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Bioactive milk peptides: an updated comprehensive overview and database

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ABSTRACT

Partial digestion of milk proteins leads to the formation of numerous bioactive peptides. Previously, our research team thoroughly examined the decades of existing literature on milk bioactive peptides across species to construct the milk bioactive peptide database (MBPDB). Herein, we provide a comprehensive update to the data within the MBPDB and a review of the current state of research for each functional category from *in vitro* to animal and clinical studies, including angiotensin-converting enzyme (ACE)-inhibitory, antimicrobial, antioxidant, dipeptidyl peptidase (DPP)-IV inhibitory, opioid, anti-inflammatory, immunomodulatory, calcium absorption and bone health and anticancer activity. This information will help drive future research on the bioactivities of milk peptides.

KEYWORDS

Peptidomics; in vitro; in vivo; hydrolysates; casein; whey

Introduction

Mammals have evolved to produce milks that meet the nutritional needs of mammalian neonates for growth and development. In addition to providing the basic nutrients, milk also contains an array of biologically active compounds, including bioactive proteins that support infant health. Enzymatic hydrolysis of these milk proteins during digestion, processing or fermentation (e.g. cheese production) releases peptides that have an array of functions, including antioxidant (Amigo, Martinez-Maqueda, and Hernandez-Ledesma 2020), antimicrobial (Ali et al. 2019), immunomodulatory (Cai et al. 2021), anti-inflammatory (Adams et al. 2020), antihypertensive (Okamoto et al. 2020), antithrombin (Liu et al. 2019), opioid (S Fernández-Tomé et al. 2016), insulin signaling (Chakrabarti et al. 2018), dipeptidyl peptidase (DPP)-IV inhibitory (Nongonierma et al. 2018), anticancer (Guo et al. 2021), osteoblast-stimulatory (Reddi et al. 2018) and wound healing (Nielsen, Purup, and Larsen 2019; Purup et al. 2019).

Thousands of research articles describe bioactivities of milk protein-derived peptides. Most of these studies describe the effects of mixed milk protein hydrolysates. However, some of this research also identifies the specific peptide sequences that exert these functions. Bioactive milk peptides can be (and, in some cases, are) applied as value-added food ingredients, supplements or medicines. Milk peptides as therapeutics likely have fewer side-effects than traditional small molecule drugs because mammalian evolution only supports compounds in milk that are safe for the neonate.

Peptide analysis of dairy products has become more common, and the advancements in mass spectrometry instruments increase the data output from these analyses, revealing hundreds to thousands of peptides present in different dairy products, such as cheese (Robinson et al. 2021), yoghurt (Nielsen et al. 2021) and kefir (Dallas et al. 2016). Human milk contains a large number of peptides released from milk proteins within the mammary gland (Nielsen, Beverly, and Dallas 2017). After feeding to infants, human milk proteins are further digested into smaller fragments and peptides in the stomach and intestine (Nielsen et al. 2020; Beverly et al. 2021). Some human milk peptides even survive to the infant stool (Beverly et al. 2020).

To understand the potential biological significance of peptides released from milk proteins in different products and at different sites of digestion, it is important to search peptidomics data against a library of known bioactive peptides. This type of analysis was made possible through the construction of the milk bioactive peptide database (MBPDB) (Nielsen et al. 2017). The initially published MBPDB included 737 unique peptide sequence-function combinations from 606 unique peptide sequences originating from the milks of ten different species (Nielsen et al. 2017). Use of the MBPDB allowed identification of hundreds of peptides with high homology to known bioactive milk peptides in human milk and infant formula (Nielsen et al. 2018) and in infant gastric digesta, intestinal digesta and stool after consuming human milk (Nielsen et al. 2020; Beverly et al. 2021, 2020).

As the initial version of the MBPDB was published in 2017, there is a need to update it to include newly discovered bioactive milk peptides. Herein, we exhaustively searched for novel bioactive peptides, added them to the

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database and provide a comprehensive overview of the current knowledge on milk-derived bioactive peptides.

Methods

Data acquisition

We searched Web of Science (www.webofknowledge.com) for research articles identifying milk protein-derived peptides with a documented biological action, as we described previously (Nielsen et al. 2017). The search was conducted with the terms "peptide" AND ("milk", "casein" or "whey") AND a biological function term. Biological function terms used were "bioactive," "anticancer," "osteoanabolic," "calcium uptake," "cytotoxic," "wound healing," "anti-inflammatory," "immunomodulatory," "insulin signaling," "satiety," "opioid," "antimicrobial," "antithrombin," "hypocholesterolemic," "antihypertensive," "antioxidant" and "angiotensin-converting enzyme (ACE)-inhibitory". We did not specify species in the search terms as we wanted to ensure that we identified all discovered bioactive milk peptides across all species. The search was performed from November to December 2022. We refined this search to only include primary research articles from 2016–2022. Based on the abstracts, we identified research articles to further review for the identification of peptides with biological function. From each paper, we extracted the following information for each identified bioactive peptide: amino acid sequence, the specific bioactivity, the protein from which it derived, position in the protein sequence, the species from which the protein derives and title, authors and DOI of the paper describing the bioactive peptide.

Data visualization

The purpose of this visualization is to provide an overview of all the peptides that were associated with the protein. A horizontal stripe is shown for each peptide, which is colored according to its bioactive function. The peptides wrapping around the edges of the plot are indicated with an arrow mark. The visualization was implemented in TypeScript using the D3.js library (ver. 7.8.2) (Bostock, Ogievetsky, and Heer 2011). More information, a downloadable version of the software, and its source code can be found at https://vis-au.github.io/psm.

Utilization of the milk bioactive peptide database

The milk bioactive peptide database is the most comprehensive database for milk-derived bioactive peptides covering all relevant functions (Nielsen et al. 2017). Inclusion of peptides in the MBPDB requires that they have 1) an appropriate primary literature reference, 2) identification of a single peptide sequence with a specific function (the functions of mixed hydrolysates are not included), and 3) *in vitro*- or *in vivo*-validated functions (*in silico* predicted functional peptides are not included). The database tool provides multiple search options, including searching based on source species, specific functions or specific protein of origin or a combination of these search terms. Peptides can either be searched as single peptide entries or *via* upload of a simple text (.txt) file with multiple peptide entries. The search tool provides a range of search options. The homology search function allows a user-determined percentage of sequence homology threshold (0-100%). A search option for "truncated peptides" allows searching for database entries that contain the sequence of the query peptide. A "precursor" search option allows searching for database entries that are contained within the input sequence. As milk proteins are one of the most extensively explored food systems within bioactive peptide research, the knowledge in this database can be used to search proteins from other sources (e.g., plants) for bioactive peptides that are homologous with the identified milk bioactive peptides. Finding homologous peptides in non-milk protein sources is especially likely for the known short milk bioactive peptides (2-4 amino acids long).

One of the main uses of the MBPDB is to search for bioactive peptides present in peptidomics datasets. The MBPDB has been used extensively by researchers to search for bioactive peptides with high sequence homology (80-100%) to identified peptides (Caira et al. 2022; Martini, Conte, and Tagliazucchi 2020; Solieri et al. 2022). For example, a study that identified 418 peptides in the intestinal samples of infants fed with human milk used the MBPDB to determine that 50 of these identified peptides have high sequence homology with known bioactive peptides (Liang et al. 2022). Similarly, a study that identified 1,473 peptides in the simulated *in vitro* gastrointestinal digestion of the cheese used the MBPDB to identify bioactive peptides with ACE-inhibitory and antioxidant activities (Abedin et al. 2022).

In the current review, we have added an additional companion tool to visualize the different identified bioactive peptide (e.g., using the MBPDB) to their parent protein. This tool can be accessed at https://vis-au.github.io/psm. The visualization tool is available for download or as online version.

A comprehensive and precise database of bioactive milk peptides is also valuable in development of machine learning methods for bioactive peptide prediction (Xu et al. 2021), for quantitative structure-activity relationship studies and for molecular docking studies (Chamata, Watson, and Jauregi 2020). These *in silico* methodologies associate the chemical composition and arrangement of the peptides with its identified biological effect (Ochoa and Cossio 2021; Z. Chen et al. 2018). These studies require training datasets collected from online bioactive peptide databases such as the MBPDB or other more general bioactive peptide databases (Qin et al. 2022; Théolier et al. 2014). The accuracy of the models depends on the accuracy of the data available in the databases used. The MBPDB provides excellent training data as all entries are validated with *in vitro* or *in vivo* studies supporting the results and only the activity of specific peptide sequences are included. Using *in silico* approaches provides opportunities for identification of novel bioactive peptides.

Overview of bioactive milk peptides

The search for newly published bioactive peptides resulted in the identification of an additional 202 peptide sequences matched to a specific function, increasing these unique

peptide sequence-function combinations within the MBPDB by 20%. These new peptides had functions including antioxidant (70 peptides), ACE-inhibitory (44), DPP-IV inhibitory (20), anti-inflammatory (15), antimicrobial (14) and a range of other functions (39). A total of 143 unique peptide sequences made up the 202 peptide-function entries. 59 peptide sequences were attributed with more than one function. Overall, the addition of these data increased the total number of unique bioactive peptides sequences annotated in the MBPDB by 14% (to 691) compared with the previously published version. Of the peptides in the database, the majority derive from bovine β-casein (total; new addition, 224; +37), bovine α_{s1}-casein (101; +14), bovine β-lactoglobulin (90; +12), bovine lactoferrin (63; +2), human β-casein (60; +2), bovine α_{s2}-casein (58; +3), bovine κ-casein (59; +15); bovine α-lactalbumin (42; +8) and human lactoferrin (27; +0). The remaining peptides (128; +16) derive from 30 different proteins.

A general overview of the number of known bioactive peptides categorized into specific health areas (cardiovascular, digestive, glycemic control, immunological, skeletal, cancer and other) is provided in Figure 1. The known bioactive milk peptide functions include, in order of abundance, ACE-inhibitory (355), antimicrobial (186), antioxidant (91), DPP-IV inhibitory (79), opioid (26), anti-inflammatory (23), immunomodulatory (23) and anticancer (18) (Figure 1).

Bioactive peptides from milk have a broad array of potential sites of action throughout the body, including the oral cavity, stomach, intestine, pancreas, liver, immune system, skeletal system, adipose tissue, muscle, nervous system and skin (Figure 2). However, for these peptides to exert their specific bioactivities *in vivo*, they would need to reach the site of bioactivity. For example, ACE-inhibitory peptides must reach the bloodstream to exert their function. In most cases, the capacity for bioactive milk peptides to reach their site of action, particularly in humans, has not been examined.

Bioactive peptides derive from an array of different sites within the parent sequences of milk proteins (as visualized in Figures 3–7). Most bioactive peptides have been identified from β-casein. The majority of these peptides derive from bovine β-casein (Figure 3), with fewer deriving from human β-casein (Figure 4). The larger number of known bioactive peptides from bovine β-casein compared with human β-casein likely reflects that bovine milk has been more intensively studied for bioactive peptides than human milk. Bovine α_{s1}-casein has three major regions for bioactive peptides (amino acids 1–35, 80–109 and 142–199) (Figure 5). Bovine α_{s2}-casein is notably dominated by antimicrobial peptides (Figure 6). Among the known bioactive peptides from bovine κ-casein, AA 106–169 is called caseinomacropeptide and has been annotated with multiple functions, including antimicrobial, ACE-inhibitory and anti-inflammatory activity (Figure 7).

Cardiovascular system

Functions of bioactive peptides that might impact cardiovascular health include antioxidant, anti-inflammatory, antihypertensive, ACE-inhibitory, antithrombin (Rojas-Ronquillo et al. 2012) and inhibition of cholesterol solubility (Jiang et al. 2020). For the most part, milk peptides would have to reach the circulatory system to have these presumed benefits (Figure 2). Herein, we examine antioxidant, antihypertensive and ACE-inhibitory peptides as these groups represent the majority of the cardiovascular-related milk peptides in literature.

Antioxidant

Overview of function and importance

Oxidative stress plays an important role in the pathogenesis of cardiovascular diseases (Mangge et al. 2014). Oxidation is caused by free radicals, which are unstable molecules that the

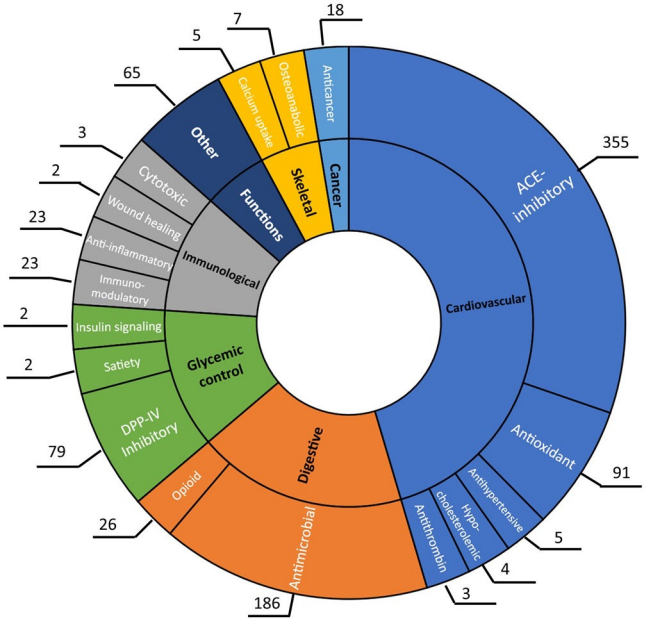

Figure 1. Current number of unique bioactive milk peptide sequences with each known function categorized by health area.

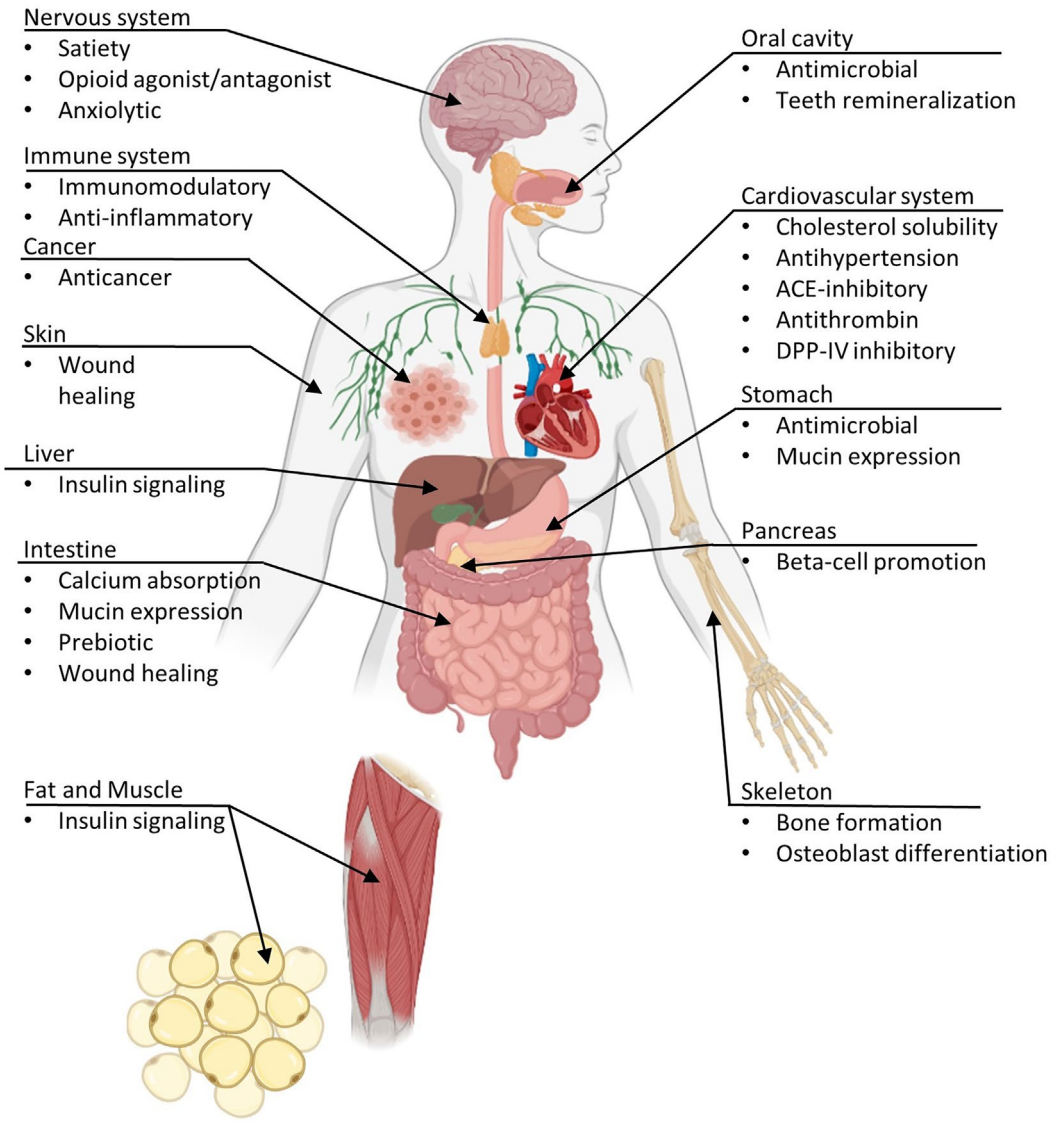

Figure 2. Proposed sites of action for milk peptides to exert their specific bioactivities.

body produces as a reaction to environmental and other pressures. Antioxidants can prevent or limit free radical-induced oxidative damage. Antioxidant bioactive peptides can scavenge free radicals in membranes, which inhibits lipid peroxidation and helps prevent overall oxidative stress. In general, known antioxidant peptides derived from milk are 5–11 amino acids long and contain a high amount of hydrophobic amino acids, such as proline, histidine, tyrosine or tryptophan (Korhonen and Pihlanto 2006). A total of 91 unique milk protein-derived peptide sequences have been found to have antioxidant activity, of which 44 was recently discovered. These peptides derive mainly from α_{s1}-casein (24), β-casein (24), and κ-casein (10) but also derive from whey proteins (Báez et al. 2021). Antioxidant milk peptides have been found from bovine (Tonolo, Folda, et al. 2020), buffalo (Sowmya et al. 2019), yak (Q. X. Liu et al. 2020) and human milk (Tsopmo et al. 2011).

In vitro evidence for individual peptides and hydrolysates

Individual milk peptides have been shown to have antioxidant functions *via* an array of *in vitro* assays, including free-radical scavenging activity (e.g., for superoxide anions and hydroxyl radicals), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) (Lorenzo et al. 2018), protecting intestinal epithelial cells (Caco-2) and macrophages (RAW264.7) from oxidative damage (Amigo, Martinez-Maqueda, and Hernandez-Ledesma 2020; Tonolo et al. 2018; Sowmya et al. 2019) and inhibiting reactive oxygen species (ROS) production in intestinal cells (IEC-6 and Caco-2) (Basilicata et al. 2018; Tonolo et al. 2018). For example, Liu et al. (2020) revealed that the β-casein-derived peptide RELEEL had high superoxide anion and hydroxyl radical scavenging activities (Q. X. Liu et al. 2020). Fermentation of milk products has been shown to release an array of antioxidant peptides (Tonolo, Fiorese, et al. 2020; Silva, Pihlanto, and Malcata 2006). Some milk antioxidant peptides alter cell protein expression to protect against oxidation. For example, a β-lactoglobulin-derived peptide (YVEELKPTPEGDL) from buffalo ricotta cheese reduced ROS release and increased Nrf2 activation and the expression of antioxidant cytoprotective factors, such as heme oxygenase, NAD (P)H:quinone oxidoreductase 1 and superoxide dismutase (Basilicata et al. 2018).

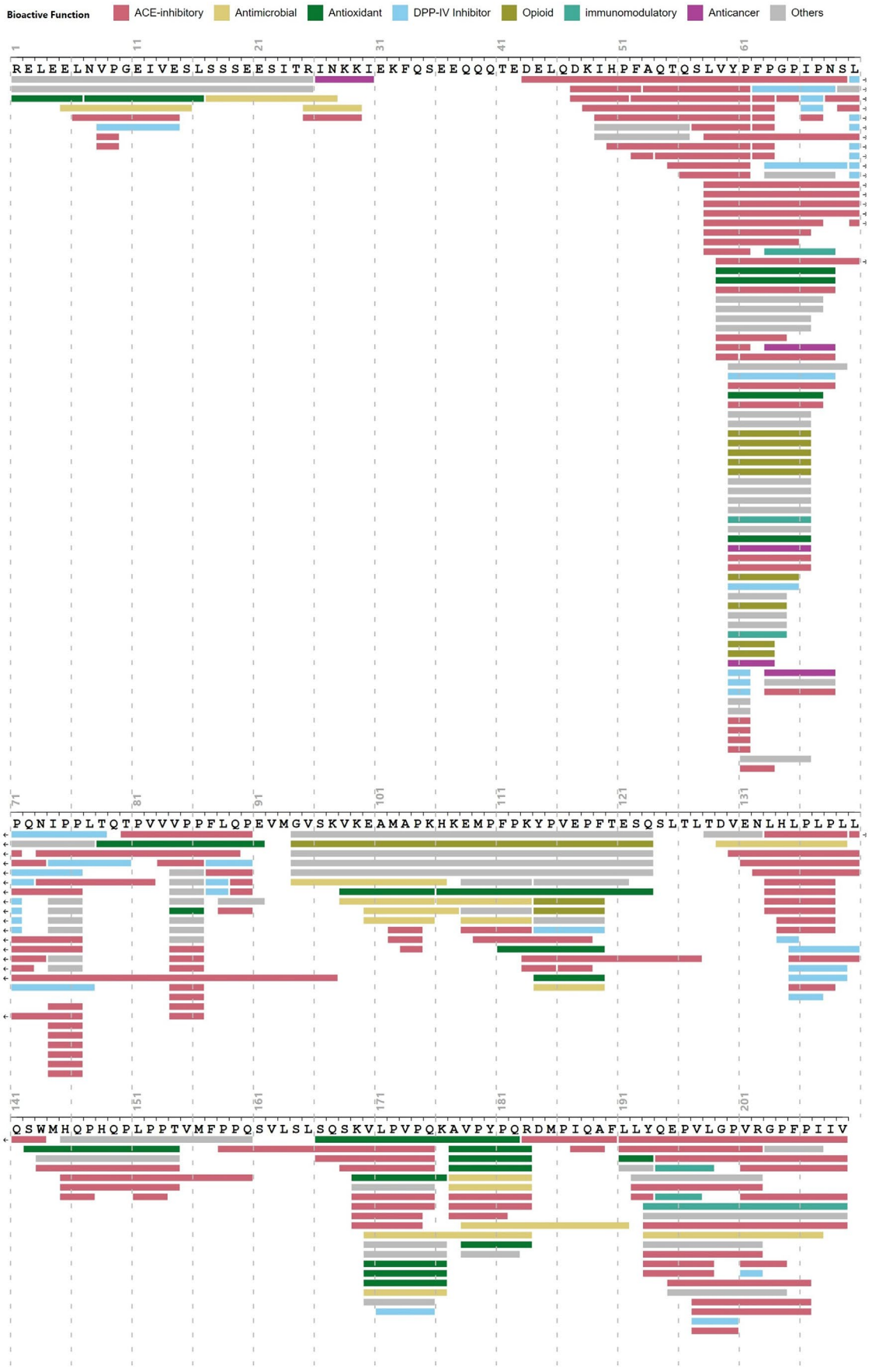

Figure 3. Bovine β-casein-derived bioactive peptides included in the MBPDB mapped across the intact protein sequence.

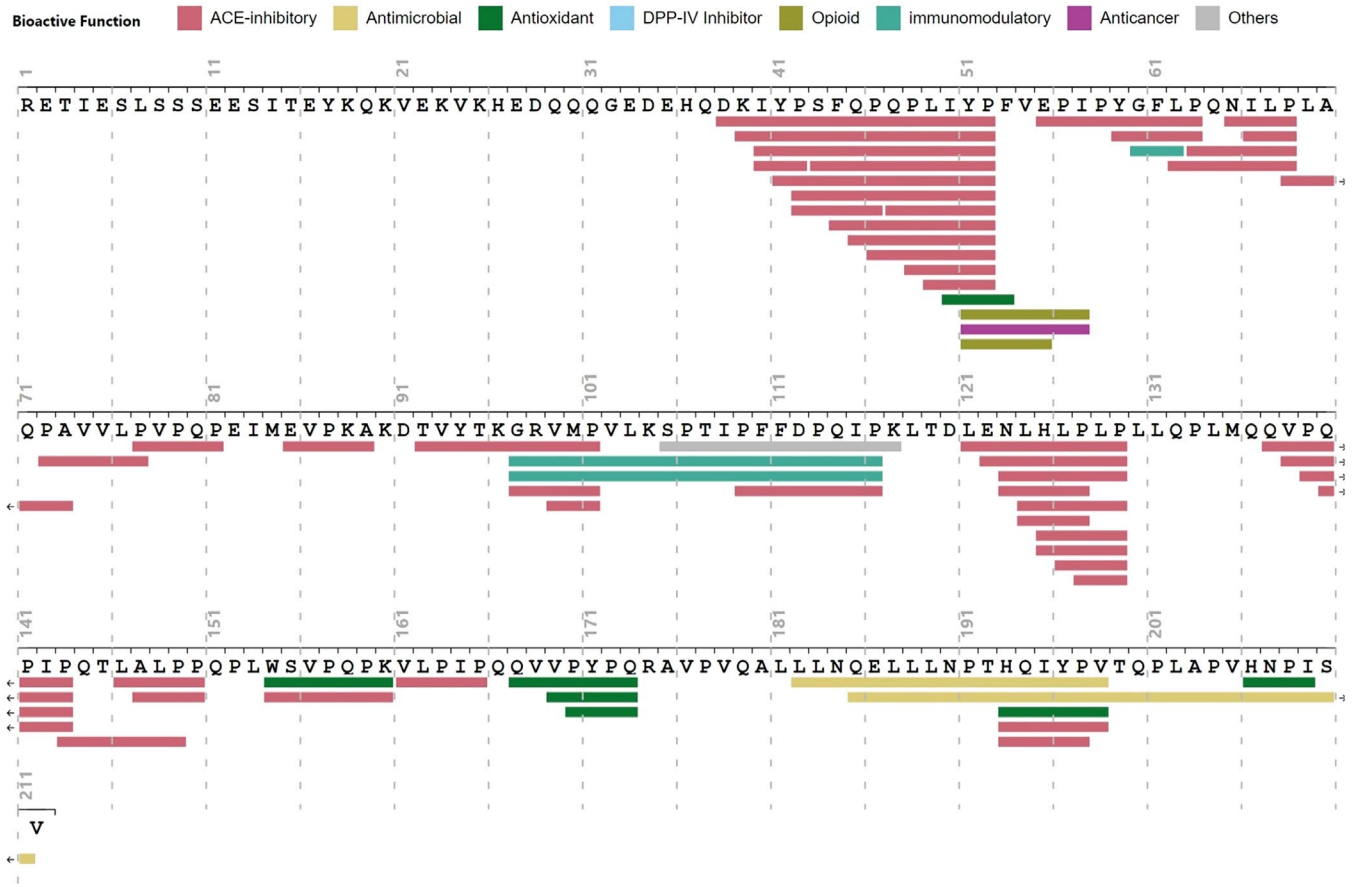

Figure 4. Human β-casein-derived bioactive peptides included in the MBPDB mapped across the intact protein sequence.

Animal evidence for individual peptides and hydrolysates

A few animal studies indicate that milk peptides can have antioxidant function. For example, Yang et al. (2020) found that the buffalo milk lactoferrin-derived peptide SVDGKEDLIW reduced the oxidative stress induced by feeding mice excessive D-galactose (Yang et al. 2020). Falkowski et al. reported that total antioxidant status significantly increased in the submandibular and parotid glands of the rats fed whey protein concentrate compared to the control group (Falkowski et al. 2018). This finding may be due to the release of antioxidant peptides during digestion but could also be due to the intact proteins themselves or other factors.

Clinical evidence for individual peptides and hydrolysates

There is currently no evidence that feeding milk protein-derived antioxidant peptides directly can exert antioxidant activity in humans. One study that fed human subjects whey proteins for 12 wk demonstrated increased plasma antioxidant capacity compared with their baseline levels (Chitapanarux et al. 2009). The observed antioxidant activity in this study could derive from antioxidant peptides released from these proteins during digestion but could also arise from the intact protein or enhanced provision of amino acids. Supplementation of whey protein isolate (WPI) in a resistance training program to healthy males increased plasma total antioxidant capacity (Sheikholeslami Vatani and Ahmadi Kani Golzar 2012). However, this finding could not be confirmed in another similar study (Brown et al. 2004). Further clinical studies are needed to assess the effects of specific antioxidant peptides.

ACE-inhibitory and antihypertensive

Overview of function and importance

Hypertension is a chronic disease represented by excessive high blood pressure due to insufficient relaxation of the blood vessels. This condition is associated with serious diseases such as arteriosclerosis, cardiovascular disease, myocardial infarction, stroke and renal failure (de la Sierra 2019). Because ACE plays an important role in blood pressure regulation, its inhibition can help treat hypertension (Aluko 2015). The structural features of ACE-inhibitory peptides are not fully determined; however, peptides with hydrophobic amino acids at their C-termini are more likely to have ACE-inhibitory actions (Ondetti and Cushman 1984). Many milk peptides have ACE-inhibitory action at least *in vitro*. The MBPDB includes 355 ACE-inhibitory peptides (of which 28 were discovered since our prior publication) as well 5 antihypertensive peptides. These peptides derive mainly from β-casein (138), α_{s1}-casein (39) and β-lactoglobulin (36).

In vitro evidence for individual peptides and hydrolysates

Several *in vitro* assay are available for ACE-inhibitory activity measurements using enzymatic reactions, including spectrophotometric and fluorometric approaches (Ahmad et al.

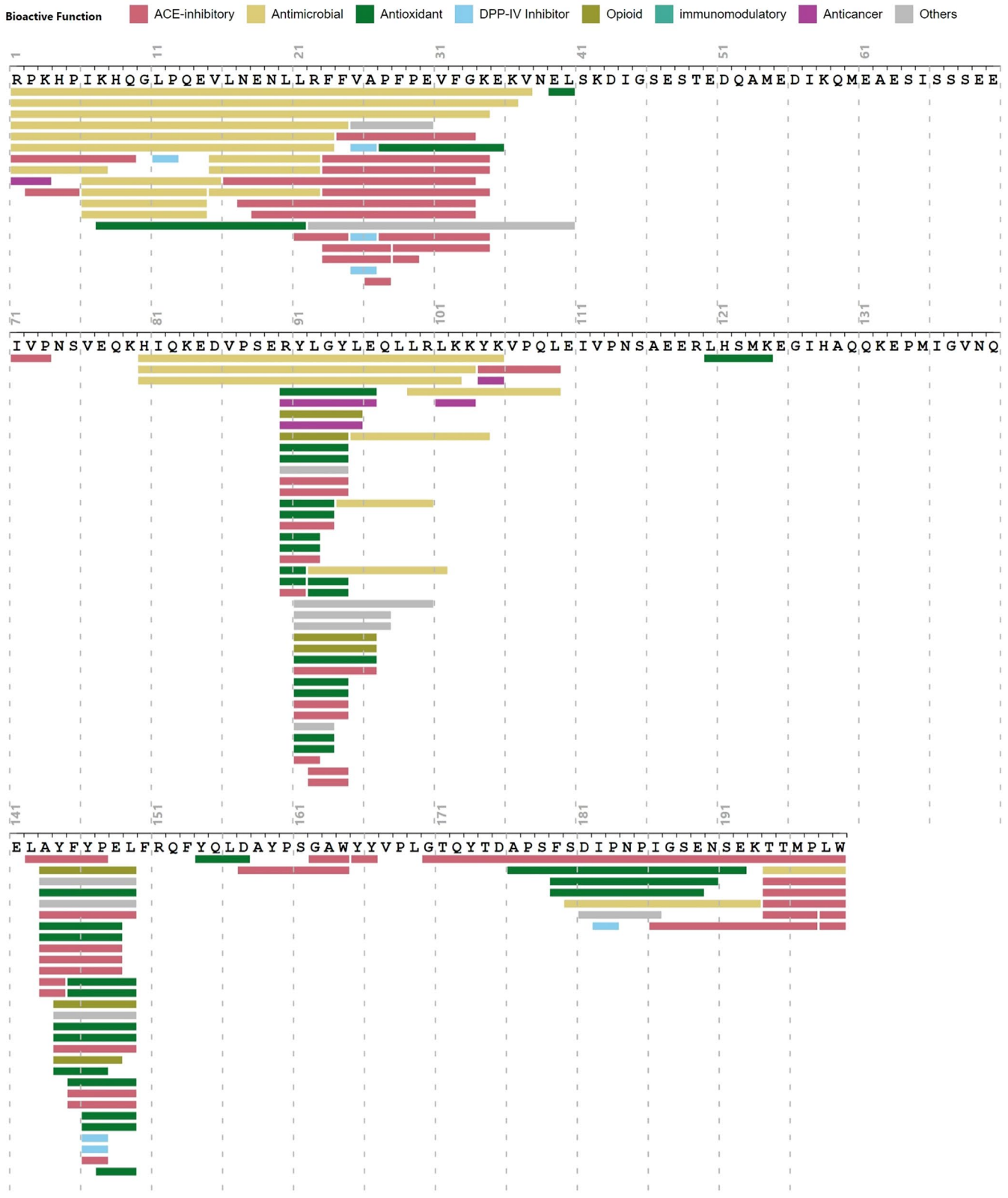

Figure 5. Bovine α_{s1}-casein-derived bioactive peptides included in the MBPDB mapped across the intact protein sequence.

2017). Using these methods, numerous milk protein-derived peptides have been identified with *in vitro* ACE-inhibitory activity. In most cases, the digestive survival and bioavailability of these peptides have not been examined. Some studies provide insight into this question. For example, Liu et al. (2020) demonstrated that β-casein-derived LLYQEPVLGPVR has strong *in vitro* ACE-inhibition, is highly stable across simulated gastric digestion, and the shorter peptide fragments produced after simulated intestinal digestion exhibit higher ACE-inhibitory activity than did the full peptide sequence (P. R. Liu et al. 2020). Similarly, Lin et al. (2017) found that

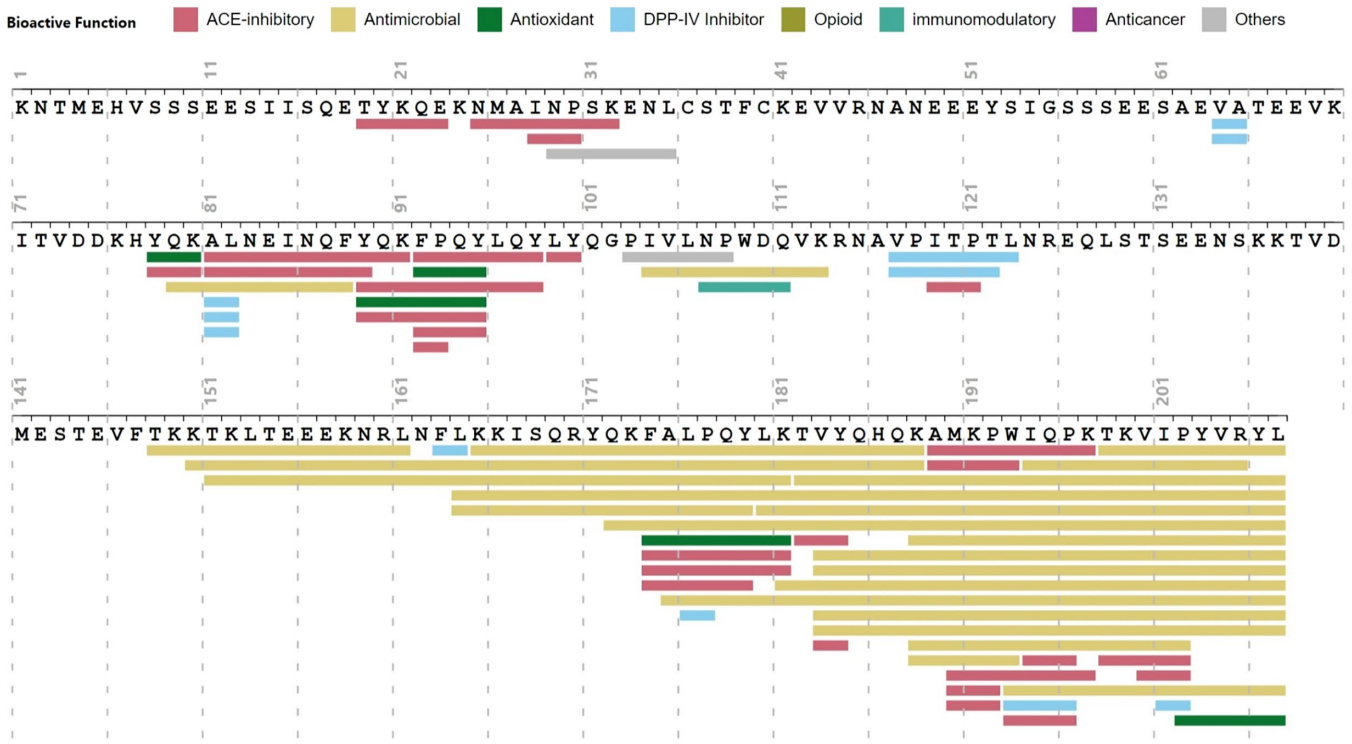

Figure 6. Bovine α_{s2}-casein-derived bioactive peptides included in the MBPDB mapped across the intact protein sequence.

Figure 7. Bovine κ-casein-derived bioactive peptides included in the MBPDB mapped across the intact protein sequence.

of two ACE-inhibitory peptides derived from enzymatic hydrolysis of yak milk β-casein (PFPGPIPN and LPLPLL), PFPGPIPN was stable across *in vitro* gastrointestinal digestion, whereas LPLPLL were cleaved into the known ACE-inhibitory peptide PLP (Lin et al. 2017; Okamoto et al. 2020). Likewise, Wu et al. found that the ACE-inhibitory peptide LPYPY from bovine κ-casein was hydrolyzed after *in vitro* pepsin (pH >1.3) digestion, yet ACE-inhibitory activity increased significantly (Wu et al. 2019). Xue et al. found that the ACE-inhibitory peptide YQKFPQYLQY from bovine α_{s2}-casein was further digested to YQK when incubated with pepsin and trypsin. However, this new peptide retained a high ACE-inhibition (Xue et al. 2018).

Animal evidence for individual peptides and hydrolysates

Several studies in animal models have demonstrated that milk peptides can lower blood pressure. For example, orally-delivered IPP and VPP deriving from bovine κ-casein and β-casein, respectively, reduced blood pressure in spontaneously hypertensive rats both short term (single dose) (Nakamura et al. 1995) and longer term (12 wk treatment) (Sipola et al. 2001). Similarly, oral administration of the bovine α_{s2}-casein peptide YQKFPQYLQY (described in the previous section as an *in vitro* ACE-inhibitor resistant to *in vitro* digestion) to spontaneously hypertensive rats significantly reduced blood pressure (Xue et al. 2018). Likewise, bovine α_{s1}-casein-derived peptides RYLGY and AYFYPEL given orally to rats significantly decreased systolic blood pressure (Sanchez-Rivera et al. 2020). Some studies have demonstrated blood pressure reduction in animals after intravenous injection rather than oral administration. For example, spontaneously hypertensive rats intravenously injected with YP and IPP originally identified from milk fermented by brown rot fungus *Neolentinus lepideus* exhibited a significant reduction in blood pressure (Okamoto et al. 2020).

Clinical evidence for individual peptides and hydrolysates

Several studies have demonstrated that feeding *Lactobacillus helveticus*-fermented bovine milk containing IPP and VPP to hypertensive human subjects decreased systolic and diastolic blood pressure. Jauhiainen et al. reported that hypertensive patients not receiving any drug treatment had reduced blood pressure when given *L. helveticus*-fermented milk with a high concentration of the IPP and VPP tripeptides compared to the control group receiving a Lactococcus sp. mixed culture-fermented milk without the two tripeptides for a 10-week intervention period (Jauhiainen et al. 2005). In a follow-up study, Seppo et al. 2003 confirmed that hypertensive patients receiving a daily dose of the *Lactobacillus helveticus*-fermented milk with a high concentration of the IPP and VPP tripeptides compared to Lactococcus sp. mixed culture-fermented milk without the two tripeptides over a 21-week intervention period (Seppo et al. 2003). In another follow-up study, Tuomilehto et al. 2004 used the same product for the test group and control group as described for the two other studies and again observed a beneficial effect on systolic blood pressure (Tuomilehto et al. 2004). However, the observed reduction in blood pressure in these studies could have been due to a range of other components in these fermented milks, rather than solely the IPP and VPP. Notably, these studies typically do not measure IPP and VPP in the blood of subjects.

Gastrointestinal system

Several biological functions of peptides can relate to gastrointestinal system health, including antimicrobial, opioid and mucin expression. The gastrointestinal tract is part of the innate immune system and a barrier for potentially harmful agents and bacteria that are ingested with our food. The gastrointestinal system is also one of the most likely sites of action for bioactive peptides, and several studies have investigated the complex mixture of peptides produced after milk or dairy product ingestion across the gastrointestinal tract.

Opioid activity

Overview of function and importance

Opioid peptides are peptides known to bind to and activate or inhibit opioid receptors μ, κ and δ. Opioid receptors are located in the nervous, endocrine, immune and gastrointestinal systems of mammals (Kaur et al. 2020). Milk-derived opioid peptides play both agonistic and antagonistic roles. Herein, we focus on the food-derived exogenous opioid peptides that have an impact on the gastrointestinal system. Two major effects opioid peptides can have on the gut are modulation of peristalsis and mucin secretion. Food-derived exogenous opioid peptides are known to modify gastrointestinal motility through interrupting neuroneuronal and neuroeffector transmission within enteric nerve pathways governing muscle activity and, consequently, alter peristalsis (Kaur et al. 2020). Opioid peptides can also stimulate gastrointestinal mucin secretion by activating opioid receptors and nervous pathways (Samuel Fernández-Tomé and Hernández-Ledesma 2020). Mucin is secreted by goblet cells and creates a thick, viscous layer of mucus which covers the surface of the epithelium and helps separate gut contents from the underlying epithelium, preventing entry of noxious substances and enteric pathogens into the body. This mucus layer constitutes a primary component of the gastrointestinal mucosal barrier and innate host defense.

Opioid peptides derived from milk have potential to interact with the opioid receptors on the surface of intestinal epithelium cells and modulate intestinal health. The common structural characteristics of both exogenous and endogenous opioid peptides are the presence of a tyrosine residue at the N-terminus and the presence of another aromatic residue, either phenylalanine or tyrosine, in the third or fourth position (Tyagi et al. 2020).

A total of 26 unique peptide sequences with opioid activity deriving from milk proteins has been identified Twenty-one peptides were identified from cow milk proteins, including caseins, β-lactoglobulin and lactoferrin. Five peptides were identified from human milk proteins, including β-casein and lactoferrin.

In vitro evidence for individual peptides and hydrolysates

Numerous studies show that milk protein-derived peptides have *in vitro* agonist or antagonist activities with opioid receptors. For example, casomorphin-7 (YPFPGPI from bovine β-casein) (Claustre et al. 2002), β-lactorphin (YLLF from bovine β-lactoglobulin) (Pihlanto-Leppälä 2000), casoxin A (YPSYGLN from bovine κ-casein), lactoferroxin A (YLGSGY from human lactoferrin) (Chiba, Tani, and Yoshikawa 1989) have opioid activity. Although not included in the database (as information on the specific functional peptide is missing), some studies have found that *in vitro* digests of milk proteins have opioid activity. For example, a recent study reported that a mixture of *in vitro*-digested bovine whey and casein proteins increased the expression of MUC5AC and MUC2 in the human intestinal goblet cell model HT29-MTX-E12 cells (Giromini et al. 2019).

Animal evidence for individual peptides and hydrolysates

A recent study found that bovine whey and casein protein hydrolysates could modulate colonic motility patterns in isolated rat large intestine as a casein protein hydrolysate could reduce motility while a milk protein hydrolysate increased motility (Dalziel et al. 2019). The authors suggest that these hydrolysates contained opioid peptides which modified gastrointestinal motility.

Clinical evidence for individual peptides and hydrolysates

The evidence of the impact of milk-derived opioid peptides on gastrointestinal motility and mucin-production is limited to pre-clinical findings. More clinical studies are needed to examine whether milk opioid peptides have effects on gastrointestinal motility and mucin secretion in humans.

Antimicrobial peptides and prebiotic peptides

Overview of function and importance

Antimicrobial treatment can help treat or prevent bacterial infections. The current crisis of rising microbial resistance to known antibiotics has led to the need for alternative, novel antimicrobials (Raheem and Straus 2019). Antimicrobial peptides have advantages over antibiotics in that they show slower emergence of resistance, broad-spectrum antibiofilm activity and the ability to modulate the host immune response (Magana et al. 2020). One of the mechanisms by which these peptides kill bacteria is by permeabilizing and disrupting the cell membrane (Corrêa et al. 2019). In general, antimicrobial peptides are typically short, cationic (positively charged) and amphiphilic (having both hydrophilic and hydrophobic components) (Lei et al. 2019). MBPDB includes 186 unique, milk-derived antimicrobial peptides, of which 11 were discovered since our previous database publication. Most antimicrobial peptides derive from bovine lactoferrin (60), α_{s2}-casein (19) and α_{s1}-casein (21). The antimicrobial peptides appear to clusters around specific regions of milk protein parent sequences, such as the N-terminal of α_{S1}-casein (Figure 4), the C-terminal of α_{S2}-casein (Figure 5) and several regions across the sequence of lactoferrin.

Some milk peptides can promote the growth of commensal bacteria (prebiotic action) (Liepke et al. 2002; Arakawa et al. 2015). The database includes three commensal-stimulatory peptides. Often, the prebiotic action of peptides is associated with glycosylation (Goonatilleke et al. 2019; Córdova-Dávalos, Jiménez, and Salinas 2019). Glycosylation state is not currently captured in the database.

In vitro evidence for individual peptides and hydrolysates

Numerous peptides from bovine and human milk proteins have been shown to have *in vitro* antimicrobial activity. Milk-derived peptides have antimicrobial activity against gram-positive bacteria, gram-negative bacteria and yeast, including *Staphylococcus aureus, Staphylococcus intermedius*, *Malassezia pachydermatis, Candida albicans, Bacillus subtilis*, *Escherichia coli, Listeria innocua*, *Micrococcus luteus, Yersinia enterocolitica* and *Salmonella enteritidis* (Biasibetti et al. 2021; Bougherra et al. 2017; Kuhnle et al. 2020; Ouertani et al. 2018; Wang et al. 2020). These findings are based on an array of tests such as disk diffusion assay, minimum inhibitory concentration (MIC) assays and colony-forming unit enumeration (Zanutto-Elgui et al. 2019). Antimicrobial peptides has also been found to be naturally present in bovine-based yogurt (Singh et al. 2020; Azizkhani, Saris, and Baniasadi 2021) and cheeses (Nalepa and Markiewicz 2023). Interestingly, GMP's anti-adhesive effect may not always be beneficial: it also inhibits the binding of certain probiotic organisms, such as *Lactobacillus pentosus*, *Lactobacillus casei* and *Lactobacillus acidophilus*, but not *Lactobacillus gasseri*. The findings that GMP has this effect for some bacteria but not others suggests that GMP binds directly to the microbe rather than to the host epithelial cell (Rhoades et al. 2005).

Animal evidence for individual peptides and hydrolysates

Relatively few studies show the efficacy of antimicrobial peptides in animals (Goldstein et al. 1998; Loury et al. 1999; Gamelli et al. 1998). Some studies have examined the antimicrobial effects of the 64 amino acid-long, bovine κ-casein-derived glycomacropeptide (GMP) produced from the cleavage of milk κ-casein by chymosin during cheese-making or by pepsin during the digestion process. In piglets, oral GMP reduced the percentage of villi with *Escherichia coli* adherence but did not reduce diarrhea (Gustavo Hermes et al. 2013). GMP's ability to reduced observed *Escherichia coli* adhesion was attributed to the prevention of adhesion, not a bactericidal effect. GMP has also been shown to bind bacterial toxins (Brody 2000). Although GMP's ability to prevent bacterial adhesion is typically attributed to its glycosylation, hydrolysis of GMP decreased this bioactivity, suggesting that the peptide moiety is also important (Azuma, Yamauchi, and Mitsuoka 1984).

Clinical evidence for individual peptides and hydrolysates

The evidence of the impact of milk-derived antimicrobial peptides is limited to pre-clinical findings.

Immune system

Immunomodulatory

Overview of function and importance

The immune system is a vital component of human health as it plays a crucial role in identifying and attacking pathogens, aging cells and tumor cells (Martínez-Medina et al. 2022). Strategies for modulating the immune response therefore has great potential to maintain an adequately functioning immune system. Immunomodulatory peptides can either stimulate or inhibit various functions of the immune system. Immunomodulatory milk peptides can interact with an array of immune-related cells. A total of 23 unique milk protein-derived peptide sequences have been found to have immunomodulatory activity, of which three were recently discovered. These peptides derive mainly from bovine milk proteins (β-casein, κ-casein and lactoferrin) and human milk protein (β -casein). A specific sequence pattern for immunomodulatory milk peptides has not been identified, likely because the mechanisms by which peptides can affect the immune system are broad. Rivera-Jiménez et al. suggested that the hydrophobic amino acids are the most frequent amino acids present in immunomodulatory peptides (Rivera-Jiménez et al. 2022). Milk protein-derived immunomodulatory peptides may be useful for immunotherapy as they likely lack negative side effects (as they are the product of millions of years of mammalian evolution to nourish infants).

In vitro evidence for individual peptides and hydrolysates

In vitro studies have shown immunomodulatory activity of both milk protein-derived hydrolysates and single peptides. The *in vitro* testing of these hydrolysates or single peptides are generally conducted using established cell lines, including the mouse macrophage cell line RAW 264.7, the human monocytic model U937, the human monocytic cell line THP-1, and the human T lymphocyte model Jurkat. Milk peptides have been found to stimulate lymphocyte and macrophage activation and proliferation, antibody production and cytokine expression. For example, bovine GMP and a bovine β-casein-derived peptide modulated lymphocyte proliferation (Otani et al. 1995; Laffineur, Genetet, and Leonil 1996). β-casomorphin-7 and β-casomorphin-10 enhanced proliferation of human lymphocytes (Kayser and Meisel 1996). The β-casein-derived peptide QEPVL also increased lymphocyte proliferation and when digested using *in vitro* digestion it was further hydrolyzed into the peptide QEPV which retained its immunomodulatory activity (Jiehui et al. 2014). A human β-casein-derived peptide GRVMPVLKSPTIPFFDPQIP (named BCCY-1) increased chemokine production in monocytes (Cai et al. 2021). Peptides produced from pepsin and trypsin caused differing effects on lymphocyte proliferation depending on protein of origin. Bovine α_{s1}-casein-derived peptides suppressed lymphocyte proliferation, whereas β- and κ-casein-derived peptides increased the lymphocyte proliferation (Sütas et al. 1996).

Animal evidence for individual peptides and hydrolysates

The immunomodulatory activity of milk peptides and milk protein hydrolysates has been evaluated in many different animal models, including a dermatitis rat model and an acute alcoholic liver injury mice model. Atopic dermatitis is characterized by significant skin barrier disruption which activates keratinocytes to develop an extreme Th2-dominant response that strengthens IgE production. Orally administered bovine GMP was shown to inhibit atopic dermatitis in rats by downregulating Th2 dominant immune response (Munoz et al. 2017). This finding suggests that GMP could be an effective alternative therapy for the prevention and management of atopic dermatitis (Munoz et al. 2017). Bovine β-casein-derived PGPIPN attenuated alcohol-induced hepatocyte damage in a dose-dependent manner in an acute alcoholic liver injury mice model (Xu et al. 2020). The bovine β-casein peptide QEPVL inhibited LPS-induced inflammation in Balb/c mice by regulating nitric oxide release and the production of the cytokines (e.g., IL-4, IL-10, IFN- γ and TNF-α) (Jiehui et al. 2014). Trypsin-digested whey protein fed to mice has also been shown to stimulate production of serum IgA and interferon-gamma (IFN-γ) (Saint-Sauveur et al. 2009). Overall, these animal studies indicate that milk-derived peptides can modulate the immune system in various animal disease models.

Clinical evidence for individual peptides and hydrolysates

The evidence of the impact of milk-derived immunomodulatory peptides is limited to pre-clinical findings.

Anti-inflammatory

Overview of function and importance

Inflammation is the immune system's defense response to toxic stimuli, such as pathogens, injury and toxic compounds. Inflammation acts to remove toxic stimuli and initiate healing processes (Guha and Majumder 2019). During acute inflammatory responses, cytokines and chemokines promote the migration of neutrophils and macrophages to the area of inflammation. This mitigation process contributes to restoration of tissue homeostasis and resolution of the acute inflammation. However, uncontrolled acute inflammation may become chronic, contributing to a variety of chronic inflammatory diseases (L. Chen et al. 2018). The common inflammatory response includes the recognition of stimuli by cell surface pattern receptors, the release of inflammatory markers and recruitment of inflammatory cells. Milk protein-derived peptides have potential to alleviate inflammation.

A total of 23 unique milk protein-derived peptide sequences have been found to have anti-inflammatory activity of which 13 were discovered since the MBPDB was first published. These peptides derive mainly from β-casein, α-lactalbumin, β -lactoglobulin, α_{s1}-casein and κ-casein.

In vitro evidence for individual peptides and hydrolysates

In vitro studies have identified individual milk peptides and milk protein hydrolysates with anti-inflammatory activity (Sowmya et al. 2019). For example, LLY from bovine β-casein showed an anti-inflammatory effect *ex vivo* on mice

splenocytes by reducing the secretion of the pro-inflammatory cytokine (IFN-γ) and increasing the production of anti-inflammatory cytokines (IL-10 and TGF-β) as well as improving phagocytosis of peritoneal macrophages. This peptide was also found to be bio-accessible as it was transported intact across a Caco-2 monolayer (Sowmya et al. 2018). The tetrapeptide IPAV from bovine β-lactoglobulin was also able to reduce TNF-α-induced IL-8 expression in Caco-2 cells (Oyama et al. 2017). In addition to individual milk protein-derived peptides, *in vitro* studies also demonstrated that some milk protein hydrolysates have anti-inflammatory activity. For example, a lactoferrin hydrolysate reduced inflammation in human cartilage and synovial cells by up-regulation of interleukin-11 (Yan et al. 2013). A whey protein hydrolysate showed anti-inflammatory activity in lipopolysaccharide (LPS)-stimulated respiratory epithelial cells (Iskandar et al. 2013) and intestinal epithelial cells by reducing interlukin-8 secretion (Piccolomini et al. 2012).

Animal evidence for individual peptides and hydrolysates

Some animal studies have demonstrated the anti-inflammatory effects of milk peptides. For example, apolipoprotein-deficient mice fed VPP and IPP had reduced atherosclerosis development and mRNA expression of inflammatory cytokines (Nakamura et al. 2013). Mice fed the bovine β-casein peptide PGPIPN had decreased levels alcohol-induced pro-inflammatory cytokines in liver tissue (Xu et al. 2020). Likewise, feeding LFP-20, a twenty-amino acid antimicrobial peptide (KCRQWQSKIRRTNPIFCIRR) from the N-terminus of porcine lactoferrin, to mice prior to a lipopolysaccharide (LPS) challenge ameliorated LPS-triggered systemic inflammatory responses (Zong et al. 2019). Bovine GMP fed to rats ameliorated indomethacin-induced enteropathy by alleviating intestinal inflammation and oxidative stress (Cervantes-Garcia et al. 2020).

The anti-inflammatory activity of milk protein hydrolysates has also been evaluated in some animal studies. For example, male Wistar rats fed *Aspergillus oryzae* protease hydrolyzed casein had reduced adjuvant-induced arthritis by inhibiting acute and chronic inflammatory reactions in comparison with controls (Hatori et al. 2008).

Clinical evidence for individual peptides and hydrolysates

The evidence for the impact of milk-derived anti-inflammatory peptides is limited to pre-clinical findings, and further research is needed.

Glycemic control system

Insulin signaling, DPP-IV inhibition and pancreatic B-cell promotion

Overview of function and importance

Type 1 diabetes involves the loss of insulin production, whereas type 2 diabetes involves reduced insulin signaling function. For type 1 diabetes, insulin is provided as treatment. For type 2 diabetes, medications that enhance insulin signaling are typically prescribed. Dairy peptides might provide an alternative treatment for type 1 or 2 diabetes. Dipeptidyl peptidase IV is an enzyme which hydrolyzes incretin hormones like glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide in the liver, muscle and adipose tissues (Rameshrad et al. 2019). DPP-IV inhibitors prevent DPP-IV from degrading those incretins, which suppresses glucagon synthesis, increases insulin release and slows stomach emptying, thus lowering blood glucose levels (Acquah et al. 2022). The MBPDB contains 79 unique milk protein-derived peptides that inhibit DPP-IV, of which 15 were discovered since the MBPDB was first published in 2017. In addition, *in vitro* studies have identified milk peptides that stimulate insulin signaling *via* different mechanisms and promote the growth and regeneration of pancreatic β-cells. The MBPDB contains two peptides that enhance insulin signaling and two peptides that promoted pancreatic β-cell regeneration.

In vitro evidence for individual peptides and hydrolysates

Many studies show *in vitro* DPP-IV inhibitory activity of milk peptides. For example, (Jia et al. 2020) found that a hydrolysate of α-lactalbumin-enriched whey protein concentrate contained several peptides with *in vitro* DPP-IV inhibitory functions, including KILDKVGINYWLAHK, EQLTKCEVFR, VGINYWLAHK, ILDKVGINYWLAHK and LDQWLCEKL. Likewise, peptides derived from trypsin hydrolysis of camel milk proteins, including ILDKEGIDY from α-lactalbumin, ILELA and LLQLEAIR from α_{S1}-casein and LPVP, MPVQA and SPVVPF from β-casein inhibited DPP-IV *in vitro* (Nongonierma et al. 2018). Lacroix et al. identified several DPP-IV inhibitory peptides in bovine milk proteins, including LKPTPEGDL from β-lactoglobulin, and LPYPY, IPIQY and IPI from κ-casein (Lacroix et al. 2017). Bovine κ-casein-derived IPP and VPP (also known ACE-inhibitors) enhanced binding of insulin to its receptor in pre-adipocytes and, thus, enhanced insulin signaling in these cells (Chakrabarti et al. 2018). Bovine κ-casein-derived IPPKKNQDKTE inhibited reactive oxygen species-mediated mitogen-activated protein kinase signaling in an insulin-resistant human liver cancer cell line (HepG2 cells), which ameliorated insulin resistance in these cells (Song et al. 2017).

Animal evidence for individual peptides and hydrolysates

β-casein-derived LPQNIPPL has *in vitro* DPP-IV inhibitory activity and when orally supplemented to rats, lowers blood glucose concentration in a glucose tolerance test compared with placebo-treated rats (Uenishi et al. 2012). This result suggests that either the LPQNIPPL peptide or a partially digested version of that peptide was able to be absorbed into the bloodstream and exert its DPP-IV inhibitory action within the rats. Zebrafish embryos that were exposed to 50 μg/mL of human β-casomorphins YPFVE and YPFVEPI from 3 to 6 days post-fertilization had higher insulin domain of expression (based on staining) compared to an untreated control,

whereas bovine β-casomorphins-5 and 7 had decreased insulin domain of expression. These findings suggests that human β-casomorphins YPFVE and YPFVEPI promote β-cell development and regeneration (Singh et al. 2020). However, the physiology of zebrafish embryos and human infants is quite different, and thus the results may not be translatable.

Clinical evidence for individual peptides and hydrolysates

Currently, there is no human clinical evidence that specific milk peptides can inhibit DPP-IV, enhance insulin signaling or promote pancreatic β-cell growth.

Skeletal system

Calcium absorption and bone health

Overview of function and importance

Osteoporosis is a condition in which bones become fragile and susceptible to fractures and is a significant public health concern. Low calcium intake or absorption and low osteoblast (bone-forming cells) activity are key mechanisms of osteoporosis development. Consuming milk proteins has been shown to promote bone formation and suppress bone resorption in human and animal trials (Aoe et al. 2001; Takada et al. 1997; Toba et al. 2000). These effects might partially be ascribed to casein phosphopeptides (CPPs), which potentially could enhance the absorption of calcium (Ahn and Je 2019), an essential mineral for maintaining bone health. Casein phosphopeptides' calcium-binding properties also enable them to support teeth remineralization in cases of tooth decay (Thierens et al. 2019). A total of eight unique milk protein-derived peptide sequences have been found to have osteoanabolic activity.

In vitro evidence for individual peptides and hydrolysates

The primary known activity of these peptides is enhancement of osteoblast differentiation. For example, the bovine lactoferrin-derived peptide FKSETKNLL increased the proliferation of mouse osteoblast cells (Shi et al. 2020). Likewise, α_{s1}-casein, α_{s1}-casein, β-casein and κ-casein-derived peptides from buffalo and bovine milk (EDVPSER, NAVPITPTL, VLPVPQK and HPHPHLSF, respectively) promoted differentiation of primary rat osteoblast cells (Reddi et al. 2018). Bovine β-lactoglobulin-derived YVEEL and YLLF, as well as α-lactalbumin-derived WLAHK, activated osteoanabolic activity in osteoblast cells isolated from rat calvaria (Pandey et al. 2018; Pandey, Kapila, and Kapila 2018). Beyond osteoblast differentiation enhancement, individual CPPs from β-casein and α_{s1}-casein (Cao et al. 2017) and a pool of CPPs enhanced Ca^{2+} uptake in Caco-2 cells (Liu et al. 2018).

Animal evidence for individual peptides and hydrolysates

Animal evidence for milk peptides enhancing bone growth in animal models is rare. One study showed that two peptides with confirmed *in vitro* activity (bovine β-lactoglobulin-derived YVEEL and YLLF) enhanced bone formation markers and suppressed inflammatory cytokines when fed to ovariectomized osteoporotic rats in comparison with the negative control group, which suggests that these peptides have osteoprotective potential (Pandey et al. 2018). CPPs have been found to enhance serum calcium levels, promote bone formation and decrease bone resorption in rats compared with control groups (Liu et al. 2021). Likewise, in a growing mouse model on a restricted protein diet, those fed a soy-based diet showed impaired bone health as measured by a lower femoral cortical thickness, bone volume, trabecular number and thickness, whereas those fed a casein-based diet mainly retained their bone health (Rouy et al. 2014). As the bioavailability of soy and casein is different, the effect cannot be directly ascribed to an effect of peptides.

Importantly, CPPs and osteoblast differentiation-enhancing peptides would likely need to partially survive digestion to act at their perceived sites of action, the gut, the bloodstream and bone. *In vitro* digestion studies indicate that CPP are partially resistant to gastrointestinal digestion (Perego et al. 2015) and these peptides have been confirmed to be present in the gastrointestinal tract and feces of rats after feeding with CPPs (Kasai et al. 1995). Whether osteoblast differentiation-enhancing peptides are absorbed in the gastrointestinal tract remains unknown.

Clinical evidence for individual peptides and hydrolysates

Cohort studies suggest that greater consumption of fermented milk products is associated with improved bone health (Biver et al. 2018; Ong et al. 2020). However, there is currently no clinical evidence for an effect of milk peptides on osteoblast differentiation and bone formation in humans. Evidence from human trials have confirmed the effect of CPP on teeth remineralization (Thierens et al. 2019; de Oliveira, Barreto, and Tostes 2022). A human clinical study found no enhancement of calcium absorption when feeding adults CPP (Teucher et al. 2006).

Cancer

Overview of function and importance

There is a need for development of therapies for cancer that have improved efficacy and lower side effect risks than current treatments. Some milk peptides can induce apoptosis in cancer cells *in vitro* and suppress tumor cell invasiveness in animal studies (Bielecka, Cichosz, and Czeczot 2022). A total of 18 milk protein-derived peptides have been found to have anticancer activity. These peptides derive from α_{s1}-casein, β-casein, κ-casein and lactoferrin.

In vitro evidence for individual peptides and hydrolysates

Numerous milk peptides have demonstrated cytotoxic effects in *in vitro* cancer cell models. For example, bovine α_{s1}-casein-derived LKK, RPK and YK and bovine κ-casein-derived FFSDK showed a dose-dependent cytotoxicity toward transformed human leukemic T and B cells; however, these peptides also killed healthy mouse T and B cells (Matin and

Otani 2002; OTANI and SUZUKI 2003). Bovine β-casein-derived peptide PGPIPN induced apoptosis in human ovarian cancer cells (Guo et al. 2021). Two peptides, YQEPVLGPV RGPFPIIV and SLPQNIPPLTQTPVVVPPF, derived from bovine β-casein reduced proliferation of human colorectal cancer cell line inducing apoptosis and cell cycle arrest (Sah et al. 2016). Bovine milk α_{s1}-casein-derived RYLGYL and RYLGYLE, human milk α_{s1}- casein-derived YVPFP, bovine β-casein-derived YPFPGPI, YPFPG and YPFP, yak milk β-casein-derived TPVVVPPFL and human β-casein-derived YPFVEPI inhibited *in vitro* human breast cancer cell proliferation (Hatzoglou et al. 1996; Kampa et al. 1996; Gu et al. 2022). Bovine lactoferrin-derived peptides induced apoptosis in various cancer cell lines; FKCRRWQWRMKKLGAPSITCVR in human myeloid leukemia cells (Roy et al. 2002), and FKCRRWQWRMKKLGAPSITCVRRAF in human breast cancer cells (Furlong, Mader, and Hoskin 2006), human monocytic leukemia cells (Yoo et al. 1997), human neuroblastoma cells (Eliassen et al. 2006) and human T leukemia cells (Mader et al. 2007). Bovine β-casein-derived peptide, INKKI, was cytotoxic to mice melanoma cells (Azevedo et al. 2012). Lactaptin, a proteolytic fragment (residue 57-134) of human κ-casein, induced apoptosis in human breast adenocarcinoma cells (Nekipelaya et al. 2008).

Animal evidence for individual peptides and hydrolysates

A few peptides from bovine milk showed anticancer effects in *in vivo* models. For example, tumor-bearing mice injected with bovine β-casein-derived INKKI showed decreased tumor volume, number of metastases and delayed tumor growth compared with control groups treated with saline only (Azevedo et al. 2012). Additionally, bovine β-casein-derived PGPIPN decreased tumor growth rate when injected into mice with ovarian cancer compared to a saline control group (Wang et al. 2013). Lactoferrin-derived FKCRRWQWR MKKLGAPSITCVRRAF injected in xenograft neuroblastoma model rats inhibited tumor growth compared with a saline control group (Eliassen et al. 2006). A recombinant analogue of peptide containing lactaptin suppressed the growth of solid tumors in mouse xenograft bearing breast cancer cell compared with a saline control group (Koval et al. 2014).

Clinical evidence for individual peptides and hydrolysates

The evidence of the impact of milk-derived anticancer peptides is limited to pre-clinical findings and further research is needed.

Future research directions

A key problem in this research field is that most work examines the *in vitro* functional activities of peptides created from *in vitro* enzymatic hydrolysis or in dairy products themselves. This strategy ignores whether these peptides will survive intact to their site of bioactivity and thus have potential to be biologically relevant. Some of the *in vitro* digests attempt to simulate human gastrointestinal digestion, whereas others use an array of non-biologically relevant digestion methods or enzymes. Whether studies that simulate human digestion can match the peptide profile of that created *in vivo* in humans is not clear. Completely matching the complex milieu of the digestive system is highly complex. Later studies identified some of these bioactive peptides in infant gastrointestinal digesta (Nielsen et al. 2018), which may indicate biological relevance within the gut. More work examining what peptides are released at various stages across human digestion is essential. Digestive samples can be obtained *via* nasogastric and nasojejunal tubes and as stool samples in humans. Once peptides are identified, the field needs to assess these peptides for functions relevant to the site at which they are collected (e.g., the interaction of peptides with gut epithelial cells and gut immune cells).

Moreover, it is particularly essential for researchers to examine which milk peptides actually survive across digestion, are absorbed and can be found in the circulatory system. The majority of identified bioactive milk peptides have functions that can only occur if the peptide can reach the circulatory system (e.g., ACE-inhibitory). Yet, we have limited evidence of milk peptides surviving to the bloodstream. Recently, Caira et al. were able to identify bovine milk peptides in the blood of adults post-consumption, including 44 that are known bioactive peptides (Caira et al. 2022). To validate the functional potential of these peptides, we must comprehensively assess the survival of peptides from various milk products to the bloodstream. For bioactive peptides that are demonstrated to not survive gastric or intestinal digestion and not reach their desired sites of action, encapsulation strategies or direct injection can be explored.

Though much milk bioactive peptide research examines hydrolysates (mixtures of peptides), identification of the function of a specific peptide within the mixture typically relies on testing synthetic candidate peptides. Though many milk protein-derived peptides are modified naturally with phosphorylation, glycosylation and disulfide bridges, these synthetic peptides used for testing commonly lack these modifications as they are more expensive and difficult to synthesize. Therefore, the effect of post-translational modifications on milk peptide bioactivity are mostly overlooked and should be the subject of future research.

Milk peptides have an array of functions in *in vitro* and in animal studies including antioxidant, ACE-inhibitory, opioid, antimicrobial, immunomodulatory, anti-inflammatory, enhancing calcium absorption, enhancing osteoblast differentiation and anti-cancer. As milk peptides are the product of more than 200 million years of evolution for infant nourishment, they likely have few, if any, side effects—which is not true for typical small molecule drugs. These bioactivities make milk peptides attractive targets for therapeutic development to prevent or treat human diseases like osteoporosis, oxidative stress-induced injury, bacterial infections,

hypertension, inflammation, chronic pain, type II diabetes and cancer. However, for the most part, there are very limited studies examining the efficacy of these peptides in human subjects (as reviewed recently (Nongonierma and FitzGerald 2015)). More clinical studies are needed to advance applications of milk peptides to human health.

Some milk peptides may have utility for enhancing food preservation such as antimicrobial peptides to prolong shelf-life of non-sterile products or antioxidants to prevent oxidative changes to foods (e.g., lipid or protein oxidation) (Rai et al. 2016; Khan et al. 2018).

Conclusion

Overall, milk and milk products contain an immense array of known functional peptides that could affect cardiovascular, immunological, digestive and skeletal health, as well as potentially glycemic control, cancer development, skin health and the nervous system. From the available literature, we were able to extract information about the functionality of milk-derived bioactive peptides and make it available through an online database. We demonstrated that most regions of major milk proteins contain encrypted bioactive peptides with a broad range of functions. Based on this collected data, we provided an updated, comprehensive review of known milk peptide functions. For each functional category, we assessed the current level of evidence, including *in vitro* assays, animal studies and clinical studies. The database is highly useful for peptidomics research and future research on bioactive peptides, as well as for development of in silico tools for the prediction of bioactive peptides.

Disclosure statement

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