Chapter 5

Milk Proteins: Processing of Bioactive Fractions and Effects on Gut Health

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Abstract

Milk is nature's most complete food. While milk clearly provides basic nutritional requirements, bioactive components within milk also impart a wide range of additional health benefits to both the neonate and the adult. However, human milk is compositionally different from cow's milk, and certain protein components of cow's milk can act as allergens to susceptible humans. One way of extracting the benefits of cow's milk proteins, while eliminating the risk of allergenicity in humans, is to hydrolyse the milk proteins. Hydrolysis of milk proteins generates smaller peptide sequences from their parent protein that can be biologically active when released. At an industrial scale, hydrolysis of milk proteins can be achieved through either enzymatic hydrolysis or fermentation. An alternative process of generating similar sized peptides is by *in silico* synthesis. These compounds can subsequently be developed as fortifying food agents.

A number of milk-derived bioactives have been characterised with a variety of health benefits in the gastrointestinal tract. These biological activities include supporting the establishment of a healthy commensal microbiome, suppressing the colonization of pathogenic bacteria and supporting barrier function. Hydrolysates of casein and whey also impart anti-inflammatory and immunomodulatory activity. This chapter gives an overview on the future potential of food grade milk hydrolysates to support homeostasis in the gastrointestinal tract.

Keywords: milk hydrolysates, anti-inflammatory, gut health, gut microbiota, gut homeostasis

1. Overall composition of milk

The overall composition of milk depends on a range of factors including genetics (species and breed), physiological state (age and stage of lactation) and environment (food and climate) [1–5]. While water is the main constituent of milk, comprising ~87% of the total volume, the remainder is composed of carbohydrates, fats and proteins in varying volumes across different species [6–8]. Among the numerous nutritional benefits of milk, milk proteins have gathered enormous attention for being a 'complete' protein as they provide all nine essential amino acids (leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, lysine) required by humans [9]. The proteins in milk are categorised into major proteins that include casein and whey fractions [1] and minor proteins that include lactoferrin, lactoperoxidases, lipases, lactase [6, 10] and miscellaneous proteins (cytokines, immunoglobulins, etc.) [11].

2. Milk protein hydrolysates

The process of breaking down milk proteins to shorter peptide sequences is termed 'hydrolysis'. This process happens naturally in the gastrointestinal tract and can be simulated in the laboratory or on an industrial scale. During the normal transit through the gastrointestinal tract, milk proteins are exposed to proteinases such as pepsin, trypsin and chymotrypsin which break them down into smaller peptides. These peptides are further digested by brush border peptidases present at the surface of intestinal epithelial cells where they produce amino acids; however, some oligopeptides still remain intact [12]. In laboratory or at an industrial scale, milk hydrolysates are released either by treatment of milk proteins with food grade enzymes or through fermentation with bacteria, which is described in detail in the following sections.

The shorter peptide sequences often possess bioactive properties beyond their nutritional contribution along with eradicating any protein-specific allergenicity [13, 14]. Processing and enriching for food grade bioactive peptides is a goal for the functional food industry. A functional food can be described as:

'a food that can beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or a reduction of risk of disease' [15].

Once the hydrolysates are released, they can potentially have bioactive properties which can exert their effects in receptive cells, including those present in the gastrointestinal tract [16]. The bioactivities of the resulting hydrolysates are variable depending on a range of factors, including the enzyme used, the processing conditions and the final size of the peptide sequence following hydrolysis [17]. The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds, i.e. the number of hydrolysed bonds per total number of peptide bonds in the protein [18]. This affects the size and amino acid composition of the peptides, which

subsequently determines the biological activity of the peptide. Hence, DH is an important consideration from the perspective of functional food research [19].

2.1. Enzymatic hydrolysis

The enzymatic hydrolysis process is conducted under mild conditions (pH 6–8, temperature 40–60°C) to minimise side reactions and to retain the amino acid composition similar to the starting material [17]. Enzymatic hydrolysis improves the solubility and heat stability of peptides, which is of benefit to the food industry. However, consumption of certain enzymes leads to allergic or toxic responses; hence, consumer safety is an important factor and requires the regulation of enzymes used for hydrolysis [20]. Enzymes that obtain 'generally recognised as safe' (GRAS) status and special approval of 'food grade' quality are legally considered as safe [20]. The food grade enzymes generally used to hydrolyse milk proteins into hydrolysates include pepsin, trypsin and chymotrypsin [21, 22]. In addition, food grade proteolytic enzymes, derived from microorganisms, can also be used to generate hydrolysates [23]. Proteolytic enzymes are of two types, depending upon their hydrolysing mechanism: endopeptidases which hydrolyse peptide bonds within protein molecules and exoproteases which hydrolyse N or C terminal peptide bonds. Post enzymatic hydrolysis, the hydrolysates usually need an additional treatment. The most common procedures include ultrafiltration, heat treatment and/or activated carbon treatment to control molecular size and elimination of bitterness in the hydrolysates [17].

2.2. Hydrolysis through microbial fermentation

Fermentation of milk proteins with proteolytic starter culture is another method of bulk production of hydrolysates. Safety measures should be considered with regard to toxicity and pathogenicity associated with the microorganisms used for fermentation. Food grade microorganisms with no related toxigenic and pathogenic response in humans are widely used. During microbial fermentation, milk proteins are subjected to 'splitting' as they are broken down by the proteolytic system of microorganisms [24]. Bacterial cultures of *Lactobacilli* spp., *Lactococci* spp. and *Streptococci* spp. are commonly used to generate hydrolysates from milk [25]. The proteolytic system of lactic acid bacteria (LAB) contains cell envelope-associated proteinases, endopeptidases, aminopeptidases, tripeptidases and dipeptidases for the production of hydrolysates [26]. LAB requires free amino acids and peptides for their growth, which they obtain from milk proteins by degradation [27]. The peptides or hydrolysates not utilised by bacteria can promote various bioactivities. LAB proteinases hydrolyse more than 40% of the peptide bonds in α-S1 and β-caseins, producing oligopeptides ranging from 4 to 40 amino acid residues [28]. Fermentation parameters such as enzyme/substrate ratio, composition of medium, heat treatment, temperature, pH and carbon/nitrogen ratio influence the release of hydrolysates from milk proteins. An alternative strategy used by food industry is starter LAB culture along with food grade enzyme to hydrolyse milk proteins. This strategy not only increases the peptide content of the hydrolysate but also diversifies the bioactivity of the hydrolysate [27].

2.3. Peptide synthesis

The *in silico* synthesis is an alternative process for generating peptide sequences modelled on various milk protein hydrolysates. Depending upon the length and quality of the desired peptide, either recombinant DNA technology or chemical synthesis methodologies can be used to synthesise peptides [29]. The application of recombinant DNA technology is preferable if the objective is to generate large peptides consisting up to several hundred amino acids. However, this is a long and expensive process. Chemical synthesis is currently used for laboratory scale peptide synthesis, especially for peptides used in therapy [30]. However, chemical synthesis uses toxic reagents that may contribute to environmental pollution and generates unwanted peptide by-products. Instead, the solid-phase synthesis approach, which is a variant of chemical synthesis, can generate peptides composed of 10 to over 100 residues in small scale, using lower amounts of chemicals [31]. Modification of peptide function by substitution of a particular amino acid in the sequence is easily done in solid-phase synthesis [30].

3. Models used for bioactivity evaluation and challenges

After the generation of milk hydrolysates, their bioactivity profile needs to be determined. In laboratory or at an industrial scale, the primary screening for the bioactivity is performed on *in vitro* platforms, using various cell culture models. Due to the difficulty in growing primary intestinal epithelial cells, cell lines derived from human intestinal tissues have been used extensively such as Caco-2, HT-29 and T-84 cells, as reviewed by Shimizu et al. [32]. Caco-2, HT-29 and T-84 cell cultures functionally resemble colonic enterocytes; however, these cultures only have a single cell type. This limits the understanding of the mechanistic effects of hydrolysates in a heterogeneous cell environment such as that of *in vivo*. This limitation is overcome by the use of intestinal tissue explants. Following the idea that pigs are good models for human research, our group used porcine intestinal tissue explants to test the bioactivity of seaweed extracts in an *ex vivo* system [33]. Further studies were carried out to establish the *ex vivo* model by our group, and Bahar et al. concluded from his study that explants from porcine gastrointestinal tract can be used to test the bioactivity of test compounds up to 3 h post-mortem [34]. Hence, the cellular heterogeneity of the tissue explants helps in better understanding of the effects of hydrolysates on cell growth, differentiation and functionality [35, 36]. Our group has applied these *in vitro* and *ex vivo* models for screening and testing the anti-inflammatory activities of milk hydrolysates [37, 38].

Although cell- and tissue-based model systems are an alternative to animal experiments, they do not reflect the *in vivo* conditions of cells and tissues in their natural state in the organism. The experimental culturing environment lacks the effect of endocrine and nervous systems that are involved in the homeostatic regulation *in vivo*, and hence animal and human trials are necessary for the full evaluation of bioactives. The difficulty to characterise the 'pharmacokinetic' and 'pharmacodynamic' properties of hydrolysates creates a challenge for researchers [39]. *In vitro* studies do not consider the degradation of the peptides by gastric, pancreatic and

small intestinal brush border enzymes. Most therapeutic peptides act systemically; however, only nano-molar or pico-molar quantities may be transported to the circulatory system due to enzymatic degradation in the gastrointestinal tract [39, 40]. Hence, findings between *in vitro, ex vivo* and *in vivo* experiments can be inconsistent. Overcoming these inconsistencies requires standardized methodologies for the analysis and the application of robust clinical trials in order to evaluate both the efficacy and metabolic fate of a particular hydrolysate [40].

4. Functionality of bioactive hydrolysates

To date, the bioactivities of a wide variety of hydrolysates have been characterised using *in vitro, ex vivo* and *in vivo* experimental platforms (**Table 1**). Hydrolysates are easily ingested in functional food offerings; however, their bioavailability might be affected due to post-ingestion break down. As seen in an *in vivo* trial by our group, milk hydrolysates with potent anti-inflammatory activity in *in vitro* and *ex vivo* platforms [37], lost this activity in the gastrointestinal tract of weaning piglets, most likely due to breakdown in the stomach (Mukhopadhya et al. *In Press*). These milk hydrolysates, combined with a viscous β-glucan, travelled to the distal section of gut with intact bioactivity (Mukhopadhya et al. *In Press*). The following sections review the potential for a spectrum of different milk hydrolysates to support the activities of the gastrointestinal tract microbiota, the gastrointestinal tract immune system and the gastrointestinal tract mucosal barrier to support homeostasis in the gastrointestinal tract.

Protein hydrolysates	Approximate content in cow milk (g/l)	Hydrolysis process	Related bioactivity in gastrointestinal tract	References
Total casein	**~80%**	EH	Bifidogenic	[51, 52]
		EH	Antimicrobial	[62]
		EH	↑Mucin	[72, 77]
		EH	↑IgG, ↑IgA	[88]
		EH, Fermentation, PS	Immunomodulation	[37, 38, 92, 93, 99]
α-S1 casein	9.1	EH	Antimicrobial	[62]
		EH, Fermentation	↑IgG, ↑IgA	[86, 87]
α-S2 casein	2.4	EH	Antimicrobial	[62]
		EH, Fermentation	↑IgG, ↑IgA	[86, 87]
β-Casein	8.5	EH	↑Mucin	[74, 75]
		EH, Fermentation	↑IgG, ↑IgA	[86, 87]

Protein hydrolysates	Approximate content in cow milk (g/l)	Hydrolysis process	Related bioactivity in gastrointestinal tract	References
k-Casein	3.0	EH	Antimicrobial	[64]
		EH	Immunomodulation	[94, 100, 101]
Total whey	**~18%**	Fermentation	Bifidogenic	[53]
		EH	Antimicrobial	[60]
		EH	↑Mucin	[78, 79]
		EH	↑IgG, ↑IgA	[89]
		EH	Immunomodulation	[89]
α-Lactalbumin	1.1	EH	Antimicrobial	[60, 65]
		EH	↑Mucin	[73, 80]
β-Lactoglobulin	2.8	EH, Fermentation	Bifidogenic	[54]
		EH	Antimicrobial	[60]
		EH	↑Mucin	[76]
Lactoferrin, lactoperoxidase, lysozyme, proteose-peptone, glycomacropeptide	~3%	EH	Bifidogenic	[55, 56, 58]
		EH, PS	Antimicrobial	[67, 68, 69]
		EH, PS	↑IgG, ↑IgA, ↑IgM	[90]

EH, enzyme hydrolysis; PS, peptide synthesis

Table 1. Milk protein hydrolysates, content in cow's milk, hydrolysis process used and their related bioactivity.

4.1. Prebioitc activity of milk hydrolysates

The World Health Organisation now recommends breastfeeding for up to 6 months, as breast milk has a major positive impact on the health and growth of the infant [41]. One of the most important benefits of breastfeeding the newborn is the colonisation of the gut by 'healthy' microbiota. 'Healthy' gut microbiota confers nutritive, metabolic and protective functions that affect intestinal physiology, immunity and whole-body metabolism. The establishment of a 'healthy' microflora in the gut during early life is crucial for the healthy development of a balanced immune regulatory network in the gut, a feature which affects the overall health of the individual [42]. Beneficial gut microorganisms aid gut health by releasing growth substrates from milk [43], improving vaccine responses [44] and decreasing gut permeability [45, 46]. After birth, the gut is colonised with bacteria from four main phyla namely *Bacteroides, Proteobacteria, Firmicutes* and *Actinobacteria*, and these phyla in turn influence the development of the gut-associated lymphoid tissue (GALT) [47, 48].

Milk hydrolysates show bifidogenic activity, i.e. they support the growth of Gram-positive anaerobic bacteria namely *Bifidobateria* spp., in the gut [49, 50]. The prebiotic characteristics of

milk hydrolysates are outlined in **Table 1**. For example, a proteolytic casein hydrolysate [51, 52] and hydrolysates of whey proteins fermented with *Lactobacillus casei* strains [53] effectively stimulate *Bifidobacteria* spp. growth. Interestingly, proteolytic digestion of β-lactoglobulin, a major whey protein of the bovine milk that is absent in human milk, generated peptides supporting both *Bifidobacterium* and *Lactobacillus* spp. growth [54]. *In vitro* peptic digestion of human and bovine lactoferrin [55, 56] and a synthetic peptide modelled on lactoferrin [57] have confirmed bifidogenic activity. Not only hydrolysates of major proteins but hydrolysates of the minor milk proteins, proteose-peptone, also supported the proliferation of *Bifidobacterium animalis* [58].

4.2. Antimicrobial activity of milk hydrolysates

Antimicrobial milk peptides prevent attachment and invasion of pathogens by either directly interacting with the pathogen and killing them or changing the host environment, leading to the inhibition of growth of microorganisms [59, 60]. The direct interaction of antimicrobial milk hydrolysates with microorganisms is specific, as they show affinity towards polarised bacterial membranes rather than dipolar membranes of eukaryotic cells [50]. There is growing evidence that the antimicrobial property of milk hydrolysates is related to the formation of α-helical structure of the peptides. The modifications of peptide's secondary and tertiary structures by phosphorylation of specific amino acid or chemical modification of C or N terminal dramatically affects the antimicrobial activity [50, 61]. Another mode of action for antimicrobial peptides is by aggregating in the cytoplasmic membrane, disrupting the membrane permeability of bacteria, and causing cell death [13, 30]. On the contrary, the indirect antimicrobial activity of milk hydrolysates is achieved by decreasing the host intestinal pH and thus limiting the growth of pathogenic microorganisms. This mechanism is also known as 'colonisation resistance' [57].

The antimicrobial effects of milk hydrolysates have been listed in **Table 1**. Casein is a major source of antimicrobial peptides, and hydrolysates of α-S1 casein exert protective effects against *Staphylococcus aureus, Streptococcus pyogenes* and *Listeria monocytogenes* [62]. Peptic hydrolysis of α-S2 casein generated two hydrolysates with antimicrobial activity against Gram-positive and Gram-negative bacteria, specifically *Saccharomyces thermophilus* and *E. coli*, respectively [62]. α-S2 casein is not present in human milk, hence hydrolysates from bovine α-S2 casein have interesting potential as a novel human gastrointestinal tract microbiota modulating agent [63]. The minor subunit of casein, κ-casein, inhibits the adhesion of *Helicobacter pylori*, an early-life pathogen, to human gastric mucosa [64]. The digestion of major whey proteins, α-lactalbumin and β-lactoglobulin, by trypsin and chymotrypsin releases peptides with antimicrobial activity against specific Gram-positive bacteria [60]. However, hydrolysates of α-lactalbumin, generated by protease treatment, have antimicrobial properties with regard to *E. coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococci* and *Candida albicans* [65]. Milk products fermented with *Lactobacillus paracasei* CBA L74 have anti-inflammatory activities in response to *Salmonella typhimurium* infection in mice [66]. Lactoferrin has gathered most attention as a source of antimicrobial peptides [67].

Pepsin digestion of bovine lactoferrin generated hydrolysates that are amphiphilic and displayed antimicrobial activity against a broad range of Gram-positive and Gram-negative bacteria including *Listeria, E. coli, Salmonella* and *Campylobacter* but not against several strains of *Bifidobacterium* [68]. Lactoferrin hydrolysates also act against the most important fungal pathogen in humans, *Candida albicans* [69].

4.3. Milk hydrolysates preserve gastrointestinal mucosal integrity

The intestinal epithelial cell layer of the gastrointestinal tract lies at the border between the gut-associated lymphoid tissue (GALT), which is the most abundant accumulation of lymphocytes in the body, and the intestinal lumen which contains a high number of dietary antigens and a varied commensal microbiota [70]. The intestinal epithelial cell layer is covered by a mucus gel, which functions as a protective layer for the gastrointestinal system. This barrier function includes the prevention of entry of pathogenic microorganisms, toxins and allergens. The mucus gel is composed of glycoproteins called mucins, with up to 20 mucin genes identified. Mucin genes are expressed by specific cells (goblet cells and enterocytes) and categorised as gel-forming secretory mucins (*Muc2, Muc5AC, Muc5B, Muc6*, etc.) or membrane-bound mucins (*Muc1, Muc3, Muc4, Muc13*, etc.) [71]. Interestingly, mice deficient in the *Muc2* gene develop spontaneous colitis and consequently colon cancer.

Milk hydrolysates can influence the expression and secretion of mucins, as outlined in **Table 1**. The modulation of mucin production by milk hydrolysates may assist in the development of dietary strategies to enhance and protect the mucus layer. In rats, jejunal *ex vivo* studies, hydrolysates of casein and α-lactalbumin increased mucin secretion, whereas the native casein did not have any effect [72, 73]. An enzymatic hydrolysate of β-casein enhanced mucin secretion by up-regulating *Muc2* and *Muc3* genes in rat intestinal cells (DHE) and *Muc5AC* gene in human intestinal cells (HT29-MTX) [74, 75]. In a similar experimental model, the enzymatic hydrolysates of β-lactoglobulin were even more effective in increasing mucus production compared to a β-casein hydrolysate [76]. Rats receiving a casein hydrolysate supplemented diet had up-regulated the expression of *Muc3* and *Muc4* genes in the small intestine and colon [77]. This β-casein fragment, which up-regulated *Muc5AC* in HT29-MTX cells, also up-regulated *Muc2, Muc4, defensin 5* and *lysozyme* expression in rat ileum [75]. In a dextran sulphate sodium (DSS)-induced colitis model in rats, diets supplemented with either cheese whey protein [78] or whey protein isolate [79] or α-lactalbumin [80] displayed gut protective effects by increased mucus production. The protection of the gut from DSS challenge by cheese whey protein was possibly mediated by its high threonine and cysteine content. In fact, specific amino acids such as threonine, cysteine, proline and serine increase the number of Muc2-containing goblet cells, up-regulate mucin synthesis and eventually help to restore *Enterobacteriaceae, Enterococcus* and *Lactobacillus* populations in the gastrointestinal tract, thus enforcing the gut defence mechanism and mucosal healing in DSS challenged rats [81].

4.4. Milk hydrolysates can modulate the gastrointestinal immune system

The intestinal mucosa exists in a non-pathological state of continuous 'physiological inflammation'. This low level of inflammation is required to prime the GALT for potential pathogenic bacteria [82]. The mucosal immune system features immune cells including neutrophils, monocyte/macrophages, dendritic cells, mast cells, B and T cells. The crosstalk between intestinal epithelial cells, gut microbiota and local immune cells is essential to maintain intestinal homeostasis, whereas, dysregulation leads to chronic intestinal inflammation [83]. Much of the experimental data come from model organisms such as mice and rats; however, a number of studies have been carried out in humans. Several examples of anti-inflammatory activity exhibited by a variety of milk hydrolysates across a range of experimental models are listed elsewhere [59, 60, 82, 84].

Several milk protein hydrolysates enhance immune cell function by increasing secretion of immunoglobulins, as outlined in **Table 1**. Immunoglobulins are glycoprotein molecules that specifically recognise antigens from bacteria or viruses and aid in their destruction through a highly complex and specific immune response [85]. Hydrolysates of αs1-casein, αs2-casein and β-casein stimulated the immune system through the enhancement of immunoglobulin G (IgG) and IgA concentrations [86, 87]. Casein hydrolysates conferred protective effects against pathogenic microorganisms in mice challenged with bacterial endotoxin, LPS, by increasing intestinal and faecal IgA and anti-LPS IgA levels [88]. Similar modulation of immune response was recorded in mice against *E. coli* infection, when receiving trypsin/chymotrypsin-digested whey protein fractions [89]. Not only strengthening the immune response but post-weaning complications were also eradicated in piglets supplemented with bovine lactoferricin and lactoferrampin fusion peptide by increasing serum levels of IgA, IgG and IgM concentrations and improved diarrhoeal scores [90].

Immune cells, such as monocytes and macrophages, play an important role in inflammatory responses and tissue repair and remodelling by either interacting directly with microorganisms during infections and/or secretion of cytokines that mediate biological effects [91]. Milk hydrolysates can modulate the gastrointestinal immune system by modulating proliferation and maturation of localised immune cells; the immunomodulatory activities of milk hydrolysates are outlined in **Table 1**. Casein peptides induced innate host immune responses in humans, by stimulating the proliferation of lymphocytes and macrophages, [92] and in mice, by activating monocytes and macrophages [93]. On the contrary, rennin-digested κ-casein fragments inhibited the proliferation of mouse spleen lymphocyte and rabbit Peyer's patch cells [94]. The mechanisms of this κ-casein fragment include acting either as an anti-IL-1 antibody or suppressing IL-2 receptor expression on CD4+ T-cells [95]. Functionally, the phagocytic activity of inflamed murine macrophages was increased by *Lactobacillus helveticus*-fermented skim milk through increasing *TNF-α* production [96].

Particular milk hydrolysates modulate the MAP kinase and NF-κβ pathways that consequently control the secretion of several cytokines that can induce inflammatory responses and strengthen the host defence mechanisms [97]. Mice supplemented with *Lactobacillus helveticus* R389-fermented milk peptides had increased circulatory intestinal calceneurin enzyme, an

activator of the gastrointestinal immune system and cytokine *IL-6* [98]. Interestingly, the immune response against LPS was inhibited by casein-derived peptides in Balb/c mice diet by increasing circulatory anti-inflammatory cytokines IL-10 and IL-14 and suppressing proinflammatory cytokines TNFα and IFNγ [99]. Similar suppression of a pro-inflammatory response by down-regulation of IL-8 in inflamed Caco-2 cells was recorded by casein hydrolysate and its size fractions [37, 38]. Further validation of the anti-inflammatory activity was performed in porcine colonic explants and the casein hydrolysate, and its size fractions down-regulated *IL-1α*, *IL-1β* and *IL-8* expression [37, 38]. Bovine κ- casein hydrolysate inhibited circulatory IFNγ secretion and suppressed *IL-10* and *FoxP3* expression in concanavalin A (ConA)-stimulated rat splenocytes [100]. This bovine κ-casein, in a human macrophage cell line U937, was associated with the suppression of circulatory pro-inflammatory cytokines IL-1β, TNFα and IL-8 production [101]. However, whey protein hydrolysate up-regulated the immune response in *E. coli* infected mice by increasing circulatory serum transforming growth factor (TGF)-β secretion [89].

5. Future developments

The potential health benefits of milk hydrolysates are a subject of growing commercial interest from a health-promoting functional-food perspective. Several commercial products are currently available in the market, and this trend is likely to continue. There are three major areas where developments can be made. The generation of milk hydrolysates is the first area of development. The generation and processing of food grade milk hydrolysates should be carefully designed to yield hydrolysates with diverse bioactivities. Novel technologies can be developed, focussing on the process of enrichment of the hydrolysates with active peptides from milk proteins. The second area of development is the research technologies used to evaluate the bioactivity of milk hydrolysates. The investigation of biochemical properties using newly developed modern analytical technologies is required to understand the cross reactivity between milk hydrolysates and the carrier food matrix. Third, robust platforms should be developed to study the molecular mechanisms by which the bioactives exert their activities. This area is the most challenging research area as the outcome from these studies forms the basis of tailored dietary formulations.

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