fermentation

Review

Bioactive Peptides Derived from Whey Proteins for Health and Functional Beverages

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Abstract: Milk serves as a crucial source of natural bioactive compounds essential for human nutrition and health. The increased production of high-protein dairy products is a source of whey—a valuable secondary product that, along with other biologically valuable substances, contains significant amounts of whey proteins and is often irrationally used or not utilized at all. Acid whey, containing almost all whey proteins and approximately one-quarter of casein, presents a valuable raw material for generating peptides with potential health benefits. These peptides exhibit properties such as antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antihypertensive, antithrombotic, opioid, mineral-binding, and growth-stimulating activities, contributing to improved human immunity and the treatment of chronic diseases. Bioactive peptides can be produced by enzymatic hydrolysis using a variety of proteolytic enzymes, plant extracts, and microbial fermentation. With the participation of plant enzymes, peptides that inhibit angiotensin-converting enzyme are most often obtained. The use of enzymatic hydrolysis and microbial fermentation by lactic acid bacteria (LAB) produces more diverse peptides from different whey proteins with α-lactalbumin and β-lactoglobulin as the main targets. The resulting peptides of varying lengths often have antimicrobial, antioxidant, antihypertensive, and antidiabetic characteristics. Peptides produced by LAB are promising for use in medicine and the food industry as antioxidants and biopreservatives. Other beneficial properties of LAB-produced, whey-derived peptides have not yet been fully explored and remain to be studied. The development of whey drinks rich in bioactive peptides and based on the LAB proteolytic activity is underway. The strain specificity of LAB proteases opens up broad prospects for combining microorganisms to obtain products with the widest range of beneficial properties.

Keywords: functional products; peptides; whey; fermentation; lactic acid bacteria; proteolytic activity

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1. Introduction

Rapid population growth on Earth, coupled with the impossibility of expanding arable land and further intensifying food production, necessitates the development of innovative methods to enhance the nutritional and biological value of existing food products. Additionally, there is a need for more efficient use of raw materials and the creation of waste-free production systems. One promising direction is the utilization of whey, which is increasingly recognized not as a mere byproduct of dairy production, but as a valuable raw material for further biotechnological processing.

Advanced methods such as chemical hydrolysis, fermentation, enzymatic treatment, and environmentally friendly technologies like ultrasonic and thermal treatments, have been successfully employed to extract peptides from whey protein [1]. These peptides exhibit excellent functional properties that positively impact cardiovascular, digestive, endocrine, immune, and nervous systems. Consequently, research on their application for health enhancement, through both the optimization of food product compositions and the development of pharmaceuticals, is highly relevant [2–4]. The development of peptide drugs is currently one of the most prominent topics in pharmaceutical research [5]. Commercial production of functional probiotic products containing bioactive peptides (BAPs) offers significant contributions not only due to their health benefits but also by reducing the risk of spoilage in dairy products. The inclusion of natural antioxidants (such as polyphenolic compounds, tocopherols, ascorbic acid, soybean and oat products, grape seeds, tea catechins, amino acids, peptides, herbs, and spices) in yogurt and other dairy products can prevent the rancidity of milk fat, thus extending shelf life and maintaining quality [6].

The use of whey in dietary and therapeutic nutrition is highly appropriate due to its high nutritional and biological value, relative cost-effectiveness, and availability. Moreover, utilizing whey waste from the dairy industry addresses environmental concerns, making this approach one of the most promising directions in the development of functional products [7]. In this review, we summarize the data on biologically active whey peptides, methods of their receiving, and the potential use in the production of therapeutic and prophylactic functional products, whey beverages in particular.

2. Milk and Whey

2.1. Milk

The value of milk as a primary food product for newborn humans and animals is due to its content of complete protein, conjugated linoleic acid, omega-3 fatty acids, vitamin D, selenium, calcium, and, most importantly, biologically active peptides [8,9] that play a key role in the physiological activity of the human body.

More recently, an inverse correlation has been shown between dairy consumption and cardiovascular diseases [10,11], as well as a direct correlation between increased dairy consumption and improved metabolic health [12]. Milk is a rich source of antioxidants, including vitamins A and E, and there is evidence that milk consumption may be associated with a reduced risk of chronic diseases [13]. The main part (80%) of the protein fraction in milk consists of caseins. Caseins are divided into four main types (αs1-, αs2-, β-, and κ-casein) constituting 40, 12.5, 35, and 12.5% of the casein fraction, respectively [14]. Caseins and whey proteins are almost completely assimilated and are extremely important in the prevention and treatment of numerous dietary, physiological, and metabolic diseases. Their amino acid composition is equivalent to that of meat proteins but does not contain harmful purine bases, unlike meat proteins. Osteopontin is found in the highest concentrations in milk—a multifunctional protein involved in immune system activation and regulation; biomineralization; tissue remodeling processes, including the growth and development of the intestine and brain; interactions with bacteria; and much more [15].

Milk is a very important source of natural bioactive components for human nutrition and health [16,17]. Various BAPs released during gastrointestinal digestion directly affect numerous biological pathways, causing behavioral, gastrointestinal, hormonal, immunological, neurological, and dietary responses [18,19]. Notably, some beneficial properties are attributed not to the original proteins of both human and animal milk but exclusively to the bioactive peptides released from them [20]. Most of these peptides remain in whey after the bulk of the casein is separated into high-protein dairy products.

2.2. Whey

Every year, more than 160 million tons of whey is generated globally [21]. Approximately half of the dry components of milk, containing a variety of compounds, are transferred to whey (including up to 100% whey proteins; up to 88% minerals; up to 25% casein; up to 12.5% fat; and up to 99% lactose, along with vitamins, enzymes, and organic acids) [22]. Since up to 25% of casein passes into whey, it has a significant impact on the properties of the total protein–peptide fraction of whey. Caseins are phosphoproteins that have the ability to bind calcium. However, κ-casein is less phosphorylated and binds calcium to a lesser extent but has stabilizing properties [23]. Whey proteins include β-lactoglobulin, α-lactalbumin, whey albumin, lactoferrin (LF), lysozyme, and other bioactivators (Figure 1). The different biological activities of these proteins are significant for the prevention and treatment of numerous dietary, physiological, and metabolic diseases. For example, LF—one of the most important bioactivators in milk—performs biological functions such as regulating iron absorption and modulating immune responses and possesses antimicrobial, antiviral, antioxidant, anticancer, and anti-inflammatory activities [3]. LF is considered one of the milk components modulating the gut flora of infants. It increases the number of beneficial bacteria, and protects the host organism from infection and inflammation, partially restoring gut homeostasis and the integrity of both the intestinal wall and lung tissue. By acting locally on the intestine and microbiota, LF is also seen as a promising candidate for the systemic treatment of influenza [24]. It has been shown that LF has anticarcinogenic activity against many human and animal tumors, modulating cell-level proliferation, differentiation, maturation, activation, migration, and immune cell function [25], as well as antioxidant and antigenotoxic activity concerning gastric and hepatic cells [26]. The rich biologically valuable composition of whey makes its loss unacceptable after separation of the main part of casein.

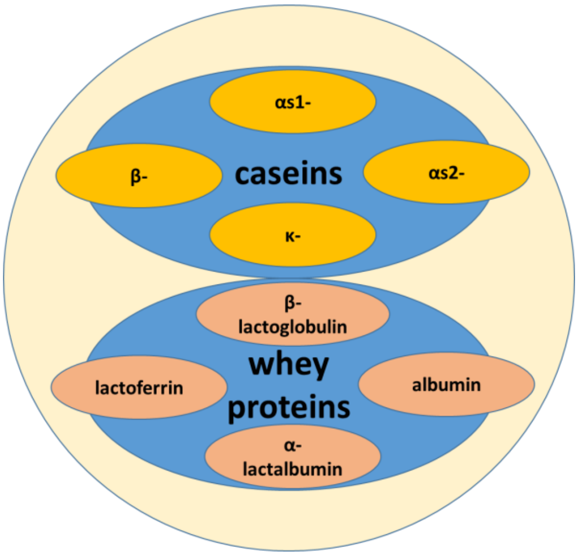

Figure 1. Whey proteins.

The increase in cheese production, Greek yogurt, and other dairy products has led to an increase in unused acidic whey, further exacerbating its detrimental effects on the environment [27]. Contrary to sweet whey (produced during hard cheese making with enzymes), acidic whey, due to its properties (elevated lactic acid and calcium phosphate levels, increased oxygen demand), necessitates higher processing expenses [28,29] and substantial efforts for its proper utilization [30].

From a nutritional point of view, whey proteins outperform others, particularly caseins, due to their abundant essential branched-chain amino acids crucial for metabolism [31]. The biological functionality of whey proteins is closely linked to their structural characteristics, allowing for them to function as intact molecules, partially hydrolyzed forms, or small bioactive peptides. Extensive research [32] has explored the biological effects of whey proteins in animal and human models, highlighting benefits such as improved nutrient uptake and decreased susceptibility to chronic diseases.

Whey protein stands as a vital byproduct of the dairy sector, yet it poses a significant environmental threat. Therefore, the enzymatic breakdown of whey protein concentrate to produce peptides presents an innovative strategy that can enhance the value of whey by transforming a low-value waste byproduct into high-quality commodities, specifically antioxidant and cytoprotective peptides. These peptides, when incorporated as functional constituents [33], have the potential to enhance consumer health by fortifying the immune, cardiovascular, nervous, and gastrointestinal systems [34]. In recent years, research related to the functions of peptides has increased significantly.

3. Peptides

Peptides produced by living organisms of various origins are currently considered as a new generation of bioactive regulators. They regulate gene expression and protein synthesis in plants, microorganisms, insects, birds, rodents, primates, and humans. Low molecular weight peptides, usually <10 kDa, with hydrophobic properties and aliphatic and aromatic chains [35], play an important role in molecular interactions to ensure necessary activity.

Short peptides consisting of 2–7 amino acid residues can penetrate the nuclei and nucleoli of cells and interact with the nucleosome and histone proteins, as well as with both single-stranded and double-stranded DNA. DNA–peptide interactions, including sequence recognition in gene promoters, are important for template-driven synthetic reactions, replication, transcription, and repair. Peptides can regulate DNA methylation status, which is an epigenetic mechanism of gene activation or repression, both in normal conditions and in cases of pathology and aging [36]. Thus, peptides are important bioregulators of important cellular functions.

In this regard, targeted enzymatic hydrolysis or microbial fermentation of whey protein concentrate is a useful innovative approach to obtain peptides for subsequent utilization as functional ingredients. In the case of enzymatic hydrolysis, the use of specific and nonspecific proteases is the most common method for obtaining bioactive peptides, requiring less time and allowing for control over the hydrolysis process to obtain peptides with specific molecular weights and amino acid compositions [37]. Then, they are further cleaved by exopeptidases into smaller peptides and free amino acids. Hydrolysis leads to the accumulation of peptides and free amino acids in food. Some of the peptides obtained may be bioactive and thus have a beneficial effect on consumer health. This process can be facilitated by in silico tools including peptide cutters, predictive modeling like molecular docking, quantitative structure–activity relationships [38], and compliance with optimal conditions for hydrolysis of whey protein concentrate [39,40]. Biologically active peptides can be produced using fungal proteases, for example, Flavourzyme produced by *Aspergillus oryzae* [41] and a new protease from *Myceliophthora thermophila* [42]. It has also been shown that the use of trypsin makes it possible to obtain bioactive peptides with high antioxidant and antimicrobial activity [43].

BAPs derived from milk are potential ingredients for health-beneficial functional food products. The wide range of physiological functions of BAPs includes antimicrobial, antihypertensive, antithrombotic, anticancer, antioxidant, opioid, anti-appetite, immunomodulatory, mineral-binding, and growth-stimulating activities [8,44,45]. They can enter the body in intact form, that is, as structures exhibiting their properties directly in the gastrointestinal tract (GIT), as well as in the form of specific protein and peptide precursors [46]. Often, these dietary proteins are first subjected to the action of digestive enzymes of the macroorganism or the residing microbiota and then enter the bloodstream as precursors of the body's bioactive polypeptides. They are targeted at treating chronic diseases related to nutrition: obesity, cardiovascular diseases, and diabetes. Peptides derived from cow, goat, sheep, buffalo, and camel milk have multifunctional properties, including antimicrobial, immunomodulatory, antioxidant, and antithrombotic, as well as inhibitory and antagonistic activities against various toxic agents. By regulating immunological, gastrointestinal, hormonal, and neurological responses, they play a vital role in the prevention of cancer, osteoporosis, hypertension, diabetes, and other diseases [1,47]. The variety of functions of peptides in the body makes them important for the development of health-supporting preparations.

3.1. Relevance of Peptide Preparations

The biological activity of peptides and their contribution to maintaining organic homeostasis, immunomodulation, and protection of the body from oxidative and other adverse processes, is well described in the literature. Reports on the activity of milk peptides in glycemic control, antihypertensive activity, and their ability to inhibit uric acid formation are summarized [35], as well as the need to address gaps related to their interaction with cellular targets and their use in human therapy.

In recent decades, the number of approved peptide drugs has steadily increased, and by 2021, the average growth rate of the global peptide therapy market was 7.7% [48]. As potential therapeutic agents, they have numerous advantages: low production cost, low toxicity, and the possibility of storage at room temperature [49]. They are used against HIV infection [5]. With the development of sequencing technologies and synthesis methods, more therapeutic peptides with two or more functional characteristics are being discovered [50], which is crucial for creating new drugs.

3.2. Antimicrobial Peptides

The antibacterial peptide-based therapeutic approach has significant potential in addressing infections caused by antibiotic-resistant bacteria, which present a growing menace to human health. In contrast to the bactericidal mechanism of conventional antibiotics with a singular target, antimicrobial peptides can eliminate pathogens by affecting multiple targets, thereby notably diminishing the emergence of antibiotic-resistant bacteria and positioning them, owing to their wide spectrum of activity, as one of the prime substitutes to intricate antibiotics [11,51]. They function through various mechanisms, augmenting natural immune responses and decreasing susceptibility to chronic diseases [52].

A favorable attribute of antimicrobial peptides is their compatibility with living organisms and degradability, thermal stability, and elevated specificity, which is highly appealing for food conservation [53]. Moreover, unlike antibiotics, peptides disintegrate entirely within the organism without manifesting adverse repercussions. One such peptide, nisin, derived from *Streptococcus lactis* [54], is presently utilized commercially (as E234). The consumer demand for food items devoid of synthetic preservatives has propelled the search for natural antimicrobial substances with a broad antimicrobial spectrum and enhanced characteristics.

Goat milk is widely recognized as an excellent source of dairy protein. The casein and whey protein BAPs derived from it also exhibit antibacterial properties [55]. Goat milk protein is a promising source for the development of high-quality protein products with high safety standards that have potential applications in the pharmaceutical and food industries.

3.3. Bioactive Peptides and Health

Over the past decade, viral infections have become a substantial health concern. The concept of antimicrobial peptides encompasses antiviral peptides, which exhibit notable potential in safeguarding the human body against various viral ailments [56]. Due to the necessity of devising experimental and computational techniques for antiviral peptide recognition, strategies for feature depiction, categorization algorithms, and assessment criteria for performance are being formulated [57]. Recently, the employment of sophisticated machine learning approaches for the design of peptide-centered therapeutic agents has become increasingly advantageous due to their noteworthy outcomes [58–61]. A web server has been established for the prediction of anti-coronavirus peptides [62].

BAPs synthesized by the cells of the macroorganism are an important part of their innate immunity. Recent investigations have shown that minor cationic peptides can regulate cell viability in diverse human cell cultures, inhibit cancer cells, and serve as therapeutic agents against cancer [63]. They exhibit immune cell functions, encompassing lymphocyte proliferation, antibody production, and cytokine control [64]; stimulate macrophages' phagocytic capability; and inhibit specific cytokine secretion [65,66]. Anticancer peptides have emerged as promising therapeutic agents for managing and preventing cancer. Their utilization may evolve into a feasible substitute for traditional treatments for this illness, as they are notably safer and more selective than conventional agents [67,68], and, owing to their heightened specificity and reduced toxicity, do not jeopardize the functionality of vital organs [69]. Nevertheless, with the rapid escalation in the number of peptide sequences, predominantly unveiled through time-intensive experimental screening, there exists a requirement for the formulation of dependable and precise predictive models. Currently, systems that outperform existing methodologies, achieving nearly 100% precision, have been devised and will prove beneficial in the development of anticancer medications [70–73]. The ability to predict the functions of peptides is a great achievement of the present time, making it possible to speed up and make more efficient the process of searching for peptides that are most suitable for specific purposes.

BAPs can also lower blood pressure by inhibiting angiotensin-converting enzyme (ACE), which plays a central role in blood pressure regulation. Angiotensin is one of two polypeptide hormones and a powerful vasoconstrictor that acts in the body to control blood pressure through the contraction of smooth muscle in blood vessels [74]. In addition, ACE inactivates bradykinin, an endothelium-dependent vasodilator that increases blood pressure [44], which forms the basis of one of the treatment strategies for hypertension. Ong and Shah [75] showed that the addition of *Lactobacillus acidophilus* to cheese starter increased the production of a number of BAPs that lower blood pressure by inhibiting ACE. Other researchers [76] used *L. acidophilus* for the fermentation of whey protein and obtained several antihypertensive and antioxidant peptides useful in the development of pharmacologically active ingredients for health in the coming years. The highest ACE inhibitory activity, as well as antioxidant activity, was demonstrated by goat milk treated with alkaline protease [77,78], which should be considered in the development of functional dairy products for the treatment of hypertension.

Due to their numerous proposed and proven health benefits of peptides, along with recent advancements in their discovery and identification [79], bioactive peptides from milk are already being used in formulations of new functional food products as nutraceuticals and natural medicines [80,81]. Fermented dairy products, such as yogurt, cheese, and sour milk, are excellent sources of milk peptides and are gaining popularity worldwide. Furthermore, both fermented and non-fermented dairy products have been shown to be associated with a lower risk of hypertension, coagulopathies, stroke, and cancer [82].

The antioxidant and ACE-inhibitory activity in fermented dairy products can be enhanced through co-fermentation with probiotic lactic acid bacteria (LAB) cultures, due to their relatively high proteolytic activity [83]. Probiotic strains (depending on their specificity) can contribute to various health benefits for the consumer, attributed to BAPs, during the fermentation of milk whey [84]. For instance, it has been shown that the probiotic *L. plantarum* subsp. *plantarum* PTCC 1896 or its components are promising for future use in treating type II diabetes by inhibiting DPP4 [40]. A proteomic profile study of kefir (an alcoholic fermented milk drink) identified 257 peptides, mainly released from β-casein [85]. Among these, 16 peptides demonstrated antimicrobial, immunomodulatory, ACE-inhibitory, opioid, antithrombotic, mineral-binding, and antioxidant activities. Yogurt peptides, when modified by treatment at pH 6.0 or above 50 °C, exhibited higher antioxidant activity compared to the control [86]. The presented achievements in the field of screening of bioactive peptides and the development of drugs with various functions contributed to the turning of researchers' attention to whey peptides as an accessible and fairly cheap source of peptides with health-beneficial and technologically valuable properties.

3.4. Whey Protein Peptides

Among the products of whey protein hydrolysis (WPH), short-chain peptides predominate, demonstrating low allergenicity and high essential amino acid content. WPH can be used in the production of various dairy products as a replacement for skim milk in the preparation of normalized mixtures, in the production of prophylactic nutrition products for people with cow's milk protein allergies, and as a primary ingredient in beverages for regular consumption [87]. Products derived from whey are also used in the manufacture of pharmaceuticals, cosmetics, and skincare products (Figure 2). They are used by athletes to stimulate muscle protein synthesis, provide energy, and enhance endurance. Moreover, bioactive components derived from whey have numerous biomedical, pharmaceutical, and therapeutic applications [88]. There are already more than 60 peptide drugs on the market, and several hundred new therapeutic peptides are in preclinical and clinical development [89].

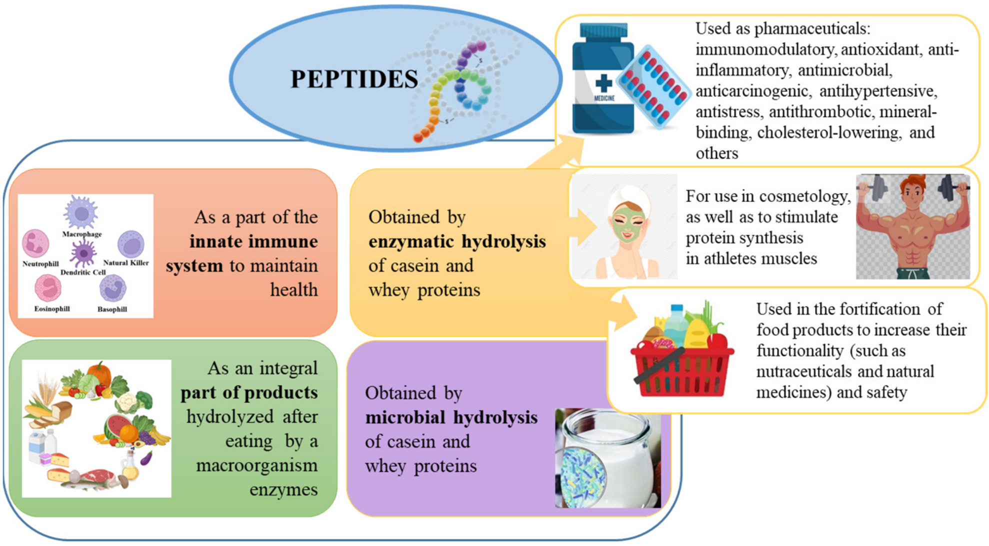

Figure 2. Sources and significance of milk and whey peptides.

Among the products of proteolytic cleavage of milk whey proteins, opioid peptides have been found to play a protective role, for example, by reducing environmental stress during breastfeeding [90]. Additionally, peptides with immunomodulatory and hypocholesterolemic effects, as well as peptides that influence intestinal motility, have been identified [45]. There is evidence that peptides derived from milk whey proteins are involved in the realization of biological functions such as calcium ion absorption, antioxidant activity, appetite regulation, and anticancer activity [91]. It has also been shown that some peptides derived from lactoferrin and casein exhibit antithrombotic activity [44,92]. According to Peipei Dou [93], WPH significantly suppresses the development of high blood pressure and tissue damage caused by hypertension by inhibiting ACE activity and reducing renin concentration, thereby lowering systolic blood pressure in spontaneously hypertensive rats. Increased *Akkermansia*, *Bacteroides*, and *Lactobacillus* bacteria also contributed to lowering blood pressure, reducing heart weight, and decreasing cardiomyocyte damage (hypertrophy and degeneration). The proteomic results showed that the expression of 19 proteins in the heart, mainly related to the Wnt and apelin signaling pathways, was altered after WPH intake. In particular, oxidative stress in serum decreased, as evidenced by reduced malondialdehyde content, increased total antioxidant capacity, and superoxide dismutase activity. The data obtained indicated that WPH exhibits promising antihypertensive capabilities in vivo and could become a potential alternative to antihypertensive dietary supplements.

Whey protein-derived bioactives, including α-lactalbumin, ß-lactoglobulin, bovine serum albumin, lactoferrin, transferrin, and proteose–peptones, have exhibited wide ranges of functional, biological, and therapeutic properties varying from anticancer to antihypertensive and antimicrobial effects [94,95].

Under the influence of trypsin and chymosin, antimicrobial peptides, mostly cationic, antagonistically active against Gram-positive and Gram-negative bacteria can be obtained from bovine αS1-, αS2-, and κ-caseins [96]. Some caseicidins had poor clinical performances compared to commercially available antibiotics, others were more effective. For example, isracidin, a 23-amino-acid fragment of αS1-casein, had a broad spectrum of activity in vitro at high concentrations (0.1–1 mg/mL) and was more effective in vivo [96]. However, the cationic nature of a significant part of casein peptides, leading to adsorption on negatively charged surfaces, and the instability in isomeric variation during fermentation have become an obstacle to the further use of many discovered peptides. Nevertheless, cappacin has been applied in oral therapy combined with zinc, increasing its antibacterial activity. At the same time, peptides derived from casein can be successfully used in the food industry.

Microbial fermentation of milk caseins yielded better results regarding the antimicrobial activity of the resulting peptides. Thus, by fermenting bovine as2-casein with *Lactobacillus acidophilus*, peptides were obtained that were effective against food-borne pathogens, such as *Listeria innocua*, *E. coli*, *Enterobacter sakazakii*, and *Streptococcus mutans* with MICs of 0.05–0.2 mg/mL [96]. The resulting peptides have great potential for use in purified form and as a casein fermentate.

Among whey proteins, lactoferrin and lysozyme have antimicrobial activity and release antimicrobial moieties upon proteolysis [97,98]. The antimicrobial peptides derived from them are the best studied. Previously obtained peptides from α-lactalbumin and β-lactoglobulin had antimicrobial activity but had also some drawbacks: a narrow spectrum of action, moderate antimicrobial activity, and potential allergenicity. However, there are now new data on highly active peptides obtained by enzymatic hydrolysis of whey. Thus, the treatment of goat milk whey with trypsin led to the production of a hydrolysate effective against toxigenic fungi of the genus *Penicillium*, which cause bread spoilage [99]. In turn, whey proteins hydrolyzed with pepsin for 45–90 min produced short peptides from β-lactoglobulin and α-lactalbumin, which exhibited significant antibacterial activity [100]. Hydrolysis of whey proteins by alcalase and savinase led to the production of peptides with high antioxidant activity, similar to lactalbumin peptides [101]. When hydrolyzing camel whey protein concentrate with trypsin, potent α-lactalbumin-derived and beta-casein-derived dipeptidyl peptidase IV (DPP-IV)-inhibitory peptides were discovered [38]. The allergenicity of antimicrobial peptides derived from milk, on the one hand, must be preliminarily assessed in each case; on the other hand, on the contrary, a decrease in allergenicity is possible due to the decomposition of initially allergenic proteins.

There is a large quantity of data on the production of antioxidant peptides from whey proteins. Thus, the treatment of whey protein concentrate with chicken and pork pepsin led to the complete degradation of α-lactalbumin (but not β-lactoglobulin) to obtain whey protein hydrolysates with high antioxidant activity [102]. Using alcalase [103] and thermolysin [104], α-lactalbumin was hydrolyzed to produce antioxidant peptides. Highly active antioxidant fractions of α-lactalbumin and β-lactoglobulin peptides were also obtained from sheep whey under the influence of pepsin and alcalase enzymes [105]. So, whey-derived antioxidant peptides can be a promising source of natural dietary antioxidants for the development of functional foods.

In another experiment with enzymatic hydrolysis of α-lactalbumin, peptides that inhibited dipeptidyl peptidase IV were obtained. Various enzymes (trypsin [106], pepsin [107], and thermolysin [108]) were used in different studies. The results obtained can be important for the prevention and treatment of type 2 diabetes.

Çakır et al.'s study differs from the bulk of research in the revealed result. By the treatment of goat milk whey fraction with trypsin, peptides were obtained as possible candidates for the treatment of SARS-CoV-2 [109]. In addition, xanthine oxidase, the pivotal therapeutic target for gout and hyperuricemia, may be inhibited by novel whey protein peptides obtained by virtual enzymatic hydrolysis of α-lactalbumin and β-lactoglobulin and tested using in silico instruments [110]; however, these data should be confirmed in practice.

In addition to digestive enzymes, proteases from plants and their fruits (cardoon, *Bromelia antiacantha* Bertol, *Bromelia balansae* Mez, *Citrus aurantium*, *Solarium elaeagnifolium*, *Cucumis melo*, *Salpichroa origanifolia*, and *Cynara cardunculus*) can also be used to hydrolyze whey proteins [111–116]. Some plant proteases hydrolyzed α-lactalbumin better, while others hydrolyzed β-lactoglobulin. When using plant enzymes, peptides that inhibit angiotensin-converting enzyme were most often obtained from both proteins. Antioxidant peptides have also been also noted. The resulting peptides can be effective in the treatment of hypertension and can also be used as functional food ingredients in food products aimed at controlling hypertension.

The production of bioactive peptides by microbial fermentation has been less studied. However, available evidence also suggests that microbial fermentation of whey proteins also often produces angiotensin I-converting enzyme inhibitor, antioxidant, and antidiabetic peptides.

In spontaneous fermentation of cheese whey [117], more than 400 peptides were identified, mainly derived from beta-casein, kappa-casein, and α-lactalbumin. Among them, 49 were bioactive and 21 inhibited angiotensin-converting enzyme. Analyzed by Pescuma et al. [118], LAB strains were able to degrade β-lactoglobulin during growth in a chemically defined medium and under starvation conditions. The different peptide profiles obtained indicate different specificities of proteases from *Lactobacillus delbrueckii* ssp. *bulgaricus* CRL 454, *Lactobacillus acidophilus* CRL 636, and *Streptococcus thermophilus*. However, α-lactalbumin was not degraded by lactic acid bacteria. In another study [119], when fermenting acidic goat whey with a mixture of dairy microorganisms, the yeast *Kluyveromyces marxianus* and LAB *Lactobacillus rhamnosus* were selected. They completely hydrolyzed α-lactalbumin and, to a lesser extent, β-lactoglobulin. Both were capable of producing angiotensin I-converting enzyme (ACE)-inhibiting peptides. ACE-inhibiting peptides were also obtained from acidic goat whey fermented aerobically for 168 h with *K. marxianus* and *Lactobacillus rhamnosus*. In this study, two novel lactokinins, resistant to the imitation of gastrointestinal digestion, were identified [120]. Hydrolysis of α-lactalbumin, β-lactoglobulin, and whey protein concentrate by serine protease of *Yarrowia lipolytica* led to a significant increase in the antioxidant activity of the hydrolysate [121]. In turn, *Lactiplantibacillus plantarum* LBBS2 and *L. plantarum* LBM2 demonstrated optimum hydrolysis of whey proteins also after 48 h of incubation [122]. At the same time, the antioxidant activity was maximum after 24 h, while the antibacterial activity was most pronounced after 48 h. In the Solieri et al.'s research [123], during the hydrolysis of whey protein concentrate with *Lactobacillus helveticus* and *S. thermophilus* strains, the strain *S. thermophilus* RBC06 was selected, which showed the highest antioxidant activity, as well as the inhibitory activity of angiotensin-converting enzyme and dipeptidyl peptidase IV. Antihypertensive lactotripeptides had the sequences valine–proline–proline and isoleucine–proline–proline. An assessment of lactic acid bacteria isolated from Iranian dairy products revealed that milk fermented for 48 h with certain strains of *Lactobacillus delbrueckii* and *Lacticaseibacillus paracasei* ssp. *paracasei* had the highest free radical scavenging [124], while the use of *L. helveticus* PTCC 1930 and *L. paracasei* ssp. *paracasei* PTCC 1945 contributed to the production of antidiabetic peptides. It has also been shown that peptide fractions with a lower molecular weight demonstrated the highest proteolysis, antidiabetic, and antioxidant properties.

The presented data indicate the high potential of microorganisms, namely, lactic acid bacteria and lactose-fermenting yeast in the production of a variety of bioactive peptides from whey, which can be of interest for the generation of functional health ingredients involved in the regulation of hypertension and diabetes and can also be used as antioxidants and antibacterial agents, especially in the food industry. Whey hydrolysates can be used as additives to create whey-based drinks with enhanced health benefits.

BAPs derived from whey protein and added to probiotic fermentation products and confectionery items (chocolate, cookies, or cream) influence human health both directly and indirectly. They increase the viable count of probiotic bacteria, enhance the stability of probiotic products, act in the human body as antioxidants and ACE inhibitors, stimulate the proliferation of intestinal epithelial cells, and more. According to Pasin and Comerford [125], the benefits of dairy products in diabetes for insulin secretion and glycemic control are attributed to the high content of essential amino acids and BAPs that stimulate insulin secretion, as well as specific combinations of macronutrients and micronutrients and the unique strains of probiotics and BAPs they contain.

4. Proteolytic Activity of Lactic Acid Bacteria

As a nitrogen source for LAB growth, a sufficient amount of free amino acids is required, and therefore, for development in high-protein environments, particularly milk, they have evolved the ability to hydrolyze proteins, producing not only free amino acids but also a wide range of biologically active peptides [126], perceived as potential therapeutic tools and important components of personalized nutrition suitable for the prevention of many civilization- and nutrition-related diseases.

In the study by Silva et al. [127], almost half of the isolates exhibited proteolytic activity. Microbial proteolytic enzymes are synthesized inside cells, are mainly secreted externally, and remain associated with the cell wall structure by covalent bonds [128]. Most LAB release amino acids and small peptides during the first six hours of incubation, whereas streptococci consume amino acids initially present in the whey to support growth.

LAB degrade whey proteins to varying degrees. In studies of various research groups, special attention is paid to the main allergenic whey protein β-lactoglobulin. It is β-lactoglobulin that is usually most easily degraded by LAB. However, the degradation of whey proteins by LAB is strain-specific [95,129]. Thus, in a study of LAB in domestic dairy products of the Balkan region, Armenia, Iraq, and Bulgaria, individual strains with relatively high proteolytic activity towards α-casein, α-lactalbumin, and β-lactoglobulin were identified [130]. As a result of hydrolysis, peptides with antimicrobial activity against *E. coli*, *Staphylococcus aureus*, *Listeria innocua*, *Enterobacter aerogenes*, *Enterococcus avium*, and *Salmonela choleraesuis* were obtained. The proteolytic activity of the isolates towards the azocasein substrate was determined in the range from 10.30 to 19.90 U/mg, with *L. plantarum* isolates having the highest proteolytic activity at various pH values [131]. Different strains of *L. plantarum* showed different levels of proteolytic activity towards milk caseins and β-lactoglobulin. Another study showed that minor whey proteins (i.e., LF, bovine whey albumin, and immunoglobulin G) were more susceptible to degradation, while β-lactoglobulin and α-lactalbumin were more resistant to native cheese whey microbiota [132]. In this case, the maximum proteolytic effect was observed at 35–42 °C. After 120 h of fermentation, ACE activity increased from 22% to 60–70%. These results indicate the promise of using mixed cultures of microorganisms, including spontaneous whey microbiota. At the same time, the selection of microorganism strains for the targeted decomposition of α-lactalbumin and β-lactoglobulin will preserve the biological value of lactoferrin and immunoglobulin.

The structure of cell wall proteases is difficult to determine experimentally due to their large size and attachment to the cell wall. However, the use of structure prediction software AlphaFold 2, the domain boundaries were clarified by the group of Christensen et al. [133] based on a comparative analysis of three-dimensional structures. The existence of two distinct domains for cell envelope attachment that are preceded by an intrinsically disordered cell wall spanning domain has been shown. Combinations of domains give LAB proteases different proteolytic activity, stability, and adhesive properties. Targeting specific domain components may allow for control of LAB proteolytic activity for use in medicine and the development of functional foods.

Among the products of proteolysis, short-chain peptides predominate, demonstrating low allergenicity and high levels of essential amino acids [88]. The resulting hydrolysate can be used in the technology of preparing various dairy products to replace skim milk in normalized blends, to produce products for the preventive nutrition of people with cow's milk protein allergy, and as the main ingredient in beverages for regular consumption.

Microbial fermentation with the release of bioactive peptides encoded in milk proteins has now been recognized as a natural, safe, and economically efficient strategy for obtaining these highly effective health regulators. The proteolytic system of LAB mainly consists of cell wall-bound proteinases (initially cleaving casein into oligopeptides), peptide transporters (transporting oligopeptides into the cytoplasm), and various intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases, and dipeptidases, converting peptides into small molecules and generating free amino acids [134,135]. In Fan et al.'s studies, proteolytic activity was most pronounced in *Lactobacillus helveticus* [136], followed by *Lact. delbrueckii* subsp. bulgaricus, *Lact. delbrueckii* subsp. *lactis/diacetylactis*, and *Lact. delbrueckii* subsp. *lactis/cremoris* [137]. Using *Lact. helveticus* as an example, it has been shown that products fermented by different strains of the same genus and species differed from each other in terms of texture, protein content, total and non-protein nitrogen, as well as proteolysis index [138].

5. Whey-Based Drinks

Native whey is rarely used as a beverage due to its unattractive taste [139]. However, developing various types of beverages based on whey is one of the most promising directions for utilizing whey for food purposes. This is not only due to the properties and composition of whey and its suitability for dietary and therapeutic nutrition but also due to its relative affordability and availability, and undeniably, its potential to address environmental pollution issues. In most cases, the range of whey beverages with improved taste is expanded by adding fruit or vegetable syrups, as well as other plant components enriched with bioactive substances, thus combining their positive properties. Without the addition of flavoring ingredients, the taste of the product can be altered using starter cultures, such as in studies by Skryplonek et al. [140,141] using *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* ssp. *lactis* Bb-12. Since acid whey virtually lacks casein, replacing part of the milk with acid whey reduces the gel strength and viscosity of the product [141]. To improve the texture, Skryplonek et al. added more milk solids to the product, either dry skimmed milk or replacing part of the milk with condensed milk. In another study, the addition of a stabilizer resulted in products with high viscosity and water-holding capacity for all tested samples [142]. Research by Oleinikova and coworkers showed that the best sensory performance and structure were achieved with a 3:2 ratio of milk to whey [143].

The biological value of whey is significantly enhanced by its use in cultivating LAB. The chemical composition of whey provides an ideal nutrient matrix for the growth and viability of LAB [129,137,144–146]. The proper selection of LAB can be used to specifically influence the functionality of the resulting beverages. To create beneficial and appealing drinks from acid whey, it is possible to modify and monitor the impact of flavoring ingredients and starter cultures with varying metabolic orientations. Additionally, research has shown that homogenization after fermentation can improve the physical stability of the product and provide a smoother taste. Lievore et al. [139] prepared a liquid beverage in which acid whey replaced water, resulting in a more homogeneous product. When developing fermented dairy products with whey, it is important to consider the addition of stabilizers, homogenization, and the type of beverage to compensate for texture changes. A notable area of interest is the growth of probiotics in the presence of whey derivatives, such as lactulose, a derivative of lactose that is a highly sought-after prebiotic in functional feeding [145]. Another approach is the simultaneous addition of prebiotics to whey along with probiotic starter cultures. For example, the addition of wheat bran [146], which imparts synbiotic properties to the beverage, also enhances the survival of probiotic microorganisms under conditions of acid and bile stresses.

Over the past two decades, a variety of whey-based beverages and similar products containing isolated whey components (mainly whey proteins) have emerged on the market. These beverages are often refreshing, using starter and/or probiotic cultures capable of metabolizing lactose, thereby defining the unique taste and texture of the fermented drink [147]. Both mono- and mixed cultures of LAB are used, and to improve the organoleptic properties, the beverages are enriched with fruit and berry additives, enhancing their consumer appeal. For developing products for children and adolescents, a formulation was created to replace milk with whey, also including iron [148]. Numerous randomized, double-blind, and placebo-controlled clinical trials have demonstrated the promising therapeutic applications of whey-derived products for preventing type 2 diabetes, obesity, cardiovascular diseases, and phenylketonuria; eliminating excessive free radicals formed as a result of oxidative stress; inhibiting tumor development; providing anti-proliferative effects; and treating metastatic carcinoma [88]. The use of acid whey in beverage formulations will not only expand the range of dairy products with high nutritional and biological value but also contribute to increased milk processing efficiency through the implementation of resource-saving technologies.

Whey drinks enriched with bioactive peptides are currently under development. However, there are already encouraging results. Thus, drinks with high antioxidant activity were obtained using kiwi powder and fermentation of *Streptococcus salivarius* subsp. *thermophilus* and probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* [78]. Also, the properties of the drink obtained by fermentation of whey using *Lactobacillus acidophilus* La-03, *Lactobacillus acidophilus* La-05, *Bifidobacterium* Bb-12, or *Lacticaseibacillus casei*-01 [84] were evaluated. It was shown that after 15 days, the drink is characterized by the greatest antioxidant activity and the presence of antihypertensive peptides. The presence of peptides with antimicrobial, immunomodulatory, and ACE-inhibiting activities was also shown. Thus, the development of whey drinks enriched with bioactive peptides opens up broad prospects for improving approaches to the treatment of many diseases.

Thus, due to their significant technological and functional potential, LAB are key microorganisms for the food industry. In fermented dairy products produced and traditionally used worldwide, LAB not only help preserve the raw materials but are also valued as bio-protective and health-promoting agents. This is due to the presence of probiotic microorganisms among them, as well as their nutritional and organoleptic properties, and they are responsible for the flavor development of the final products [69,149]. The choice of specific starter cultures depends on the particular phenotypes that benefit the product, ensuring the shelf life, safety, texture, and taste of the final product. Studies have shown that whey promotes the production of bacteriocins by LAB [150,151]. It has also been shown that the introduction of acetic acid bacteria into the starter culture contributes to anti-*Candida* antagonism in whey-based fermented beverages [152]. Rationally selected LAB can also be used to develop functional beverages with reduced beta-lactoglobulin whey protein content [129], which is the main cause of milk allergy. Thus, the use of proteolytic bacteria can make a significant contribution to reducing milk allergenicity.

6. Perspectives

In view of the necessity to establish technology that produces minimal waste and enhances the profitability of the dairy sector, the need for improving the effectiveness of whey utilization and conversion into a broad array of valuable products and food supplements is steadily increasing. This is supported not only by its health-promoting food elements but also by the marketability of the products created, positioned as Generally Acknowledged as Safe (GRAS) [4,153,154]. The ideal collection of vital amino acids found in whey proteins, which elevate their biological worth in comparison to proteins from other sources, enables them to act as a reservoir of BAPs under the influence of digestive proteolytic enzymes and microbial fermentation. To enhance the process of producing BAPs, hybrid approaches, presently utilized in biopharmaceuticals, have been suggested, founded on computational simulation combined with heuristics and mechanistic simulation. Criteria for industrial procedures concerning the liberation and resilience of peptides based on multiple process variables have been consolidated, and certain techniques for enriching peptides derived from whey, potentially suitable for industrial use, have been examined [34].

The worldwide market for BAPs is progressively expanding due to rising consumer consciousness of the favorable correlation between functional food items and health, alongside the potential to use them as replacements for conventionally marketed medications, particularly those subject to stringent legislation or those that have become ineffective.

Different nations have established diverse regulatory frameworks to shield consumers from hazards and misleading assertions regarding BAPs. Scientific verification of their safety and effectiveness is a crucial factor that can substantially influence the introduction of BAPs into the market. Assertions regarding the health advantages of BAPs must be substantiated by substantial human research findings. Regulatory standards for the utilization of BAPs obtained from various food protein sources, including milk, whey, fish, and soybeans, are present in numerous countries' markets [155].

A scientometric evaluation of studies published between 2011 and 2021 by Brazilian researchers [127] indicated a rising interest in fundamental investigations involving BAPs from various protein origins, encompassing food items and agro-industrial remnants. The most extensively studied characteristics of these biomolecules are their antioxidant properties and their linked antimicrobial capabilities. Furthermore, there are accounts of other impacts, such as anxiolytic, anti-adipogenic, anticoagulant, anticonvulsant, anti-sclerotic, and cyto-regulatory functions, which ascertain the potential for utilizing these biomolecules in the innovation of novel products.

Considering the continuous growth in the number of peptide sequences, predominantly identified through laborious experimental screening, coupled with several challenges, there is a necessity to devise a dependable and precise model for their forecasting. Systems have now been devised that outperform existing techniques, achieving almost complete accuracy, which will be particularly advantageous in the design of anticancer medications [70–73]. One method to enhance the effectiveness of BAPs release investigations and diminish the quantity of labor-intensive experiments is the utilization of computational modeling of enzymatic protein hydrolysis. Through the application of bioinformatics modeling methodologies, a digital representation of the peptide complex of various kinds of whey with anticipated biological activity, safety, and sensory characteristics has been formulated [156]. The investigation was carried out using proteomic database instruments founded on a formerly established algorithm for amalgamated bioinformatics modeling. With respect to the positioning of the protein proportion in the protein profile, hydrolysis by the protease complex chymotrypsin C-subtilisin was identified as the most effective technique for liberating peptides with both antioxidant and ACE-inhibitory functions. It was additionally observed that bioactive peptides procured as a consequence of in silico hydrolysis modeling following gastrointestinal digestion can be deemed safe in relation to allergic responses and toxicological impacts.

An effort to develop an extensive repository of bioactive peptides delineated in the literature, graphically correlated to the sequences of precursor proteins, which could be employed for seeking particular roles of bioactive peptides derived from milk proteins from any mammalian origin, was executed by Nielsen et al. [157].

7. Conclusions

Recently, there has been growing recognition of the key role of food and beverages in the prevention and treatment of diseases. In recent years, beverages based on whey have gained prominence among health foods due to their nutritional and health-promoting qualities [158].

The growing interest in BAPs prompts the scientific community and the food industry to develop new food additives and functional products based on them. Their use is facilitated by the simplicity of the procedure for their production and separation [159]. Currently, such products are recognized as a rational option for improving health status [160]. They provide nutritional benefits beyond a normal balanced diet and can rightfully be called "products of the future" [161]. Foundations have already been laid for developing new commercial dairy products with antihypertensive and antidiabetic effects and high antioxidant activity, which opens up excellent opportunities for obtaining functional dairy products that improve heart health by reducing blood pressure and heart rate.

For the production of fermented beverages based on whey enriched with biologically active components, the selection of a strain of LAB with high proteolytic and peptidolytic activity towards milk proteins is a crucial parameter. Other beneficial properties of whey protein peptides produced with lactic acid bacteria have not yet been fully explored and remain to be studied.

A deep understanding of the functionality and regulation of the proteolytic system of LAB opens up great opportunities to influence the final result of obtaining food products with the desired potential properties contributing to health reinforcement.

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