucose levels in a dose-dependent manner, with 90 mol/kg BW being effective and 45 mol/kg BW being ineffective in an oral glucose tolerance test (OGTT) [86]. Thus, these in vivo studies signify that the peptide from cow and/or buffalo milk possesses potent antidiabetic activity and immensely supports the prevention of the symptoms and diseases associated with metabolic syndrome in vitro and in in vivo animal models. But still, there is a dearth of information that directly demonstrates that dairy milk–derived bioactive peptide ingestion attenuates DPP-IV activity in humans, and thus would positively affect type-2 diabetes.

Antihypercholesteremic

In Vitro

Pancreatic lipase and cholesterol esterase are the two lipolytic enzymes responsible for the hydrolysis and digestion of fat and cholesterol esters. When these enzymes are inhibited, intestinal fat and cholesterol absorption are suppressed, which instigates a slow but steady decline in body weight. Peptides from different sources, including cow and buffalo, exhibit inhibitory actions on pancreatic lipase and esterase. The antihypercholesteremic activity and its associated enzyme inhibition by bovine and buffalo milk peptides are shown in Table 1. Mudgil et al. [92•] investigated camel and cow casein–derived peptides with potential inhibitory activity of key lipid-digesting enzymes. The results showed that cow milk casein hydrolyzed by enzyme alcalase and pronase-E increased inhibition of PL. Peptides generated through enzymatic hydrolysis or upon simulated gastric digestion (SGD) from cow casein hydrolysates MMML, FDML, and HLPGRG were prospective pancreatic lipase inhibitors, while peptide LP showed potent cholesterol-esterase inhibition. It has been reported that the enzyme-specific inhibition of two vital hypercholesteremic enzymes, cholesterol-esterase and pancreatic lipase, aids in the prevention of hypercholesterolemia and obesity by inhibiting the uptake of fatty acids, thus limiting the deposition of fatty acids in the body. The lipid regulating function of bovine alpha-lactalbumin and its peptide in HepG2 cell lines was explained by a cell viability assay, triglyceride levels, and peroxisome proliferator–activated receptor-α (PPAR-α) levels in hepatic cells. Upon simulated

gastrointestinal digestion (SGID) of bovine alpha-lactalbumin, the peptide sequences GINY and DQW were obtained. These two peptides increased PPARα levels, activated the PPARα signaling pathway, increased expression of β-oxidation-related genes CPT-1a, PPARα, and ACOX1, and decreased expression of lipogenesis-related genes SCD-1, ACC1, and FASN, improving lipid metabolism and decreasing lipid accumulation. Hence, α-lactalbumin and peptides from lactalbumin show potential sources to ameliorate obesity [93].

Exploration of the role of casein-hydrolyzed peptide in the initiation of trans-intestinal cholesterol excretion (TICE) and the hyperlipidemic effect of the peptide and cell lines treated with isolated peptide were studied. Bovine casein hydrolysate–derived peptides SQSKVLPVPQK and HPHPHLSF induced TICE via regulation of the liver X receptor-α (LXR-α) signaling pathway, and enhanced expression of $ABCG_5$ was recorded. Induction of $ABCG_5$ expression in the intestine could contribute to elevated fecal cholesterol excretion. Moreover, these peptides induced fibroblast growth factor 15/19 (FGF15/19) exudation from enterocytes, which diminished hepatic bile acid synthesis involved in adjusting hepato-biliary cholesterol, thereby aiding in the maintenance of cholesterol homeostasis [94]. Consistently, bioactive peptides LQPE, VAPFPE, TDVEN, and VLPVPQ from milk casein hydrolyzed with neutrase facilitated cholesterol-lowering activity by diminishing cholesterol micellar solubility and absorption. The phenomenon of reduced mRNA expression of acetyl-CoA-acetyltransferase-2 and microsomal triacylglycerols (MTP) in proximal intestinal cells was shown to influence the cholesterol-related proteins and enzymes' expression, which affects cholesterol absorption [95].

Lastly, xanthine oxidase (XO) is presumed to be the source of reactive oxygen species that cause atherosclerosis and cholesterol crystals. The XO inhibitory assay is crucial to understand uric acid biosynthesis. Peptides ALPM and LWM interactions with XO result in stable complexes and inhibit XO. The IC_{50} values of these peptides to inhibit XO were 7.23 and 5.01 mM, respectively, with both contributing via non-competitive routes [96]. These studies carried out to date did indicate that specific peptide sequences can play a role in lowering cholesterol and fatty acid absorption via different modes of action; however, these studies are not sufficient to draw a strong conclusion, even when considering the effect demonstrated in the in vitro model systems.

In Vivo

Hyperlipidemia, a condition of excess blood lipids or fats, is one of the speculated risk factors of metabolic syndrome and is closely associated with obesity, atherosclerosis, and thrombosis. Although the exact pathophysiology of the metabolic syndrome is unknown, evidence-based experiments have

proven that controlling excessive cholesterol prevents mortality. Therefore, maintaining blood cholesterol levels is pivotal and could be beneficial both physically and medically. Several studies have shown that peptides from different sources, including milk, exhibit cholesterol-lowering effects by signaling inhibitory pathways, gene expression, and/or attenuating lipid absorption (Table 2). Hence, the antilipidemic roles of bovine casein–derived peptides generated via pepsin and trypsin were investigated against a high-cholesterol diet-induced hyperlipidemic mouse model. The results demonstrated diminished serum cholesterol, suppression of hepatic CYP_7A_1 and CYP_8B_1 expression, and inhibited hepatic bile acid synthesis in the treated group. Meanwhile, there was an increased level of fecal cholesterol and serum fibroblast growth factor [94]. Exploration of the effect of hexapeptide PGPIPN (0.04, 0.4, and 4.0 mg/kg) in AALI mice revealed that PGPIPN had a significant reduction in the serum and liver TG and TC levels. Interestingly, the low-dose (0.04 mg/kg) ingestion of peptide PGPIPN sufficiently decreased TG (0.5 µmol/mL) and TC (0.8 µmol/mL) levels. Thus, it was speculated that PGPIPN attenuates alcohol-induced liver damage by regulating lipid metabolism [53].

Hyperuricemia (HUA), on the other hand, is a metabolic disease that is closely associated with metabolic syndrome. The enzyme involved in the metabolic pathway of purine is a xanthine oxidase (XO), which catalyzes the oxidation of hypoxanthine and xanthine to form uric acid and liberate superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and ROS. Inhibition of the enzyme XO is necessary to prevent uric acid and lipid crystal formation [97]. An experiment in the potassium oxonate-induced HUA rat model displayed that the milk-derived peptides are the prospective XO inhibitors. Their serum uric acid levels and XO activity were considerably lowered by ALPM and LWM interventions (the latter more so), particularly in comparison to the model group, even though the uric acid–lowering effect was not as strong as that of the commercial drug allopurinol [96]. These studies conducted in vivo for investigating the antihyperlipidemia effect are again not sufficient to draw solid conclusions. Therefore, more robust studies involving peptides with different physiochemical properties on various metabolic targets playing roles in hyperlipidemia should be carried out.

## Digestive Stability of Dairy Milk–Derived Peptides

Throughout the digestive tract, protein breakdown, modification, and digestion take place, while the main organ for protein and peptide absorption is the small intestine. Peptides, either newly formed or surviving from gastric and intestine enzyme hydrolysis, are transported into the bloodstream. On ingestion of the readily hydrolyzed peptide, it encounters the brush border membrane peptidase before passing through the intestinal epithelium and being absorbed. The brush border membrane peptidase hydrolyzed the ingested peptide further, potentially altering its bioactivity and functional properties [98]. Thus, protecting or preserving the functional molecular characteristics of the peptide through the digestive system is crucial for their bioactivity [76].

To study the digestibility and stability of the peptides, mostly three gastrointestinal proteases, pepsin, chymotrypsin, and trypsin, are employed to mimic the human gastrointestinal tract under simulated gastrointestinal digestion conditions of food. This helps to study the retention of functional activities of peptides when orally administered in a human sample study. However, in the Caco-2 cell digestive stability study, the cell is subjected to several membrane peptidases, such as dipeptidyl peptidase IV, endopeptidases, aminopeptidase, enteropeptidases, and aminopeptidase [99, 100]. According to a study by Xia et al. [101], ACE inhibitory activity declined throughout the various stages of simulated in vitro digestion but was still high after trypsin treatment, which gives plausible insight that the Gly-Ala (GA) dipeptide may have some in vivo stability. Also, when the peptide was fed to SHR rats at a dose of 15 mg/kg, it showed a consistent decrease in blood pressure with a drop of 17.48 mmHg. Another study by Xue et al. [76] demonstrated that on the administration of peptide YQKFPQYLQY (YOK) at different pHs (3–9), ACE activity was still stable, and when subjected to in vitro digestion by digestive enzyme pepsin (1–2 h) and trypsin (4 h), the ACE activity of peptide remained unaffected. Casein hydrolysate–derived peptide VLPVPQK was hydrolyzed by cellular peptidases before efflux, resulting in the new peptide VLPVPQ on the apical surface, which rapidly reached the basolateral chamber [98]. Similarly, peptidases on the surface of Caco-2 cells hydrolyze five milk protein–derived peptides LPYPY, LKPTPEGDL, IPIQY, WR, and IPI, with 8 to 30% of the peptides hydrolyzed after 2 h [99]. Tripeptide transport across the Caco-2 cell layer, however, revealed that it was transported from the apical to the basal chamber in the trans well at a concentration of 6.9 µg/ml in its intact form without being hydrolyzed [19].

For the effective and potent delivery of the functional peptide, different multifactorial and complex approaches are under scrutiny. Delivery of the bovine lactoferrin in conjunction with the solid lipid particles and biopolymer-encrypted liposomes showed improved stability, the solid lipid particles being found to be the primary medium for oral delivery of the bovine milk lactoferrin [102]. Certain emerging technologies such as encapsulation, double emulsion with and without Pickering, liposomes, niosomes, or enteric-coated capsules are being prepared for the safe and optimal delivery of these peptides (Fig. 3) [103, 104]. A comparative study of IPP peptide-loaded niosomes and

Fig. 3 Digestive stability with various approaches to enhance stability of the milk-derived peptides (description: free form indicates the peptide solely without nano-carriers for delivery, whereas carrier-based indicates the peptide incorporated in the nano-carriers such as nano emulsion and liposomes)
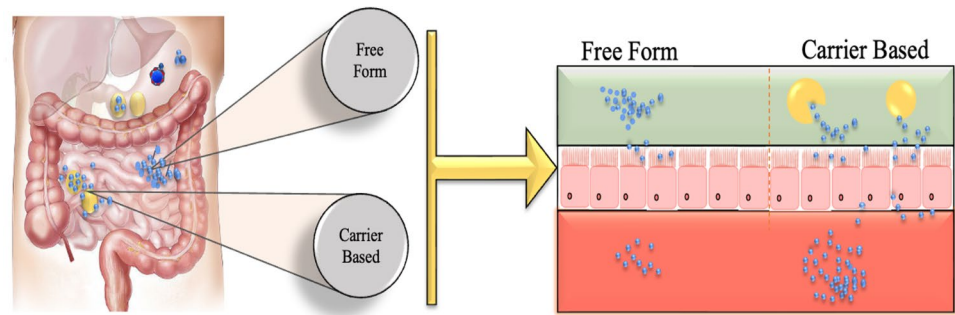
liposomes, for instance, provides a clear insight into a more stable and effective delivery. During long-term preservation, a niosomal composite–loaded functional beverage displayed better palatability, biological activity, and physicochemical characteristics than a liposomal one [103]. When compared to nonencapsulated peptide, an optimized double emulsion loaded with DPP-IV inhibitory peptide decrypted from hydrolyzed α-lactalbumin of Gir cow milk revealed approximately four times better functionality, i.e., DPP-IV inhibition activity [104].

Allergenicity

Food allergy is an IgE-mediated phenomenon that causes a clinical condition or a combination of complications in the respiratory tract (edema in the larynx, rhinorrhea, sneezing, and wheezing), gastrointestinal tract (nausea, vomiting, diarrhea, and abdominal pain), cutaneous region (urtication and angioedema), and cardiovascular (hypotension and tachycardia). Most of the common foods that have allergenicity are peanuts, shellfish, wheat (gluten), eggs, milk, and soybeans [105]. Individuals with impaired digestion are vulnerable to developing food allergies after ingestion; however, allergic reactions can occur through sensitization via other routes, such as skin contact and inhalation (respiratory tract) [106•].

During milk protein hydrolysis, apart from health-beneficial physiologically active peptides, the speculation of cytotoxic or allergenic peptides is very much under consideration, which has placed a clinical safety assessment as a mandatory step. Despite its beneficial property, the mass commercialization of the bioactive peptide is still not up to par with its potential, which could be linked to the infant allergenicity profile of the various peptides resulting from different means of proteolysis [107]. The discrete heterogeneity of bioactive peptides resulting from the hydrolysis is huge, and assessing the beneficial bioactive peptides among all the resulting peptides is complex. Identification of the beneficial bioactive peptides and bioactive peptides with potential allergenicity is a crucial turning point in the commercializing of milk peptides [14].

However, complete and reliable allergenicity profiling of the resultant peptide is not possible, as the study of allergenicity in a clinical setting is unethical; thus, assessments are limited to rodents and cell lines. This only gives the proximal estimation, which does not fully correlate with human physiology, leaving a gap from theoretical benefit to actual implications arising after human consumption [108]. To bridge the gap results from various assessment approaches, such as in vitro, in vivo, and in silico must be performed and integrated to overcome the absence of human clinical trials [9••, 109]. The safety evaluation for peptide-like functional foods still does not have a robust guideline, hence in vivo trials on rodents are considered to be the standard [110]. Because avoiding allergen-containing foods is the only known intervention, safety evaluation for possible allergenicity should be prudent [105]. In the case of milk-derived food products, the prevalence of cow milk allergy is 2–3%; however, enzyme-induced partial hydrolyzation might diminish its allergenicity, as the resultant hydrolysate has an immunoregulatory effect. Based on the degree of hydrolyzation, completely hydrolyzed proteins could be administered to treat the allergic reaction. Similarly, partially hydrolyzed proteins could be administered to prevent allergic reactions [111].

Future Perspectives and Conclusion

The worldwide annual production of milk climbed by an average of 1.6% (838 million tons) in 2018, with India's production leading the way at 3% (174 million tons). However, India is the least milk-exporting country. Major exporters such as New Zealand, the European Union, and the USA had production increased by 3.2%, 0.8%, and 1.1%, respectively. The projection of annual milk production worldwide was postulated to grow at 1.8% (1060 million metric tons) by 2031. Despite the COVID-19 pandemic, global milk production is still trending up to meet the anticipated growth. However, regardless of the production curve, dairy consumption is declining due to the emergence of plant-based dairy substitutes, which may

have negative effects on dairy demand [112], resulting in surplus milk. Hence, commercialized production of the milk protein–derived peptide would be an effective way of relieving the economic burden for the producers.

A possible best way of utilizing milk-derived bioactive peptides would either be the optimization of the yogurt fermentation for increased production of functional peptides or fortification, which might also be an option to supply peptides as per the demands of consumers' clinical conditions [113]. But, this could only be envisioned if the novel production technologies work in conjunction with the computational (in silico) approach for discovering resultant bioactive peptides, as in silico analysis utilizes bioinformatics to virtually simulate the occurrence of biological systems [35, 39].

Even though the production and purification of milk peptides are challenging, there is growing interest in these bioactive peptides due to the wide range of functionality of milk-derived peptides. As articulated in this review, these peptides have the potential to minimize the effects of metabolic syndrome. In vitro or in animal model systems, indications are that the majority of milk peptides would or have a significant effect on alleviating symptoms of various diseases associated with metabolic syndrome; however, there are still only a handful of human studies published. Given this, extensive research into the promising peptides in human health is required before commercializing milk peptide–based antihypertensive, antidiabetic, and antihypercholesteremic products. Although some peptides are being studied in human trials, determining the mechanisms underlying their physiological effects remains difficult. The major challenge in peptide research is to investigate and get reliable data on the fate of peptides when consumed or ingested and on the changes occurring during their transit through a gastrointestinal phase of digestion. Synthetic peptides of known sequence can be validated for their biological properties in different systems (in vitro, cell lines, ex vivo); however, what effect will transit of the peptide through the human gastric and intestinal phase of digestion have on the bioactive property is a crucial challenge. Research to address this challenge is very important for designing effective peptide therapies for the treatment of various metabolic syndromes.

In conclusion, MPDPs appear to be potential therapeutics, nutraceuticals, and natural medicines in the pharmaceutical industry for those with metabolic syndrome. Furthermore, research is needed to investigate the optimal production and isolation approaches of MPDPs. Additionally, designing stable and efficient delivery systems to be utilized in peptide therapy with enhanced bioavailability is also equally important, which calls for novel research in this area.

Declarations

Conflict of Interest The authors report no conflict of interest.

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Papers of particular interest, published recently have been highlighted as:
- Of importance
- • Of outstanding importance

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