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Bioactive Peptides from Milk

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Abstract

Milk is a major source of dietary energy, protein and fat. Due to their specific biological properties leading to health benefits, bioactive peptides (BAPs) derived from milk proteins have been a subject of intensive research during past two decades. These peptide sequences, encrypted within proteins, are liberated *in vivo* during gastrointestinal digestion or *in vitro* by fermentation with proteolytic starter cultures or using proteases. BAP generally comprises 2–20 amino acid (AA) residues. Isolation and characterization of BAP of different bioactivities from milk protein hydrolysates of cow, buffalo, camel, goat, mare, sheep, donkey and yak milks have been reported. Bioactivities of BAP, which depend on constituent AAs and the sequence, include mineral binding, opioid, angiotensin-converting enzyme (ACE) inhibition, immunomodulatory, cytotoxicity, antibacterial and antithrombotic. This chapter focuses on the methodologies adopted to produce BAPs and their prospective role in health enhancing nutraceuticals/pharmaceuticals.

Keywords: bioactive peptides, bioactivities, casein, milk proteins, nutraceuticals, whey

1. Introduction

One of the achievements of mankind over a century is the remarkable progress in the field of healthcare including management of communicable diseases leading to increase in life expectancy of population around the world. However, there is a tremendous upsurge in non-communicable diseases (NCDs) such as cardiovascular diseases (CVDs), diabetes, obesity, etc., which are associated more to life style changes and eating habits than to hereditary [1]. About two-thirds of the 57 million deaths that occurred globally in 2008 were due to NCD [2]. Further one-fourth of these deaths occurred before the age of 60. One among the four major behav-

Journal risk factors identified for NCD is unhealthy diet. Healthy diet and physical activity were an integral part of the WHO's 2008–2013 action plan of the global strategy for the prevention and control of NCDs. Essential nutrients, standards and guidelines for diet and food preparation were considered prime for nutrition in the twentieth century [3]. New challenges now arise due to increased life expectancy, drastic life style modifications and elevated costs of healthcare. This has directed to new nutritional concepts which target maximum well-being and good health along with minimum disease risk throughout the lifespan. Nutraceuticals and functional foods are an attempt towards resolving these health issues and to create a healthy society [4]. The idea of nutraceuticals/functional foods is more often mentioned as an emerging field. However, the concept has been well acknowledged in ancient Indian Vedic texts and Chinese traditional medicine where it quotes "food and medicine have common source." Egyptians and Sumerians are just a few other civilizations that show evidence of food being used as medicine and in disease prevention [5].

There is no agreement on the definition of nutraceutical and functional foods. The term nutraceutical was first mentioned by Defelice in 1989 [6]. It was defined by Zeisel as "those diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix and used to enhance health in dosages that exceed those that could be obtained from normal foods" [7]. European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) originally proposed that a food can be considered "functional" if it provides positive impact upon one or more physiological functions in addition to its nutritional effects and improves health via reducing disease. Nutraceutical is also defined as an isolated or purified product from food that is generally in medicinal form and not generally related to food where as a functional food is defined as foods that offer more than basic nutrition by providing additional physiological benefit [8]. The biological active substances in functional foods (designer foods/medicinal or therapeutic foods) can either be an essential macro/micronutrient, a non-nutritive component or a component whose nutritive value is not listed as essential [9]. Functional foods are generally grouped into conventional foods containing natural bioactive substances, enriched or modified foods with bioactive substances and synthesised food. These foods as alone or in combination are considered functional foods. Foods/food product could be prepared using different approaches to make it functional: (a) by removing a component that may be identified to cause harmful effect when consumed, (b) elevating a constituent naturally present to induce targeted effect, (c) introducing a micro/macro nutrient for its positive effect and (d) substituting a constituent usually with macronutrient or increasing bioavailability of a food component [5]. Japan was the first country to emerge in production of functional foods to cope with accelerating cost of healthcare in 1980, mainly to improve the health condition of the aging population [5, 10]. Scientific studies have catered to the growing awareness on the relationship between nutrition and health issues that have led the end users' attention towards healthy foods. Functional or biologically active molecules from food have been widely studied and assessed for moderating these conditions as well as to restore their normal physiological functioning.

Biopeptides from dietary proteins have exhibited potential benefits to intervene these abnormal biophysiological conditions [11]. Bioactive peptides (BAPs) have been defined as

protein fragments that have a progressive impact on body functions or conditions ultimately influencing health [12]. When BAPs are encrypted within their parent proteins, they are described as “cryptide” or a hidden peptide and are released by gastric digestion of food proteins or by exogenous/endogenous proteolysis by plant, animal or microbial proteases (especially during fermentation) [13]. These peptides demonstrate diverse physiological functions including metabolic functions, immunomodulatory, microbicidal, thrombolytic and pre/probiotic functions in human system [14]. Existing knowledge about milk-protein-derived BAPs as pharmaceutical constituent for potent drug or dietary supplement in the form of formulations of nutraceuticals is reviewed in detail in this chapter.

2. Sources of BAPs

Food proteins are not only a source of nutrients for maintenance of proper body functions but are also considered as a source of BAPs which can promote health and prevent diseases. BAPs are released from food proteins by endogenous proteolysis during gastric digestion or by exogenous hydrolysis using physical (heat), chemical (acid/alkali) or plant/animal/microbial proteases during food processing [15, 16]. Unlike physical/chemical treatments, enzymatic hydrolysis has an advantage of producing intact BAPs without any residual/toxic chemicals in the end products [17]. The size of active sequences may vary from 2 to 20 amino acid (AA) residues and generally have molecular weight <6000 Da [18]. BAP from different sources are found to possess common structural properties with hydrophobic AA residues in addition to lysine, proline or arginine [19]. Low molecular weights (dimer–heptamer) of BAPs are resilient to gastrointestinal (GI) tract enzymes and are easily absorbable in an intact form into the blood stream, hence suitable for therapeutic formulations or as functional foods. Higher molecular weight BAPs, in contrast, are possibly degraded during their passage through GI tract making them either inactive or less active. However, in few cases they may also become more active and thus more absorbable to be transported to target organs. Although milk proteins are considered as the most important source for BAPs, animal, plant and marine proteins also contain potential bioactive sequences [13, 20, 21].

2.1. Milk as a source of BAP

Milk is a characteristic secretion by female mammals with an array of bioactive substances to meet whole nutritional requirements, defensive and physiological functions of the young ones of that particular species. It is considered as one of the nearly complete single foods available in nature for maintaining human health and growth. Although the major mammalian milk that is consumed by humans is that from bovine, milk from sheep, goats, yak, horses and camels is also used. Constituents of milk are similar among species but differ significantly between species [22]. Major constituents of milk include carbohydrates, proteins, lipids, micronutrients and traces of various other organic and inorganic compounds. Lactose is the key carbohydrate present in milk, which is a disaccharide formed from glucose and galactose with β 1-4 glycosidic linkage. Fat content of cow's milk is ~3.25%. Bovine milk fat composition is distinct from other sources due to their diet and the presence of a rumen.

Normal bovine milk comprises about 3.5% of protein, 80% and 20% of which are constituted by casein and whey proteins, respectively [23]. Sub-fractions of casein and whey possess specific biological properties.

Milk from different animal sources, such as bovine, goat, yak and donkey, has been extensively studied for production of BAPs and for their potential positive impact on health, and physiological benefits have been experimentally investigated [24]. Among these, cow's milk has proven to be the best source. To effectively produce biofunctional peptides, a combination of such processes is also preferred [15]. Expression of dormant bioactivities of BAPs, encrypted within the sequence of parent proteins, depends on the site of proteolysis, i.e., in mammary gland or at a GI location as well as the specificity of proteases. Milk-protein-derived BAPs have claimed to be health enhancing components that can be used to reduce the risk of disease or to enhance a certain physiological function, such as potential antimicrobial, antihypertensive, antioxidative, cytomodulatory, immune stimulating, opiate effects, etc., in humans. BAPs derived from milk protein may function as exogenous regulatory substances at diverse intestinal and peripheral sites of mammals. They may be absorbed via carrier-mediated or paracellular transport to regulate activities of target organs [12].

The primary physiological role of casein was considered to be a source of AAs required for neonatal nutrition. However, subsequent studies have shed light on its diverse physiological roles including prevention of pathological calcification of mammary gland by micellar caseins [25]. It is a phosphoprotein that precipitates at 20°C from raw skimmed milk at pH 4.6. The electrophoretic separation and AA sequence homology evaluations of different casein fractions revealed its heterogeneous nature with four major families of casein: α_1 , α_2 , β and κ [26]. Individual casein differs in the phosphate content and calcium-binding properties. Generally, α_1 -, α_2 - and β -caseins bind calcium strongly and precipitate at relatively low calcium concentrations and hence referred as "calcium-receptive caseins" having concentrated phosphoserine and proline regions. In contrast, κ -casein is "non-receptive casein" (not sensitive to these calcium concentrations), which can stabilise up to 10 times its mass of the calcium-sensitive caseins and possesses one phosphoserine [27]. Caseins α_1 and β have potential to liberate more than 20,000 biofunctional peptides each. Biopeptide from casein was the first identified food-derived BAP that boosted Vitamin D-independent bone calcification in rachitic infants in 1950 [28]. Few milk-derived peptides reveal multi-functional properties, i.e., specific peptide sequences having two or more different biological activities, as casein's primary structure possesses overlapping peptides or "strategic zones" in certain regions. These zones are generally protected from proteases [29]. For example, a strategic zone containing immuno-stimulating and opioid peptides has been reported to reside between residues 51–63 and 60–70 of human and bovine β -casein, respectively [30, 31].

Whey is the chief by-product or even considered as a co-product of cheese and other dairy industries. Concentration of protein in whey depends on various factors, such as milk source, time of the year, type of animal feed, lactation stage, processing quality and whey type [32]. Whey proteins constitute 20% of the whole milk protein, which are heterogeneous in nature. Discovery of whey dates back to 3000 years ago. Seventeenth and eighteenth centuries observed whey being used for its medicinal purpose [33]. Whey proteins are now consid-

ered as the highest quality natural protein available [34]. In recent past, there has been an exponential research on whey proteins due to the presence of nutrients that are found to have an active role in the betterment of human health [35, 36]. Whey proteins constitute 20% of whole milk protein which are heterogeneous in nature. All whey proteins are soluble, perform functions like carrier ligands and are involved in various biological activities. Elevated nutritional values of whey proteins are attributed to their diverse AA composition [15]. Globular whey proteins contain acidic/basic and hydrophilic/hydrophobic AAs along their polypeptide chains, well balanced throughout α -helix motifs [35]. With increased magnitude of dairy industry, enormous quantity of whey is produced across the globe. A substantial portion of it is wasted or underutilised as animal feed, in turn increasing the cost of its disposal. Use of whey as a source of BAP provides economic health potential for global population [37, 38]. Under controlled conditions, hydrolysis of whey releases peptides that could be a part of preventive or therapeutic applications [39]. Recent literature reports some biologically important whey peptides: α - and β -lactorphin, lactoferricin B, glycomacropeptide (GMP) to name a few. Commercial formulations with whey as an ingredient are also available, making it a value-added product [40].

3. Production and purification of milk BAPs

Selection of appropriate food protein followed by its enzymatic hydrolysis is the general process used for BAP production. BAPs can be released by *in vivo* or *in vitro* hydrolysis of the parent proteins. They can be produced *in vitro* by (1) enzymatic hydrolysis with digestive enzymes, (2) fermentation of milk with proteolytic starter cultures and (3) proteolysis by enzymes derived from animals, plants or microorganisms [14]. Once the AA sequence is known, it is also possible to synthesise BAPs through the chemical methods, enzymatic synthesis and/or by recombinant DNA technology [15].

3.1. Protein hydrolysis under simulated gastrointestinal tract conditions

In vivo digestion of protein, although possible, is rarely used for the production of BAPs as it requires removal of intestinal contents from animals after feeding protein diet. However, it can be simulated. The protein of interest is first treated with pepsin for a few hours to stimulate digestion, followed by treatment with pancreatin or with a combination of trypsin and chymotrypsin mimicking digestion in stomach at specific pH. This method also enables estimation of BAPs released from food proteins though the stimulation of intestinal proteases after food intake is not taken into consideration [12]. BAPs produced via *in vitro* stimulation of gastric enzymes from milk are mainly ACE inhibitory peptides. Digestive enzymes in combinations with other proteases from bacterial/fungal sources are also used in the generation of BAPs from various food proteins [22].

3.2. Fermentation with proteolytic starter cultures

The presence of lactic acid bacillus (LAB) in milk fermentation can be either as spontaneous or as inoculated starter cultures. Although under spontaneous fermentations the growth of LAB cannot be predicted or controlled, this procedure has been practiced and carried out traditionally for years [41]. The fermentation process by lactic acid bacteria starter cultures, used in the dairy industry, involves the hydrolysis of milk proteins (particularly casein) into peptides and AAs which are used as nitrogen sources necessary for their growth. Several lactic acid bacteria are widely used and their role can be divided into starter and non-starter cultures. The proteolytic system of lactic acid bacteria consists of a cell wall-bound protease and other intracellular proteases [42]. Different starter cultures and fermentation conditions affect the composition and nature of released BAPs. Studies have exhibited that LAB proteases especially from *Lactococcus* sp. and *Lactobacillus* sp. could hydrolyse $\geq 40\%$ of peptide bonds of α_1 and β caseins resulting in the release of various oligopeptides that are further hydrolysed by complex peptidases [43]. Pure cultures as well as mixed cultures are used for fermentation. Apart from lactic acid bacteria, yeast and other microflora may also be present in a few commercial starter cultures. Peptide profiling of bovine kefir, which is a fermented milk beverage, revealed 236 unique peptides that were released from caseins during its production by kefir grains [44]. BAPs with physiological benefits, such as ACE inhibition, immunomodulation, anti-oxidative, antimicrobial, etc., have been identified from microbial fermented products [45–47]. An endopeptidase from *Lactobacillus helveticus* improved the production of casein-derived ACE inhibitory peptides, VPP (Val-Pro-Pro) and IPP (Ile-Pro-Pro) via carboxy terminal processing mechanism [46]. Even with insufficient evidence to establish the cause-effect relationship between the maintenance of blood pressure (BP) and the consumption of fermentation-derived peptides, food products fortified with such BAPs could prove valuable as nutritional therapeutics at least in certain sub-population [42].

3.3. Enzymatic hydrolysis by proteases

Plant, animal and microbial proteases have been used for the production of BAPs from the protein source. Enzymatic hydrolysis is more suitable for the production of BAPs, especially in food and pharmaceutical industries, due to the lack of residual organic solvents or toxic chemicals in the end product. Further hydrolysis takes place under mild controlled conditions and yield-predictable end product. The widely used enzymes of animal and plant origin include pepsin, trypsin, chymotrypsin, bromelain, papain or ficin. One of the best and cheap sources of animal origin proteinases is pancreases, by-products of the meat industry. Recently, proteases from plant sources are gaining attention. ACE-inhibitory peptides and anti-ulcerative peptides were studied from peptides synthesised from whey protein hydrolysis by *Cynara cardunculus* [48, 49]. Proteases from extracts of celery, fennel, parsley and ficin latex have shown potential in releasing peptides from skim and whole milk with ACE inhibitory and antioxidant properties [50, 51]. However, these preliminary observations need further validation through clinical studies involving human volunteers to confirm such promising *in vitro*/animal study results [49]. Microbial proteases widely used are those obtainable from the *Bacillus* sp., *Bifidobacterium*, proteases from the LAB and few fungi [14, 17]. Furthermore, LAB and their

products are considered safe (generally recognized as safe, GRAS). Microorganisms are a relatively cheap source of proteases. Microbial proteases, especially from bacteria, provide few advantages over proteases from other sources. A recent study showed that *Bifidobacterium bifidum* MF 20/5, *Arsukibacterium ikkense*, *L. helveticus*, *Bacillus thermoproteolyticus*, etc., could release peptides from milk and milk products that were potent ACE inhibitors, antimicrobial and antioxidant agents [52–55].

3.4. Chemical synthesis and recombinant DNA technology

Enzymatic production of peptides generally does not produce adequate quantities and is not always economical. Chemical and recombinant technologies are used in such conditions to produce peptides in large quantities. However, they require specialised equipment, skilled labours and incur high cost. Further these techniques can be employed only when the peptide sequence is known. The size of peptide determines the kind of technology to be used. Chemical synthesis is preferred for producing medium and small peptides (5–80 AAs) and recombinant technology for larger peptides and proteins [56]. Numerous such peptides have been approved to be used as drugs in the past decade. This application greatly depends on chemical modifications of peptides to improve membrane permeability, receptor affinity and stability along with decrease in hepato-renal clearance of the peptide. It is also proven that bioavailability of peptides improves with chemical modification, such as bond replacement and conformational modifications including peptide cyclisation. Chemical synthesis has proved that large-scale peptide synthesis at reasonable cost can be achievable through solid phase [57]. Easy peptide separation from other products and impurities remain its major advantage. However, requirement of side-chain protection, racemisation, decrease in yield during removal of protective chains and toxic nature of solvents used are a few drawbacks associated with this method. Chemical synthesis is still considered as one of the best suited procedures to synthesise medium-sized peptides of therapeutic significance [16].

Recombinant DNA technology involves gene expression in microorganisms using modern techniques of cloning to synthesise one or more peptides concurrently. This method requires high cost involving research; however once established it will lead to their economic yield. The *Escherichia coli* based bacterial expression system is widely preferred. Human and bovine β -casomorphins were produced through this technique [15]. Particularly, this technology finds superiority in synthesis of large peptides and proteins [16]. Additionally, it comparatively offers effective alternative for bulk production of peptides [58].

3.5. Purification and characterisation

Purification of peptides after its production from any of the above-mentioned process is prerequisite for its industrial use. Peptides with more than 95% purity is required for NMR studies, enzyme studies, monoclonal antibody production, in treatment of disease, clinical research and structure–function relationship studies. Immunological studies and PAGE analysis require purity of >70–80% [58]. Ultra-filtration is performed using series of membrane filters with different molecular weight cut-off resulting in minimising non-peptide concentration in the hydrolysate. Purification procedure often used is RP-HPLC. This enables

rapid purification and detection of peptides in a mixture. Ion exchange, affinity, size-exclusion chromatography and capillary electrophoresis are also being preferred [12]. Peptide characteristics are evaluated through mass spectrometry [58].

4. Absorption of BAPs

BAPs produced after their release from parent proteins by GI enzymes at times exert their activity locally or after absorption at the peripheral organs [14]. Some peptides may undergo further hydrolysis by peptidases in the course of transport of brush border and cytoplasm. Intestinal absorption plays an important role in the effectiveness of their biological functions. Even with little unambiguous *in vivo* evidence, the absorption of BAPs is suggested to be possibly operating through any of the following pathways: carrier-mediated transporter system, passive diffusion and transcytosis, paracellular pathways via tight junctions and endocytosis [21].

4.1. Carrier-mediated transport system

Carrier-mediated transport system actively transports bi- and tripeptides. Cytoplasmic peptidases hydrolyse peptides to AAs in the intestinal epithelial cells; however, some peptides resist this hydrolysis and are transported across the membrane [59]. PepT1 (transmembrane protein) is an intestinal proton-dependent peptide transporter with a wide range of substrate specificity for more than 400–800 di- and tripeptides. L-AA-containing peptides are preferred over D-AA/D-stereoisomers for transporting across membranes [60]. It is a bidirectional transporter where membrane potential and proton gradient decide the rate and direction of absorption. About 70–80% of peptides are absorbed through PepT1 transport system due to its high transporting capacity and intestinal expression [61].

4.2. Passive diffusion and transcytosis

Maternal colostrum-derived γ -globulin permeability associated with passive immunisation by mammalian neonatal small intestine has long been established. Lipid-soluble peptides easily enter enterocytes and are hydrolysed by the action of cytosolic peptidases releasing AAs for intercellular metabolism. Some AAs from enterocytes are moved into portal circulation through transporters on basolateral membrane [62]. Role of this system in absorption is considered small, as peptides are most likely to be hydrolysed by cytoplasmic peptidases [63]. Large polar proteins (>600 Da) are hydrolysed before they enter into enterocytes. Vesicles are formed by invagination of apical membranes that capture the large peptides. These vesicles fuse with lysosomes to form phagolysosomes where enzymatic digestion of the macromolecule occurs. Proteins that resist this hydrolysis could be drawn into enterocytes by means of receptor-mediated, absorptive or fluid-phase transcytosis to be secreted into basolateral membrane. Di- and tripeptides are generally hydrolysed by cytosolic peptidases; however, those that resist are hydrolysed by vascular endothelial tissue/plasma peptidases [63–65].

4.3. Paracellular pathway through tight junctions

Paracellular pathway is considered important in BAP absorption in intact form. Tight junctions are formed by adhesive membrane proteins (claudins, occludin, tricellulin and junctional adhesion molecule A) between intestinal epithelial cells creating small pores to enable passive diffusion of peptides. Diffusion through tight junctions depends on permeability and elasticity of these junctions that are controlled by these proteins [65, 66]. Tight junctions generally allow diffusion of cations and inert small molecules (<600 Da) and also larger oligopeptides [67].

4.4. Endocytosis

Intestinal epithelium of adult mammals could absorb proteins in small quantities by endocytosis even though such absorption is much smaller (<0.1%) in comparison with other absorption system; however, they are biologically significant. Larger peptides are generally internalised by endocytosis [fluid phase and/or absorptive (receptor)]. Fluid phase (pinocytosis) is non-specific endocytosis that begins with plasma membrane “pinching off” to vesicle that contains dissolved peptides in extracellular (EC) fluid. Vesicle or pinosome drifts inwardly to perinuclear region to bind with lysosome. Peptides are hydrolysed during this and vesicle is recycled back to plasma membrane. In absorptive or receptor endocytosis, the membrane binding site is specific for the peptide to be transported. Receptor–ligand binding results in clustering of this complex into clathrin-coated regions of plasma membrane followed by endocytosis. These complexes are transported to endosomes where low pH of the endosome disassociates receptor–ligand complex and receptor is either recycled to plasma membrane or may be degraded [67, 68].

5. Bioactivities of BAPs from milk

Naturally occurring bioactive molecules are preferred in comparison with chemosynthetic components for functional food preparation. Protein-derived BAPs are presently gaining attention due to their safe and economic benefits [69]. Food technologists and scientists have been concentrating in understanding relationship between casein- and whey-derived BAPs with their bioactivities. Deciphering their AA sequences along with an understanding on their bioavailability and stability *in vivo* have helped in the development of health-promoting milk protein [70]. **Figures 1 and 2** illustrate various bioactivities of milk-derived peptides. However, relationships between their structural properties and functional activities have not been completely elucidated. Many BAPs from milk proteins are relatively small (e.g., 2–9 AAs), possessing hydrophobic AA residues in addition to proline, lysine or arginine groups. Several *in silico* approaches including quantitative structure activity relationship (QSAR) as an application of chemometric and bioinformatics methods have been used in an attempt to predict the activity of these peptides. Structure–function relationship elucidating studies highlighted the significant contribution by specific AA residues, such as tyrosine (Y), leucine (L), proline (P) and tryptophan (W), present in BAPs for diverse physiological activities [71]. Recent studies have shown that many tryptophan(W)-containing peptides originate from milk

proteins, which have been shown *in vitro* to display a wide range of bioactivities such as ACE inhibition along with antioxidant, antidiabetic and satiating-related properties [71].

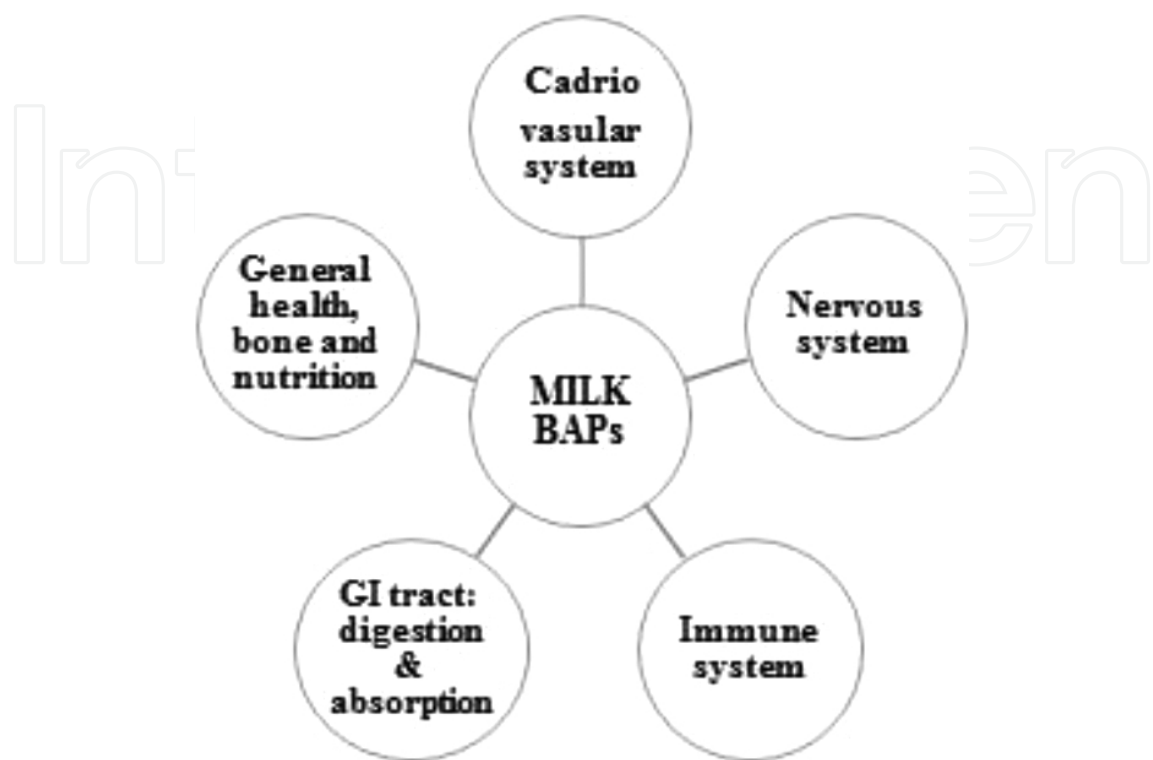


Figure 1. Physiological role of milk bioactive peptides [72, 26].

BAPs from Milk proteins (casein and whey)			
ACE Inhibitor	Antioxidant	Anticancer	Antimicrobial
• VPP • RYLGY • AYFYPEL • YQEP • VPKVK • YQEPVLGP • RPK • RPKHIKH • LQKW • LLF • IIAEK • IPAVFK	• YFYPEL • SSSEE • VAGTWY • INYW • LDQW • WYSL • DMPIQ • LPLPLL • VPYPQR • DKIHPEA • GPFPIIV • KVLPPVQK • QPHQPLPPT • YPFPGPIPNS • WYSLAMAASDI • LQKW • LDTDYKK	• VYQHQAAMKPWIQPKTKVI PYVRYL • VYQHQAAMKPWIQPKTKVI PYVRY • AMKPWIQPKTKVIPYVRYL KPWIQPKTKVIPYVRYL • LKKISQRYQKFALPQY • FKCRRWQWRM	• IKHQGLPOE • VLNENLLR • SDIPNPIGSEN • SEK

Figure 2. Representative BAPs from milk proteins and their prominent bioactivities [40, 73, 74]. *AA represented by one letter code.

5.1. Functionalities of casein and whey peptides

5.1.1. Mineral binding property

Normal bone development in humans requires adequate calcium supply. Low ingestion of calcium levels are prevalent among infants (mostly pre-term), young and post-menopausal women, elderly who have low calcium absorption and those in treatment or prevention of osteoporosis and children with rickets [75]. Enhancement of Vitamin D-independent bone calcification was the suggested role of casein-derived phosphorylated peptides in rachitic infants [14]. Specific caseinophosphopeptides (CPPs) that are phosphorylated regions released from α - and β -caseins can form soluble organophosphate salts functioning as mineral carriers, especially for calcium. CPPs were the first BAPs to be mentioned by Mellander [28] in 1950. CPPs have been shown to possess mineral binding properties thereby improving bioavailability of metal ions [76]. CPP-ACP (amorphous calcium phosphate) exhibited anti-plaque, remineralisation and demineralisation prevention of teeth [77]. Increased bioavailability of iron in the presence of CPP has also been reported [78]. Whey peptides also exhibited mineral-binding properties [79]. Tryptic hydrolysates of whey proteins inhibited calcium phosphate formation thereby favouring calcium absorption. [80]. Significant elevation in mice calcium absorption in the presence of tryptic hydrolysate of whey (Ser-Thr-Glu-Tyr-Gly) suggested their possible utility as functional food or dietary supplement to prevent calcium deficiency [81].

5.1.2. Antioxidant activity

Aerobic metabolism inherently releases free radicals which are scavenged by antioxidants in the biological system that protects against the damage caused by reactive oxygen species. Free-radical-mediated reactions play a major role in cellular damage and aging via lipid oxidation and production of secondary lipid products contributing to major degenerative diseases, such as CVD, diabetes and even in neurological disorders [82]. A number of milk BAPs have been shown to possess antioxidant property. This ability of peptides to bind/interact with radical species and/or inhibit oxidation could be a boon to boost human health [83]. Casein peptide, derived through pepsin hydrolysis, bearing the sequence Tyr-Phe-Tyr-Pro-Glu-Leu (YFYPEL) was found to show superoxide anion scavenging activity [84]. Further they identified that the preferred sequences were EL>YFYPEL>FYPEL>YPEL>PEL, suggesting that Glu-Leu in the sequence is important for this activity. Casein-derived peptides exhibited antioxidant property via inhibition of lipid peroxidation. It was further observed that proteolysis or dephosphorylation of casein did not impair its antioxidative potential [85]. Peptide with SerP-SerP-SerP-Glu-Glu domain has proven to possess free radical scavenging, hydroxyl and metal chelating properties [19, 86]. A recent study on peptides derived from sodium caseinate by proteases (papain, trypsin and pancreatin) demonstrated the contribution of proteolytic conditions on the extent of antioxidant activity [87]. Antioxidative potential of whey protein hydrolysates (WPHs) was associated with both low and high molecular mass fractions [88, 89]. α -Lactalbumin (α -LA) hydrolysed by thermolysin-yielded peptides with antioxidative properties. Peptides with Trp or Tyr at their extremities were found to

possess higher antioxidative activity in comparison with gallic acid and Trolox [90]. Trp-Tyr-Ser-Leu purified from whey protein displayed high radical scavenging and superoxide radical scavenging activities among peptides obtained through other enzymes [91]. Many peptides from whey have been shown to be multifunctional and possess antioxidant property along with ACE inhibition and antimicrobial properties [92, 93]. IIAEK and IPAVFK peptides, derived from tryptic hydrolysate of β -lactoglobulin (β -LG), possessed potent ACE inhibitor potential while VAGTWY was found to be an effective antioxidant having 1.7-fold higher activity in comparison with commercial antioxidant [94]. Hydrolysates derived from sheep cheese whey through hydrolysis by protease from *Bacillus* species P7 were effective as antioxidant and antihypertensive agents. They exhibited 3.2-fold increases in radical scavenging activity and could potentially be engaged in retarding oxidation in food products or as a nutraceutical [95].

Lipid peroxidation leads to spoilage, reduced shelf life along with release of free radicals that possibly decrease the nutritional value of food. Independent or combined antioxidant or antimicrobial roles by whey BAPs have extended their utility as natural food preservation agents. Ability of lactoferrin (Lf) to retard growth of microbes or limit lipid oxidation finds its application in food preservation as it acts as antimicrobial and physical barrier between food and microbes. It can also be used as spray or directly applied to beef carcasses improving its shelf life [96]. Peptide sequence Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile from α -LA and β -LG hydrolysed by commercial proteases was found to possess radical scavenging activity higher than butylated hydroxyl anisole indicating its utility as antioxidant in food industry [97]. Alcalase-treated whey protein isolate (WPI) hydrolysates proved to possess antioxidant property by acting as metal ion chelator, radical stabiliser and hydrogen donor thereby inhibiting lipid oxidation ascertaining their potential to replace synthetic antioxidants [98]. Radical scavenging activity of fragments LQKW and LDTDYKK of β -LG-enriched WPC were identified [99]. Antioxidant peptides and their application have been reviewed by Power et al. [18]. Critical contribution of thermal processing contributing to maintenance of milk protein bioactivity was delineated. Lf and β_2 transforming growth factor were significantly high in low-heat-treated WPC mainly from acid whey that could induce immune response via proliferation and cytokine responses of intestinal epithelial cells. These findings could be vital in maintaining optimal bioactivity infant's formula thereby influencing maturation and immune modulation of developing intestine [100].

5.1.3. Angiotensin-converting enzyme inhibitors

CVD is the leading cause of mortality and morbidity globally. BAPs from milk have gained prominence in protection against CVD owing primarily to their antihypertensive effects along with hypocholesterolaemic, anti-inflammatory and anti-oxidant potentials [101]. Multifunctional ACE regulates blood pressure via catalytic conversion of angiotensin 1 to angiotensin 2 which is a potent vasoconstrictor. ACE inhibition is known to be the major strategy in BP treatment using pharmacological drugs. Due to their side effects, there is continuous search for safe and possibly natural inhibitors [102]. Extensive research has been focussed on ACE inhibitory biopeptides. Casokinins that are casein-derived peptides, found in α_1 -, β - and κ -

caseins, are very effective in inhibiting ACE [72]. Casein peptide, VPP, is a potential inhibitor of ACE. Research suggests that monocyte adhesion to inflamed endothelia is well moderated by VPP that may prove vital in the prevention of atherosclerosis [103]. Pepsin hydrolysed bovine casein (HBC) with molecular mass <3000 were studied for their antihypertensive property. HBC exhibited nearly 10 times decrease in DBP substantiating its potential as ACE inhibitor and an antihypertensive agent. Pepsin hydrolysate of casein has been commercialised and patented as potential hypotensive agents containing α_1 peptides [99, 104]. Structure–activity studies have shown that three residues in the C-terminal region of BAPs seem to interact with active centre of ACE. This interaction may enhance the ACE inhibitory activity if hydrophobic/aromatic AAs (Trp, Tyr and Phe) or imino acid Pro are situated in this location. Additionally, Arg and Lys residue's positive charge may increase the inhibition [105]. Further C-terminal region's importance in ACE inhibition was delineated through QSAR model [106, 107]. A recent study on α -casein-derived peptides identified a potent ACE inhibitor using endoproteinase from *Aspergillus niger* [108]. Enzymatic hydrolysis of β -LG obtained from chemical hydrolysis of ovine and caprine whey exhibited consistently high ACE activity. It was postulated that end product of thermolysin hydrolysates, LQK from LQKW and LL from LLF, possibly possessed ACE inhibitory action [109, 110]. Stage 1 hypertensive young adults treated with dietary intake of WPC beverage daily for 6 weeks resulted in a significant decline in diastolic and systolic blood pressure [111]. Hydrolysates of camel milk, colostrum and colostrum whey proteins by pepsin and pancreatin to mimic intestinal digestion presented increased ACE inhibition along with multifunctional effects like radical scavenging and antimicrobial effect. Biopeptides α -LA and immunoglobulin (IgG) exhibited multifunctional activity including ACE inhibition [92]. Recently, optimisation studies to produce whey hydrolysate from WPC containing 70% protein with commercial enzyme through response surface methodology demonstrated up to ~65% inhibition of ACE and 50% antioxidant property [93].

5.1.4. Opioid activity

Opioid peptides are short sequence AAs required by the brain for important functions and these mimic the effect of opiates in the brain. Opioid peptides are peptides that exert affinity towards opiate receptors. Some of the functions of these peptides are to increase analgesic action, moderate social behaviour, stimulate endocrine secretions, increase GI transient time and thereby inhibiting intestinal peristalsis, motility, etc. [112]. Activity of opioid agonist peptides is inhibited by the opioid antagonist naloxone both *in vitro* and *in vivo*. All opioid receptor ligands are proved to possess some characteristic features, such as N-terminal Tyr residue, another Phe, Tyr or another aromatic AA at the third or fourth position from the N-terminal end. These features ensure appropriate fit between the peptide and receptor for the specific target activity [113]. One of the earliest discovered BAPs from milk was casein opioid peptide, β -casomorphins. Water buffalo, sheep and human milk also contain analogues of β -casomorphins. Exorphins derived from α -casein of milk corresponding to bovine α_1 -casein and casoxins from κ -casein mimic as opioid antagonists [114]. Recent research on casein-derived β -casomorphins-7 has shown to have positive influence on neurogenesis through redox-based epigenetic effects [115]. Enzymatic hydrolysis of whey by GI enzymes individu-

ally or in combination was used *in vitro* to release an agonist peptide, serorphin, from bovine serum albumin (BSA). It also led to the production of antagonist peptides, lactoferroxin A, B and C from Lf, α -lactorphin from α -LA and β -lactorphin from β -LG [116]. α -Lactorphin was shown to upregulate mucin gene (MUC5AC) expression and secretion in human colonic goblet-like cells [117]. Trypsin hydrolysates of β -LG and β -lactorphin exhibit similar results possibly operating via opioid pathway. Results confirm that mucin secretion could be induced via opioid receptor and whey peptides as promising gastrointestinal protective agents [118].

5.1.5. Antithrombotic activity

Rennin hydrolysis of κ -casein during milk clotting releases caseinomacropeptide (CMP) that contain peptide sequences aiding blood clotting by inhibiting platelet aggregation and binding of γ chain of fibrinogen to fibrinogen receptors [119]. The sequences LSFMAIPPK, MAIPPKKNQDDK, MAIPPKK and NQDK in primary structure of κ -caseins were found to be involved in antithrombotic activity [119, 120]. κ -Caseinoglycopeptide from sheep casein exhibited antithrombotic property [121]. Undecapeptide (106–116) of bovine κ -casein exhibits structural similarities to dodecapeptide of human fibrinogen γ chain at C-terminal. Ile¹⁰⁸, Lys¹¹² and Asp¹¹⁵ residues of undecapeptide are positionally homologous with r-chain of human fibrinogen for platelet receptors thereby inhibiting clot formation [122].

5.1.6. Antimicrobial activity

Conventional antibiotics use is increasingly turning deterrent due to antibiotic resistance by microorganisms resulting in treatment impediments and augmented healthcare expenses. Antimicrobial peptides (AMPs) resulting from milk have the advantage due to their precise infected cell targeting, broad spectrum nature, safe and economical source with vast industrial potential [17, 20, 71]. Antimicrobial properties of milk was first identified in 1930 when lactenin was identified to possess antibacterial property against scarlet fever *Streptococcus* [123]. Research has shown that milk proteins contain several motifs that can be released by proteolytic enzymes to enhance the antimicrobial potential of these proteins [124]. Disease prevention/control through BAPs primarily involves microbial interaction as AMPs or immunomodulation [125]. α_1 -Casein-derived peptides, caseicins, were the first purified antibacterial peptides from casein and were effective against a wide range of Gram-positive bacteria including *Staphylococci*. Isracidin, N-terminal peptide of α_1 -casein was found to be effective against *Lactobacilli* and Gram-positive bacteria *in vitro* and yielded a strong protection against *Staphylococcus aureus*, *Listeria monocytogenes* and *Streptococcus pyogenes* *in vivo*. Isracidin is comparable in its antibiotic action to standard commercial antibiotics in protecting cows and sheep against mastitis [126]. Caseicin A, B and C from α_1 -casein share common characteristics with isracidin in their AMPs. Caseicin A and B were effective against *E. coli* and *Enterobacter sakazakii* while caseicin C only showed minor activity against *Listeria innocua* [127]. Peptides obtained from bovine α_1 -casein by *Lactobacillus acidophilus* DPC6026 fermentation have been active against infectious pathogenic strains in neonate, *E. sakazakii* and *E. coli* suggesting the possible bioprotective applications of these AMPs in infant milk formula [128]. Digestion of casein with pepsin yielded two AMPs, Cp1 from α_1 -casein and Cp2 from α_2 -casein.

Cp1 was effective against both Gram-positive and negative bacteria whereas Cp2 exhibited inhibition generally more towards Gram-positive bacteria [129]. Casocidin-I (165–203) characterised and isolated from bovine α_2 -casein was effective against *E. coli* and *S. cornosu* in infants. Human milk does not contain α_2 -casein and hence it was proposed that human intestinal flora was influenced by casocidin-I ingestion [29]. Chymosin digestion of sodium caseinate resulted in the release of five antibacterial peptides (Cr1, Cr3, Cr4, Cr5 and Cr7) that were found potent against Gram-positive bacteria [130]. Peptide from human β -casein (184–210) showed broad spectrum antibiotic activity against Gram-positive and negative bacteria. Fragment from rabbit β -casein (64–77) exhibited potent action against Gram-positive bacteria [105]. Ser(P)149, a mono phosphorylated sequence of κ -casein (138–158), also displays antibacterial effects against *Porphyromonas gingivalis*, *E. coli* and *S. Mutans* [74]. κ -Casein-derived AMPs, kappacin, is a non-glycosylated and phosphorylated form of CMP. These peptides limit GI tract infections in neonates possibly by transmembrane cation gradient collapse and by elevating bacterial sensitivity to gastric acids [105]. CMP by itself is a powerful health booster. It is effective against cholera toxins as well as influenza virus. Prevention of bacteria adhesion to oral cavity by CMP controls dental plaque consequently promoting teeth remineralisation. CMP has also been patented in hygiene products for dental caries prevention [131]. κ -Casecidin, trypsin digest of κ -casein, exhibited both antimicrobial potential and mammalian cell cytotoxicity including cell lines of human leukaemia probably through apoptosis [127]. Whey protein Lf has been considered as a good source of AMPs. A cationic peptide, from Lf N-terminal region, lactoferricin 27, binds to microbial membrane and increases its permeability causing cell death. Potential to bind iron, a pathogenic virulence factor of many bacteria, and depriving it from cell vicinity has been another mechanism by some whey-derived AMPs especially on *E. coli* and *L. monocytogenes* [132]. Three α -LA hydrolysates by trypsin and chymotrypsin was found potent against many Gram-positive bacteria, especially *Bacillus subtilis*. Four peptide fragments from trypsin digests of β -LG exerted bactericidal property only against *B. subtilis* [132]. Targeted modifications after partial hydrolysis by pancreatic enzymes improved antimicrobial property by β -LG towards Gram-negative bacteria [132]. Whey proteins and their chemically modified analogues were effective against *Helicobacter pylori* infections and exhibited antiviral activity towards human herpes simplex virus type 1 (HSV-1) during and after infection [35]. Fungal stasis and antifungal role of Lf and its synthetic peptides are well established against *Candida albicans*, *Candida glabrata* and other *Candida* species [39]. Whey from goat milk on hydrolysis with gastric and duodenal juices exhibited antimicrobial properties. Nature of protein substrate and proteases affect AMP generation and their efficacy. Camel milk whey protein exhibited higher inhibition of *E. coli* in comparison with that of bovine. Camel whey hydrolysates by trypsin, thermolysin and chymotrypsin showed increased antimicrobial activity [133]. Peptic and pancreatic hydrolysates of camel colostrum proteins were inhibitory to growth of *E. coli* and *L. innocua* signifying their natural antimicrobial potential [92].

The biggest challenge faced by researchers in food-borne pathogen control is tolerance to high salt and low pH by a wide range of microorganisms (bacteria, fungi and viruses). Novel antimicrobial agents from natural sources are therefore the need of the hour to overcome this challenge [134]. Recent past has witnessed a rise in interest towards the use of BAPs as bio-

preservative for food or as functional food that is more prophylactic, nutritious and healthy [128]. This extended application of AMPs is advantageous over chemical food preservatives as they introduce fewer antagonistic effect, retain the organoleptic flavours of food along with their nutritional benefits and require decreased heat treatment for minimal processing [74, 105]. Milk-derived AMPs find immense potential in therapeutic healthcare and food preservation.

5.1.7. Immunomodulatory property

Body's immune system protects it from pathogens through its specialised cells, antibodies and lymphatic system. Dietary composition and their intake play a vital role in body's immune system. Immunomodulation includes stimulation or suppression of immune functions in the human body. BAPs from innumerable sources have revealed their immunomodulatory role in humans. They are identified to boost the immunological activity by regulating antibody production, cytokine regulation and reactive oxygen species induced immune functions [127]. Neonates' resistance to microbial infections is through physical transmission of passive immunity through breast feeding via a number of multifunctional factors including casein, which on digestion in GI tract releases peptides with immunomodulatory functions [72]. β -Casomorphin-11, a hexapeptide corresponding to Val-Glu-Pro-Ile-Pro-Tyr possessing immunostimulatory effect, was isolated from tryptic digest of β -casein of human milk. Several other hydrolysates from β -casein (191–193) and α_1 -casein (194–199) including β -casomorphin-11 exert protection against *Klebsiella pneumoniae* in mice *in vivo* and activate phagocytic action in human and murine macrophages *in vitro* [30]. β -Casokinin-10, obtained via pepsin-chymosin hydrolysis of bovine casein, stimulated proliferation of peripheral blood lymphocytes in rats. Stimulatory effect on proliferation of human peripheral blood lymphocytes was presented by synthetic peptide sequence analogue from bovine κ -casein (Tyr-Gly) [135]. Chymosin-mediated bovine casein hydrolysis released fragments (1–23 and 193–209 of α_1 and β -caseins, respectively) with immunomodulatory capacities. CPP III, a commercially available CMP containing mainly α_2 -casein (1–32) and β -casein (1–28), increases immunoglobulin production in spleen cell cultures of mouse. Further, it induces proliferation by lipopolysaccharide, concavalin A (Con A) and phytohaemagglutinin. O-Phospho-l-serine residue of CPP III was found to be responsible for this immunostimulating activity and therefore study suggested relative stability of this peptide even with GI tract proteinase action. These observations were considered vital for developing infants' formula with optimised immunomodulatory activities [72]. Enhanced mucosal immunity was observed in mice with oral administration of CMP [15]. Immunotherapy against HIV virus displayed positive results in decreasing the tendency to progress to AIDS (in patients with pre-AIDS) probably by inhibiting the infection development [136]. Eight casein hydrolysates' (titled a–h) bioactivities were assessed for their antioxidant and immunomodulatory response. Hydrolysates (d–h) exhibited increased Con A-stimulated IL-2 production; however, IL-10 was least stimulated in Jurkat cells in culture [47]. Further studies revealed cross-linking of sodium caseinate with transglutaminase before hydrolysis by Prolyve 1000 (microbial proteolytic preparation) had a positive impact on anti-inflammatory functions in Jurkat cells [137]. Peptides with immunosuppressive property are utilised during medical conditions like grafting and transplantations and

also during autoimmune disorders whereas stimulative immune peptides are required for overall immune strength. Immunopeptides released after digestion in adult human are not significant to stimulate immune response for therapeutic purposes [138]. Whey peptides have exhibited its positive impact on immune system through various studies both *in vivo* and *in vitro* [15]. GMP and its peptic hydrolysates were found to stimulate proliferation and phagocytic activity of human macrophage-like cells U 937 [139]. Lymphocyte activation, antibody production, non-immune responses like NK cell and macrophage functions are all reviewed extensively by Gauthier et al. [138]. Cow and goat whey protein samples revealed *in vitro* a dose-dependent inhibition of T-lymphocyte proliferation indicating the possible role of whey peptides probably hindering some important proliferation activating signals [140]. Synthetic peptides hydrolysed by trypsin or chymotrypsin on theoretical basis from α -LA (fragments 10–16 and 104–108) and β -LG (fragments 1–8, 15–20, 55–60, 78–83, 84–91, 92–105, 139–148, 142–148 and 102–105) were evaluated for their immunomodulatory functions. Different extent of proliferation stimulation was observed by fragments 15–20, 55–60, 84–91, 92–105, 139–148 and 142–148 from β -LG and α -LA 10–16, while inhibition and/or stimulation on cytokine secretion was also observed by fragments 15–20, 55–60 and 139–148. Cytotoxic effect was predominant in β -LG 1–8, 102–105 and α -LA 104–108. Studies show whey-derived peptides to have prospective to modulate specific immune response [141]. Rat and mice models have shown that dietary intake of whey adds positive influence in combating small bowels by reducing ulcers and having high IgA in gut advocating their protective role in patients with intestinal disorders and perioperative damage to small intestine [142, 143].

5.1.8. Anticancer property

In spite of promising results from anticancer studies on casein peptides, casein is not regarded as chemo preventive. β -Casein derivatives, β -casomorphin-7 and 5, along with their opioid properties, have displayed arrest of cell cycle and anti-proliferative properties in colon and breast cancer cells [144, 145]. CPPs inhibit intestinal tumour proliferation through activation of calcium channels and stimulate apoptosis [146]. Calmodulin (CaM), a calcium activated protein, plays a major role in physiological maintenance of the body functions, such as cell proliferation and neurotransmission. CaM binding properties of food-derived peptides prove vital in preventing many chronic diseases including Alzheimer's and cancer [12]. Nitric oxide synthases (NOSs), protein kinase II and phosphodiesterase I (PDE) are some of the major enzymes that require CaM for their activation. Nitric oxide produced from arginine by CaM-dependent NOS is involved in neurotransmission, immune response and other vital muscle movements along with several other functions in the body. In excessive levels, it may lead to degenerative nervous disorders, such as stroke, Parkinson's, Alzheimer's, etc. NOS exists in three isoforms and depends on CaM for their activation. Inhibitors of CaM are effective against these isoforms. One isoform is involved in tumour necrosis factor production by macrophages and hence CaM inhibitory substances during diseased conditions could provide therapeutic benefits [147]. Similarly, CaM-PDE catalyses the breakdown of cAMP to linear nucleotide phosphate. Apoptotic cell death of normal and cancer cells is induced by cAMP in human body. CaM-PDE in excess causes depletion of cAMP leading to physiological imbalance in cell death. Inhibitors of CaM-PDE increases cAMP cellular levels that could offer therapeutic relief

during cancer treatment. BAPs can exhibit effective CaM inhibition when they have hydrophobic AA residues and positive charge at physiological pH to interact with CaM [148]. Pepsin mediated hydrolysis of α -casein released such five peptides that were potential CaM-PDE inhibitors [12]. Whey protein and its derivatives suppress tumour growth by elevating glutathione levels and consequent free radical scavenging. The significant abundance of cysteine, an essential AA required for glutathione synthesis, in β -LG may be contributing to peptide's tumour protective role [15]. Tumour preventive role by peptides could also be through their modulatory effect on immunity and cell viability [149]. Tumour development and progression of lung, colon and oesophagus was found to be inhibited in both rat and mouse models after oral administration of bovine Lf. However, studies could not resolve its mode of action [15]. Apoptosis-inducing property of α -LA was exhibited on tumour and immature cells but not on mature cells. This property was attributed to its 3D protein folding that is unlikely of its native form [150, 151]. Case reports presented anti-tumour effect by whey proteins on urogenital cancer when administered as dietary supplements. However, further clinical trials were required to confirm its efficacy as therapeutics [152]. N-terminal peptides of Lf are investigated for their anticancer activity. Fragments of bovine Lf 17–38 induced apoptosis in HL-60 cell lines and fragments 17–41 exerted cytotoxicity in melanoma, colon carcinoma and fibrosarcoma cell lines. Cytotoxicity of Lf was found to be located within FKRRWQWRM sequence. *In vivo* and *in vitro* cytotoxic effects on neuroblastoma cells and breast cancer cells were exhibited by lactoferricin B and lactoferricin, respectively. Lf further displayed capacity to inhibit angiogenesis required for tumour growth [74]. Human α -LA made lethal to tumours (HAMLET) and bovine α -LA made lethal to tumours (BAMLET) are complexes of calcium depleted or apoform of α -LA that exhibited anti-tumour activity in human studies. Skin papillomas treated with HAMLET (skin application) for 3 weeks exhibited reduction in lesion volume in relatively 75% of test group and further complete lesion resorption with 2-year symptom-free period [153]. Apoptosis along with decreased size and surface atrophy of tumours were observed in bladder cancer patients treated with HAMLET [154]. Daily supplementation of bovine Lf for a year significantly arrested growth of colorectal polyps in animals possibly through its iron chelating property [155]. Bovine Lf-treated colorectal cancer patients undergoing chemotherapy showed relief from chemotherapy side effects (kidney and liver toxicity, mucositis and anaemia) compared to the controls with no impact on normal cells; however the results did not achieve statistical significance [156]. WPH exhibited low tumour multiplicity in rats with chemically induced mammary tumours when fed as diet in comparison with casein hydrolysates, 20% casein and intact whey [157].

5.1.9. Miscellaneous properties

Various other health potentials of casein/whey peptides are also explored by researchers worldwide. Type 2 diabetes, a globally prevalent metabolic disorder characterised by impaired insulin secretion by pancreatic β cells is considered an epidemic in recent past. Its association with increased risk of chronic health issues including CVD and hypertension makes it a public health burden resulting in elevated healthcare cost globally. This has led to research towards finding an economic nutritional approach to improve postprandial glycaemia. Dietary modifications alone in numerous million cases could prevent this disorder [158, 159].

Epidemiological studies support positive correlation between intake of milk and dairy products with its lesser incidence. The positive impact of milk on insulin resistance is majorly attributed to its proteins and peptides that is validated through *in vitro* and *in vivo* studies [101]. Human studies on metabolic effects of milk proteins on glycaemia and insulinaemia exhibited higher insulinotropic activity by whey in comparison with casein, other protein-rich food and supplements possibly owing to faster rate of whey digestion [158]. The positive effect of WPHs as an insulinogenic agent has also been evaluated. Whey proteins are considered as an excellent source of insulin regulators due to its rich (40–50%) essential AA composition. These AAs are primarily involved in inducing and modifying insulin secretion by β cells of pancreas [160]. Experimental evidence suggested faster gastric emptying and subsequent stimulation of incretin, an insulin secretagogue produced by small intestine (SI), in the presence of hydrolysates of whey [71, 161]. Gastric emptying studies revealed influence of different protein sources and their degree of hydrolysis on insulin response. WPHs lacking carbohydrate stimulated rapid release of AAs, dipeptides (Val-Leu and Ile-Leu), along with a significant increase in insulin secretion. Insulinotropic effect of whey hydrolysate is attributed to increased postprandial concentration of AAs, such as Ile, Leu, Lys, Thr, Val, all of which stimulate insulin secretion [162]. Animal studies on diabetes and β cells of pancreas were also appraised [163]. Incretin [glucagon-like peptide (GLP 1) and glucose-dependent insulinotropic polypeptide (GIP)] hormones contribute ~70% insulin release from pancreatic β cells. Dipeptidyl peptidase (DPP IV), an abundant enzyme present in circulation and on cell surfaces, acts on incretin decreasing their plasma levels. Inhibitors of DPP IV could improve half-life of GLP-1 whose activity is preserved even during type 2 diabetes thereby stimulating its insulinotropic effect. BAPs of whey from bovine, caprine and ovine are considered insulinogenic by acting as potential inhibitors of DPP IV [94]. Brandelli et al. have extensively reviewed DPP IV inhibitors from whey proteins and their hydrolysates [40]. Yet another approach in diabetes management involves inhibition of membrane bound α -glucosidase (carbohydrate hydrolysing enzymes) from SI that catalyses release of monosaccharides from complex carbohydrates. Inhibition of α -glucosidase delays carbohydrates degradation and thereby decreasing the postprandial plasma glucose levels [164]. WPI, α -LA, β -LG and Lf hydrolysates from peptic digestion were investigated for their DPP IV and α -glucosidase inhibition potential. α -LA, β -LG and WPI hydrolysates were found to be potential inhibitors for both, whereas Lf and serum albumin could inhibit DPP IV alone suggesting WPHs potential antidiabetic property [165]. β -LG- and WPH-derived peptides by serine protease from *Cucurbita ficifolia* were found to be potent natural inhibitors of α -glucosidase, DPP-IV and ACE. The study supported antidiabetic nature of these peptides through inhibiting incretin and carbohydrate digestion [166].

Muscles play a key role in whole protein body metabolism. They serve as reservoir of AAs, which are required by vital organs and tissues for their protein synthesis when adequate AAs are not available from gut absorption. It also provides hepatic glycogenic precursors. Any impairment in muscle metabolism may thus check pathologic and chronic disease conditions. In order to maintain balance in rate of breakdown and synthesis of protein in vital organs, postprandial absorption of proteins become vital especially for elders and sportsmen [167]. Protein homeostasis by a suitable diet strategy is critical for growth, maintenance and/or limit

loss of protein after stress. The quantity and quality of protein ingested could modulate protein metabolism by its AA composition and digestibility [168]. Dietary protein from milk, meat, etc., provides AAs that intensely stimulate protein synthesis in skeletal muscles [169]. Milk proteins were investigated for their role in muscle mass maintenance and their possible role as anti-aging factor. Whey proteins have proved to possess greater postprandial retention than casein proteins. Improved anabolic properties of whey than that of casein was mainly attributed to their faster digestion and absorption leading to increased availability of AAs to stimulate protein synthesis [170]. Leucine has been recognized as important nutritional signal to stimulate postprandial muscle protein deposit [171]. During aging, impairment of protein synthesis in skeletal muscle postprandial results in a progressive decline in muscle mass leading to sarcopenia [172]. Anabolic resistance towards muscle protein synthesis is considered a major causative factor in sarcopenia [173]. Clinical trial has been registered for whey proteins' effective stimulation of protein accretion in muscle after ingestion of food in comparison with casein [174]. Recent research on myofibrillar protein synthesis after ingestion of whey in young men during rest and after exercise exhibited a 49–56% increase. The study concluded that 20g dose of whey protein is adequate for maximum stimulation of myofibrillar protein synthesis [175].

5.2. Dairy products

Starter cultures employed traditionally in dairy industry for milk fermentation release BAPs. End products of dairy industry, such as fermented milk, cheese, etc., are found to possess peptides that are biophysically active. Cheese is the oldest and widely used dairy product. It contains abundant casein-derived peptides formed during its production due to rennet-based proteolysis. Subsequent ripening associated secondary proteolysis contributes to the formation of diverse biologically active peptides whose function depends on the nature of proteolytic enzyme used and the ripening stage of cheese [176]. Hydrolysates produced by proteases differ according to their casein specificity. CPPs have been identified in Cheddar, Comte and Herrgard cheese [21]. Various biological activities similar to casein and whey peptides have been associated with varieties of cheese. **Table 1** represents compilation of antihypertensive peptides from selected commercially available cheese. VPP and IPP sequence of several hard, semi-hard and soft cheese were also studied for their ACE inhibitory properties [15, 177–182].

Antioxidant and antimicrobial activity were presented by cheddar and artisanal Coahlo cheese peptides [182, 183]. Studies identified radical scavenging peptides from Burgos cheese made by different proteases of animal (rennet), plant (*C. cardunculus*) and microbial (*Mucor meihei*) origin. Cheese made by animal rennet could yield an antioxidant peptide from α_1 -casein [184]. Antihypertensive peptides from Calpis, a Japanese soft drink, were identified as VPP and IPP from α_1 -casein and β -casein, respectively [136]. Peptides from fermented milk and yogurt from bovine and sheep's milk were evaluated for their antihypertensive and antioxidant activities [97, 185]. A recent study on fermented camel milk (chal) and bovine milk using protease from LAB exhibited significant antioxidant property in camel milk in comparison with its bovine

counterpart [186]. Research stays on to find novel BAPs from various dairy products and to disclose their probable values and health benefits.

Cheese type	Source and peptide information
Cheddar	α_1 -casein:1–6, 1–7, 1–9, 24–32, 102–110 β -casein: 47–52, 193–209
Fresco cheese	α_1 -casein:1–15, 1–22, 14–23 and 24–34 β -casein:193–205, 193–207, 193–209
Spanish cheese	Several fragments of α_1 and β casein
Gouda cheese	α_1 -casein: 1–9, β -casein: 60–68
Herrgard cheese	β -casein: 29–105, 29–107, 1–105, 1–107, 29–93, 30–93
Italian cheese mozzarella, crescenza, Italico and Gorgonzola	β -casein: 58–72
Parmigiano-Reggiano cheese	β -casein:8–16, 58–77, α_2 -casein: 83–33

Table 1. Antihypertensive peptides from diverse commercial cheese [180–182].

6. Commercial products

Extensive evidence found in literature suggests potentiality of BAPs as therapeutic or dietary supplements/functional foods. Commercial products enriched with BAPs are available in the market with empirical knowledge from research [187]. Abundant potential is found in peptides to be used as dietary supplements or as functional foods; however, they may not always be included in regular intake as additional information regarding safety, cytotoxicity and hypersensitivity as mandatory [47]. Food and oral hygiene products along with various ingredients enriched with BAPs from milk proteins are available commercially. **Table 2** represents selected products from milk-derived peptides with their claimed potential health benefits. Various other food-derived BAPs are also available in market with substantial health benefits associated with them. A few have been approved by FDA for therapeutic uses. One of the major challenges for existing and future commercial peptide products is valid documentation of the functional and health promoting properties associated with them [26, 70].

Protein	Peptide sequence	Commercial product	Food type	Health claim	Website/ manufacturer
Milk proteins	Dipeptide and tripeptides	PeptoPro®	Ingredient	Boosts muscle protein synthesis	http://www.dsm.com/markets/foodandbeverages/en_US/products/nutraceuticals/peptopro.html DSM, Netherlands
α_1 -casein	YLGYLEQLLR	Prodiet F200	Confectionary,	Stress reliever	www.ingredia.com/

Protein	Peptide sequence	Commercial product	Food type	Health claim	Website/ manufacturer
		Lactium	drink		Ingredia, France
α_1, β -Casein	IPP and VPP	Calpis	Sour milk	Hypotensive	www.calpis.net/ CalpisCo. Japan
β and κ -casein	IPP and VPP	Calpico/ Calpis AMEAL s	Fermented milk	Hypotensive	www.calpis.net/ Calpis Co., Japan
Casein-derived dodeca peptide	FFVAPFPE VFGK	C12 peptide	Peptide ingredient	Hypotensive	www.dmv.nl/en/ DMV, Netherlands
Casein	Casein hydrolysate	Excellion Calcium Caseinate A	Powder	Nutraceutical	www.dmv.nl/en/ DMV, Netherlands
	Sodium caseinate from curd	Excellion OPTIMIX 100	Dried milk protein	Nutraceutical	www.dmv.nl/en/ DMV, Netherlands
	Casein hydrolysates	PROTARMOR™ 80	Ingredient	Weight loss Sports nutrition	http://www.armor-proteines.com/en/products/protarmor-80/ Armor Proteines, France
	CPP	Caploc	CPP ingredient	Mineral absorption	www.arlafoodsingredients.com/ Arla Foods, Denmark
	CPP	CE90CPP	ingredient	Mineral absorption	www.dmv.nl/en/ DMV, Netherland
	CPP	Kotsu calcium	Soft drink	Mineral absorption	www.asahiinryo.co.jp/ Asahi Soft Drinks Co. Ltd, Japan
	CPP	Tekkotsu Inryou	Soft drink	Mineral absorption	www.suntory.com/softdrink/ Suntory, Japan
	CPP-ACP	Recaldent	Tooth paste	Remineralisation of enamel	http://www.recaldent.com/p_welcome.asp Cadbury enterprises
Whey protein isolate	GMP (106–109)	BiPRO WPI	Ingredient and infant formula	Antithrombic, anticariogenic Antimicrobial	www.daviscofoods.com/specialty/bipro.html Davisco foods, Minnesota
Whey protein hydrolysate	β -LG fragments	Biozate 3 and 7	Ingredient	Hypotensive	www.daviscofoods.com/specialty/biozate.html Davisco foods, Minnesota
α -Lactalbumin	α -LA fragments	Lacprodan® ALPHA-10 and	Health food	Improves sleep and memory	www.arlafoodsingredients.com/products/alpha-lactalbumin/

Protein	Peptide sequence	Commercial product	Food type	Health claim	Website/ manufacturer
		Lacprodan® ALPHA-20			health-foods/, Arla foods, Denmark
Whey protein	WPC	Hiprotal® Whey Protein 35 and Hiprotal® Whey Protein 45	Ingredient for infant nutrition	Muscle building	www.domo.nl/en/markets- ingredients/list/infant-nutrition- ingredients-list/ FrieslandCampina Domo, Netherlands
Whey peptide	CMP	Lacprodan® CGMP-10	Infant food	Cognitive and neuronal development	www.arlafoodsingredients.com/ products/ caseinoglycomacropeptide/ infant-nutrition/ Arla foods, Denmark
Whey protein	WPH	Peptigen® IF-3087/3090/3012/3080	Infant food	Mineral binding	www.arlafoodsingredients.com/ products/whey-hydrolysates/ infant-nutrition/Arla foods, Denmark
	WPH	Lacprodan® DI-3071/3021 Lacprodan® HYDRO.milk	Sports nutrition	Vitamins and minerals uptake; oral hygiene improvement	www.arlafoodsingredients.com/ products/whey-hydrolysates/ sport-nutrition/ Arla foods, Denmark

Table 2. Commercial products from milk protein/peptides [70, 187].

7. Summary and future prospects

BAPs derived from milk proteins have been a subject of growing interest as health-supporting foods due to their varied nutritional and biological properties. Several studies have shown the role of BAPs as functional food and nutraceuticals. Still, the existence of milk-derived peptides in the gastrointestinal tract has been demonstrated through only a handful of studies. In addition, very little or no information about the stability, bioavailability and efficacy of these BAPs generate a main breach in knowledge to allow an improved understanding on their role in human health. There is still a need for the development of integrated research platforms involving inter-disciplinary skills to bring more clarity on the role and mechanism of action of milk-derived BAPs in humans. Developing tools to preserve/augment the activity of BAPs and favour their optimum utilisation in food systems appear to be yet another prerequisite at this stage. Human-volunteer-based clinical studies on standard recommendations to improve the oversight and safety are also required to confirm the likely results obtained at the *in vitro* and animal model systems. To conclude, milk BAPs with their multifunctional assets

appear to offer substantial upcoming prospective for the development of products to support health.

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