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Milk-Derived Antimicrobial Peptides: Overview, Applications, and Future Perspectives

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Abstract

The growing consumer awareness towards healthy and safe food has reformed food processing strategies. Nowadays, food processors are aiming at natural, effective, safe, and low-cost substitutes for enhancing the shelf life of food products. Milk, besides being a rich source of nutrition for infants and adults, serves as a readily available source of precious functional peptides. Due to the existence of high genetic variability in milk proteins, there is a great possibility to get bioactive peptides with varied properties. Among other bioactive agents, milk-originated antimicrobial peptides (AMPs) are gaining interest as attractive and safe additive conferring extended shelf life to minimally processed foods. These peptides display broad-spectrum antagonistic activity against bacteria, fungi, viruses, and protozoans. Microbial proteolytic activity, extracellular peptidases, food-grade enzymes, and recombinant DNA technology application are among few strategies to tailor specific peptides from milk and enhance their production. These bioprotective agents have a promising future in addressing the global concern of food safety along with the possibility to be incorporated into the food matrix without compromising overall consumer acceptance. Additionally, in conformity to the current consumer demands, these AMPs also possess functional properties needed for value addition. This review attempts to present the basic properties, synthesis approaches, action mechanism, current status, and prospects of antimicrobial peptide application in food, dairy, and pharma industry along with their role in ensuring the safety and health of consumers.

Keywords Antimicrobial peptides · Antibiotic resistance · Bacteriocins · Dairy · Food preservation · Lactic acid bacteria · Milk · Probiotics

Abbreviations

AMP	Antimicrobial peptides
AMR	Antimicrobial resistance
DNA	Deoxyribonucleic acid
LAB	Lactic acid bacteria
MDR	Multi-drug-resistant
HPLC	High-performance liquid chromatography
RP-HPLC	Reverse-phase liquid chromatography
IEC	Ion-exchange chromatography
SEC	Size-exclusion chromatography
FPLC	Fast protein liquid chromatography
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
ESI	Electro spray ionization

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NALDI	Nanostructure laser desorption/ionization
LC-MS	Liquid chromatography-mass spectrometry

Introduction

Good and safe food has always been a priority for humans and numerous strategies have been employed over the years to achieve it. This includes various food preservation processes and techniques to increase the shelf life and usability of the food items. Most preservation processes aim at decreasing the growth of microbes by limiting their vital life requirements of moisture, temperature, pH, osmolarity, etc. Some processes also include use of preservatives using chemical additives which act as antioxidants or as growth limiters to the microbes. This has been very successful in increasing shelf life of food items and we see a humongous increase in number of processed and canned foods in the past decades. Lately, some of these chemical preservatives are being identified as carcinogenic and public awareness against it has increased [1, 2]. Now, research has gained attention towards developing safer food preservatives while maintaining food quality, shelf life, and palatability using novel bioactive agents. One of such agents are antimicrobial peptides (AMPs) which are gaining interest as broad-spectrum antimicrobials with activity at micromolar concentration, and microorganisms are less likely to develop resistance against them. Predominantly, AMPs are a family of small peptides found throughout nature and plays an important role in organisms' innate immune system. Apart from that, AMPs can be produced from a variety of protein sources such as milk via enzymatic hydrolysis or microbial fermentation. AMPs have found application in food and pharmaceutical industry. Primarily, they can be used as biopreservatives in food products and have no appreciable effect on applicability across different food matrices.

In the modern world, foodborne infections have emerged as one of the most common public health concerns which are caused by either live pathogenic organisms or their toxins. Owing to their negative impact on the health and economic condition of the individuals, assurance of microbiologically safe food is very important. Also, the food recalls due to foodborne disease outbreaks and food spoilage are decreasing the consumer confidence leading to significant food and economic losses. Adoption of food preservation techniques using the natural substances is very crucial to prevent food losses and diseases spread to consumers. AMPs present an interesting alternative to chemical preservatives used for food preservation. Also, AMPs have potential to replace conventional antibiotics employed in animal welfare as prophylactic or therapeutic. AMPs are unique in the sense that they target the cell membrane of microorganisms, while conventional antibiotics target specific cellular activities such as DNA, protein, or cell wall synthesis. AMPs are more advantageous compared to conventional antibiotics owing to their ability to bypass common resistance mechanisms, thereby limiting microbial resistance [3]. AMPs are amphipathic molecules composed of 6 to 100 amino acids with a net positive charge of + 2 to + 9. This net positive charge guides them towards negatively charged bacterial cell membranes and promotes their ability to rupture and destabilize the cell membranes [4]. After the discovery of lysozyme, the scientific community shifted attention towards the detection, isolation, purification, and characterization of AMPs. AMPs combat low and high-affinity pathogen targets which confer them with the ability to overcome pathogen resistance leading to their prominence in the new era of antimicrobials [5].

Milk serves as a readily available source of precious bioactive peptides with diverse biological activities such as antimicrobial, antihypertensive, anti-oxidative, antithrombotic, and immunomodulatory [6, 7]. Fermented dairy products including yogurt, sour milk, and cheese include a variety of naturally produced bioactive peptides. Milk-derived AMPs, in particular, are an important component of the innate defense, especially on mucosal surfaces such as the lungs and small intestine, which are constantly exposed to a wide range of pathogens [8]. Lactoferrin, among other milk proteins, exhibits bacteriostatic and bactericidal activity against a variety of bacteria by binding to iron [9]. Infant milk lactalbumin has previously been shown to have antagonistic effect against *Escherichia coli* O127, and also a reduction in the incidence of diarrhea [10]. In a series of successive clinical trials, half (1–11) displayed antibacterial activity against antibiotic-resistant *Staphylococcus aureus* [11–14]. Moreover, CAMP211-225, a milk-derived peptide, was recently found to have antibacterial action against *E. coli* and *Yersinia enterocolitica* [15]. As an outcome of these research findings, milk-derived AMPs are gaining attraction as a safe and effective alternative to antibiotics, with the added benefits of application in food targeting shelf life extension. Therefore, this review aims to delineate the recent developments in milk protein-derived AMPs and their potential application in food, dairy, and pharma sector with reference to emerging antibiotic resistance. An attempt has also been made to present reader with an overview of production, characterization, and mode of action of AMPs.

Food Chain and Antibiotic Resistance

For almost a century, antibiotics and other antimicrobial formulations remain a prominent weapon to deal with infections both in humans and other animals. Over some time period, microbes started developing resistance against these antimicrobials, leading to decreased efficacy of drugs and a

burden on the global economy. AMR is prevalent across both species and geographical boundaries due to the food chain web, relaxation in international trade barriers, and lack of dose accuracy acquaintance. Animal husbandry and dairy sector are also reeled into this as the animals reared for milk and meats are frequently exposed to these antimicrobials. The possible factors responsible for emerging antibiotic resistance in the dairy sector include unrestricted availability of drugs, unchecked usage, improper diagnosis of disease, poor infection prevention and control measures, and lack of adequate surveillance and monitoring system [3]. There is an estimate of about a 67% increase in usage of antimicrobial compounds in the dairy sector by 2030 [16] especially in developing countries such as India due to increasing population, the gap in demand and supply, and greater chances of bacterial growth and survival due to tropical environmental conditions. Preservatives are usually added to keep the food protected from spoilage. Milk and milk products being a rich source of proteins, sugar, and vitamins are an excellent medium for growth of microbes. In countries with tropical climate with temperature exceeding 30 °C and where refrigeration or cold chain facilities are limited, milk is often preserved using chemicals [17]. However, their use in milk is debatable due to the reported bad effects on health. A comparative list of common chemical preservatives used for milk and milk products preservation is presented in Table 1. Available reports have raised hopes of overcoming the menace of antibiotic resistance and adverse health effects of other chemical preservatives via application of AMPs considering their high propensity towards resistant bacteria. The use of AMPs as therapeutic adjuncts due to least chances of resistance development offers a promising policy in the pharmaceutical industry as well [28].

Milk and Whey-Derived Antimicrobial Peptides

Antimicrobial peptides can be classified based on their origin, activity, structural characteristics, and synthesis process. Antimicrobial peptides can be isolated from microbes, amphibians, and insects. Various microorganisms such as bacteria and fungi can serve as a source of AMPs [5]. Nisin and gramicidin are classic examples of AMPs originating from *Lactococcus lactis*, *Bacillus subtilis*, and *Bacillus brevis*. The relatively high cost involved in chemical synthesis of AMPs leads to more interest in microbial origin peptides. Mammalian milk is a very potent source of antimicrobial peptides. α-Lactalbumin, β-lactoglobulin, lactoferrin, and other casein fractions are the major identified AMPs of milk

Table 1 Common preservatives used in milk and milk products

Name of preservative	Effective against	Legal safe amount	Adverse health effects of overdose	References
Hydrogen peroxide	Pathogens, aflatoxins	Maximum 0.05%	Irritation to GI track, nausea, vomiting, etc	[18]
Salicylic acid	More effective against fungi and yeast compared to bacteria	Not defined due to its heat instability	Gastric irritation, bleeding, diarrhea etc	[19]
Benzoic acid	Bacteria, yeast, and fungi	300 mg/kg in dairy based desserts and 1000 mg/kg in dairy fat spreads	Asthma, urticaria, metabolic acidosis, convulsions, and pseudo-allergy	[20, 21]
Sorbic acid	Bacteria, yeast and molds	1000 mg/kg in dairy based drinks, 3000 mg/kg in cheese	Metabolic acidosis, convulsions and hyperpnoea	[21, 22]
Boric acid	Pathogens	Declared unsafe by FAO/WHO	Nausea, vomiting, bloody diarrhea, severe colic, renal failure	[23]
Sodium carbonate/bicarbonate	Neutralizes bacterially developed acidity	Less than 0.3% as stabilizer in condensed, evaporated and powdered milk	Gastrointestinal problems	[24]
CO_2	Bacteriostatic effect against Gram-negative bacteria	-	May reduce body mineral density	[25]
Formaldehyde/formalin	Antiseptic nature	Not allowed	Potent carcinogenic, vomiting, diarrhea	[26]
Bronopol	Lowers somatic cell count	Not defined	Irritant reactions and allergic contact dermatitis	
Potassium dichromate	Bacteriostatic effect	-	Carcinogenic, skin irritation, rhinitis, and allergic contact dermatitis	[27]

origin [29]. On the basis of their activity, AMPs can be classified into 18 major categories (in light of ADP3 database). These categories include antimicrobial (antibacterial, antiviral, antifungal), antiparasitic, anti-human immunodeficiency virus, and antitumor peptides. A significant number of AMPs have antibacterial activity against common pathogenic bacteria (Table 2). Number of synthetic antimicrobial peptides display inhibitory activity against both Gram-positive and Gram-negative pathogens. In an in silico study, goat milk proteins were highlighted as a potential source of AMPs having application in food sector. Different online tools were used to predict the physiochemical properties, toxicity, and allergenicity of peptides [44].

Antimicrobial peptides can also be classified on the basis of constituent amino acids such as proline-rich peptides, tryptophan-arginine-rich peptides, histidine-rich peptides, and glycine-rich peptides. The amino acid composition primarily regulates the antimicrobial activity and probable mechanism of action. For example, AMPs rich in proline (non-polar amino acid) enter bacterial cells through the non-invasive pathway. In contrast, arginine and histidine (basic amino acids)-rich peptides are attracted towards anionic bacterial membrane. On the basis of structural conformation of AMPs, they can be divided into four major categories, viz., linear, α-helical peptides, ß-pleated sheet peptides, linear extension structure, and both α-helical and ß-sheet peptides. This section focuses on milk and whey as source of AMPs. Casein makes up 80% of milk protein. Casein hydrolysis results into generation of diverse number of antimicrobial/bioactive peptides. Isracidin was the first antimicrobial peptide obtained from bovine casein hydrolysate. Casein hydrolysate-based AMPs, such as casecidin (caseicin A and caseicin B), lactenin, isracidin, and kappacin, are derived from α-, ß-, and κ-casein fractions [9]. αs1-casein f(99–109) obtained from pepsin-mediated hydrolysis of bovine sodium caseinate protein displayed antimicrobial activity against Gram-positive (*B. subtilis* and *Listeria innocua*) and Gram-negative bacteria (*Salmonella typhimurium*, *E. coli*, *Salmonella enteritidis*, and *Citrobacter freundii*) [45]. Caseicin A αs1-casein f(21–29) and caseicin B αs1-casein f(30–37) from bovine casein checked the growth of *Cronobacter sakazakii* in powdered infant formula trials [30]. In later studies, these peptides were found to inhibit *Klebsiella* spp., *Salmonella* spp., and *Staph. aureus* [46]. The chymosin digest of bovine sodium caseinate results into release of αs2-casein f(181–207), f(175–207), and f(164–207), which showed potential inhibition of a wide variety of Gram-positive and Gram-negative bacteria [44]. Another κ-casein-derived peptides such as kappacin, k-casein A (138–158) exhibited potential activity against *Streptococcus mutans*, *E. coli*, and *Porphyromonas gingivalis* [31].

Whey proteins are obtained following casein precipitation and constitute about 20% of the remaining protein in milk. Hydrolysis of whey proteins can generate bioactive peptides having antioxidant, antimicrobial, antihypertensive, and antidiabetic activities [47]. Whey lactoferricin (Lfcin) is a well-identified multifunctional peptide obtained from pepsin hydrolysis of bovine lactoferrin protein. Chemically synthesized lactoferrin domain peptide lactoferrampin f(268–284) exhibits anti-*Candida* activity and antibacterial activity against *B. subtilis*, *E. coli*, and *Pseudomonas aeruginosa* [32]. β-Lactoglobulin, another fraction of whey protein, composing 50% of whole protein can be found in the milk of many mammals, but not in human milk. Upon tryptic digestion, it produces four fragments including f(15–20), f(25–40), f(78–83), and f(92–100) displaying activity mainly against Gram-positive bacteria [48]. This protein component resists proteolytic enzymes and gastric digestion and serves as a stabilizer in yogurt and cheese due to its heat-gelling capacity [49]. α-Lactalbumin, another fraction of whey protein (14.4 kDa), results from trypsin or chymotrypsin digestion. Its presence in bovine milk is just 20%, while in human milk, it is the most abundant whey protein [50]. This protein component has high nutritional value leading to its commercial application in infant formula. Furthermore, it displays high antagonistic activity against Gram-positive bacteria including antibiotic-resistant variants.

Synthesis, Purification, and Identification of AMPs

Antimicrobial peptides that are encrypted in an inactive form within the protein can be released either through enzymatic hydrolysis or via microbial fermentation. Enzymatic hydrolysis, especially used in the food and pharmaceutical industries, is the most common approach for decrypting bioactive peptides from whole protein sources. Proteases used for hydrolysis may be of the gastrointestinal origin or from microbial or plant source. Trypsin and pepsin are the prominent proteases commonly used to obtain bioactive peptides with diverse activities. In particular, trypsin and pepsin hydrolysis have contributed to most of the recognized antimicrobial peptides [51]. In addition, alcalase, chymotrypsin, pancreatin, and thermolysin are used individually or in combination to release bioactive peptides from diverse protein sources. Shorter reaction time and ease of scalability select enzymatic hydrolysis over microbial fermentation. Proteolytic enzymes of microbial origin can be the source of many new peptides with unique bioactivities. Microbial proteases represent one of the most important tools in the modification of protein structure, development, and production of new protein hydrolysates to obtain specific peptides that can be commercially exploited. The peptide bonds cleaved by the proteolytic enzymes are surrounded by the amino acid sequence having some degree of substrate specificity. For instance, when bovine milk casein is hydrolyzed using

Table 2 Milk-derived AMPs and their activities

Antimicrobial peptide	Amino acid sequence	No. of amino acids	Biological role and mode of action	Reference
β-Casein$_{211-225}$ from human milk	YPVTQPLAPVHNPIS	15	Effective against *E. coli* and *Y. enterocolitica* Effectively reduced ileal mucosa damage in an experimental necrotizing enterocolitis mice model	[16]
α-s2-casein peptides from camel milk	IKEVESPAE, VPTENKISQ, AEVPTENKISQ, NIKEVESPAE, AVRNIKEVESPAE, VESPAEVPTENKISQ, VAIHPSKED	9–15	Effective against *Strep. faecalis*, *Sh. dysenteriae*, *Staph. aureus*, and *E. coli*	[33]
PDC$_{213}$ (derived from β-casein$_{213-226}$ of human milk)	VTQPLAPVHNPISV	14	Effective against *Staph. aureus* and *Y. enterocolitica* Permeabilize bacterial membranes and cell wall leading to cell death	[34]
Bovine β-casein	YQEPVLGPVRGPFPIIV, YQEPVLGPVRGPFPI	15–17	Antimicrobial activity against *E. coli* DH5 α	[35]
Bovine k-casein	AIPPKKNQDKTEIPTINTIA-SGEPTSTPTTE	63	Antibacterial against Gram-positive and Gram-negative pathogens	[31]
Kappacin A$_{106-169}$	AVESTVATLEDSPEVIESP-PEINTVQVTSTAV			
Isracidin$_{1-23}$ (α-casein)	RPKHPIKHQGLPQEVLNENLLRF	23	Broad-spectrum activity displayed under in vitro and in vivo conditions	[36]
Caseicin A $_{21–29}$ (α-casein)	IKHQGLPQE	9	Effective against Gram-negative bacteria	[30]
Caseicin B $_{30–37}$ (α-casein)	VLNENLLR	8	Effective against Gram-negative bacteria	[30]
Casocidin-I$_{150-188}$ (α-casein)	KTKLTEEEKNRLNF-LKKISQRYQKFALPQYLKTVYQHQK	39	Antimicrobial activity against Gram-positive and Gram-negative bacteria, and yeast	[37]
Bovine isracidin	RPKHPIKHQGLPQEVLNENLLRF	23	Antimicrobial activity against *E. coli* DH5 α	[35]
Bovine lactoferrin (LF)	FKCRRWQWRM KKLGAPSITCVRRAF	25	Displays antibacterial, antiviral, antifungal, and anti-parasitic activity	[38]
Lactophorin camel milk	IYMESPQPTDTSPAQ, FRNTATQSEETKE, VIMSNHQVSPSED, SSFRNTATQSEETKE, LHPVPQESS, SSFRNTATQSEE	9–15	Effective against *Strep. faecalis*, *Sh. dysenteriae*, *Staph. aureus*, and *E. coli*	[33]
Lactoferrampin bovine LF	WKLLSKAQEKFGKNKSR	17	Bacterial membrane disruption	[32, 39]
Peptide from yak milk	RVMFKWA	7	Antimicrobial activity against *B. subtilis*, *Staph. aureus*, *L. innocua*, *E. coli*, *E. cloacae*, and *Salm. paratyphi*	[40]
Sheep αs2-casein	LKKISQ, LKKISQYYQKFAWPQYL, PYVRYL, VDQHQKAMKPWTQPKTNAIPYVRYL	6–25	Antimicrobial	[41]
Cow β-lactoglobulin	ALPM, LKP, VAGTWY	3–6		
Cow β-casein	GPIHNS, GPVRGPFPIIV, KIHPFAQT, MPIQAFLL, PVLGPVRGPFPIIV, YQEPVLGPVRGPFPIIV	6–17	Antimicrobial	[42]
Cow κ-casein	TVQVTSTAV, MAIPPKKNQ, MAIPPKKNQD	9–10	Antimicrobial	
αs2-Casein$_{151–181}$ bovine milk	TKLTEEEKNRLNFLKKISQRYQKFALPQYLK	31	Antibacterial activity against *B. subtilis* and *E. coli*	[43]

pancreatin, the rate of peptide fragments produced is highest for ß- and α-casein [52]. In contrast, papain most effectively hydrolyzes sodium caseinate, followed by trypsin and pancreatin [53]. Thus, different milk sources differ in susceptibility to hydrolytic enzymes, which indicates differences in the number, type, and concentration of bioactive peptides.

Microbial fermentation to obtain bioactive peptides is gaining recognition due to being a natural, safe, and cost-effective strategy. Lactic acid bacteria (LAB) have developed the ability to hydrolyze proteins to compensate for their amino acid requirement. LABs not only generate free amino acids for their own use but also produce a wide range of biologically active peptides [54]. The proteolytic system of LABs mainly comprises of cell wall-bound proteinases (initially degrade casein into oligopeptides), peptide transporters (transfer oligopeptides into the cytoplasm), and distinct intracellular peptidases, including endopeptidases, aminopeptidases, tripeptidases, and dipeptidases which convert peptides into small molecules and generate free amino acids [55]. The proteolytic activity of LABs is exerted in a species- and strain-dependent manner. To name a few LABs, *Lactobacillus helveticus*, *Lact. delbrueckii* subsp. *bulgaricus*, *Lact. delbrueckii* subsp. *lactis/diacetylactis*, and *Lact. delbrueckii* subsp. *lactis/cremoris* display effective proteolytic activity for milk protein hydrolysis [56]. Especially, *Lact. helveticus* strains are most extensively studied and known to possess high extracellular proteinase activity. Fan and coworkers identified 212 peptide sequences from casein fermented with *Lact. helveticus*, among which 44 previously identified peptides possess antimicrobial activity [57]. In another study, *Lactobacillus acidophilus*-generated peptides (IKHQGLPQE, VLNENLLR, and SDIPNPIGSENSEK) displayed antibacterial activity against pathogenic *Enterobacter sakazakii* and *E. coli* [30]. Ebner and coworkers investigated the proteomic profile of kefir (alcoholic fermented milk beverage) and identified 257 peptides mostly released from β-casein [58]. Among them, 16 peptides were previously reported to have antimicrobial, immunomodulatory, ACE inhibitory, opioid, antithrombotic, mineral binding, and antioxidant activities. Fungal strains, e.g., *Aspergillus oryzae* and *Aspergillus flavipes*, have also been used for generating bioactive peptides from bovine and goat milk via solid-state fermentation. The generated peptides displayed potent antimicrobial activity [59]. Caseicin A (IKHQGLPQE) and caseicin B (VLNENLLR) generated from sodium caseinate fermentation by *Bacillus cereus* and *Bacillus thuringiensis* effectively inhibited *C. sakazakii* [60].

Recombinant DNA technology (RDT) is extensively used for the production of proteins and hormones having wide application in medical sciences. RDT has now also been explored for scaling up both the production and yield of the specific bioactive peptides. This method allows simultaneous production of several peptides by expression of peptide coding region into microbes [61]. AMPs of milk origin have been produced by over-expression of bovine lactoferricin B-W10 (LfcinB-W10), a novel derivative of cationic antimicrobial peptide lactoferricin Lf(f17–41) in *E. coli*. Likewise, by combining bovine lactoferricin and the inducible insect antimicrobial peptide thanatin, a hybrid antimicrobial peptide was developed [62].

Membrane filtration technology is the method of choice for the isolation and purification of peptides based on molecular weight. It has several advantages such as being cost-effective, non-chemical, energy-saving, and easy to set-up. Protein hydrolysates obtained following hydrolysis are fractionated through molecular weight cut-off membranes of varying sizes (0.5 to 100 kDa). Different fractions are later screened for their antimicrobial activity. Goat milk casein hydrolysates low molecular weight (< 3 kDa) fractions showed high antimicrobial activity against *E. coli* and *B. cereus* as compared to 3–10 kDa fractions [63]. Nanofiltration technique could also be applied for isolation of peptide with molar mass < 1 kDa, as it consists pore size of 0.5–2 nm [64]. More recently, an electrodialysis with filtration membrane technique has been adopted for increased efficiency based on peptide molecular weight and net charge [65]. For analytic purpose, there are many powerful techniques for separation, purification, detection, and isolation of bioactive and novel antimicrobial peptides. These are one-dimensional electrophoresis, high-performance liquid chromatography (HPLC), reverse-phase liquid chromatography (RP-HPLC), ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), fast protein liquid chromatography (FPLC), gel-filtration chromatography, affinity chromatography, or multidimensional system. RP-HPLC is the most widely used purification method for milk-derived bioactive peptides due to its quick detection and separation potential from the sample mixture. Peptides are fractionated using an analytical silica-based RP-C18 column in the stationary phase [66]. The peptides fraction obtained are collected between retention and gradient time and the data is analyzed using an ultra-violet detector [63]. This technique has been preferably used to fractionate protein and peptide content in both one-dimensional and multidimensional separation systems based on peptide polarity and molecular weight. Size-exclusion chromatography also separates peptide molecules based on molecular size. Earlier, Morais and coworkers used SE-HPLC and separated whey protein concentrate hydrolysates into four fractions and characterized peptides according to chain length with molar mass < 1000 Da [67]. Parameters such as pore size/volume, ionic strength, and mobile phase nature affect the performance of the SE-HPLC column [68]. A multidimensional separation system combining more than one separation tool provides high-resolution power and peak capacity as compared to the one-dimensional purification and separation approach. Using this approach, Rahimi and

coworkers hydrolyzed camel milk casein protein enzymatically and the peptides obtained were fractionized by multidimensional technique using ultrafiltration membranes and semi-preparative RP-HPLC [69]. However, multidimensional separation techniques are not always effective, due to variable nature of operational techniques which makes the solvent incompatible, while proper selection of combination of different multidimensional separation system will lead to potential outputs as well as purify and identify peptides of complex samples with high desirable bioactivity [65].

Identification and Characterization of Peptides

The antimicrobial peptide sequence can be determined using various approaches such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), electro spray ionization (ESI), and nanostructure laser desorption/ionization (NALDI). In recent years, the most frequently used method for profiling and identification of peptides from several food sources including milk is through liquid chromatography coupled with mass spectrometry (LC–MS) [70]. The chromatographic technique combined with mass spectrophotometry termed as liquid chromatography/tandem mass spectrometry (LC–MS/MS) identifies and characterizes complex mixtures of peptide sequence, based on their molecular mass, and has high resolution and separation efficiency [71]. Fractionated peptides are desalted on a RP-C18 trap column and further separated using reverse-phase C18 analytical column. The eluted peptides are collected at different time intervals, on particular flow rate and the data monitored using MS analyzer instrument such as Q-Exactive Orbitrap and Q-TOF ion-trap. Peptide databases like BIOPEP, UNIPROT, SWISS PROT, and MBPDB are used to identify amino acid sequence for discovery of novel peptide [72]. Nowadays, in silico techniques like homology modeling, hidden Markov models, and support vector machine are also explored for modeling of identified protein fragments to confirm their bioactivity. Figure 1 represents the different antimicrobial peptides synthesis, purification, and identification strategies.

Antimicrobial Peptide Database

Peptides derived from food proteins, due to their biological and functional properties, are considered valuable health beneficial and functional food components. The research interest in bioactive peptides is reflected by the huge spike in the number of articles published annually on these peptides [73]. The latter has ramifications in terms of the necessity to collect and store the huge amount of data being generated in databases. A variety of databases have been developed in the past to keep track of various types (antiviral, antimicrobial, antitumor, hemolytic, and cell penetrating peptides) of bioactive peptides [74]. Most of the information about these bioactive peptides is available in various databases such as TumorHoPe [75], Biopep-UWM, StraPep, FeptideDB, ACEpepDB, BioPD, APD, BACTIBASE [76], CAMP, PenBase [77], RAPD [78], Hmrbase [79], PhytAMP [80], PeptideDB, ACEpepDB [81], Amper [82], and BAGEL3 [83].

Several antimicrobial peptide databases have been created over the past several years including Peptaibol Database [84], PenBase [77], Defensins Knowledgebase [85], PhytAMP [80], BACTIBASE [76], CAMP [86], YADAMP [87], DAMPD [88], Milk AMP [89], CAMPR3 [90], DBAASP [91], APD [92], MBPBD [93], FeptideDB [94], and FermFooDb [74]. Despite the huge potential of antimicrobial peptides from food especially from milk, only a few databases are dedicated to them. Food-derived bioactive peptides have been widely reported since the 1970s. However, in databases of food-derived antimicrobial peptide, the information including sequence, function, source, and references is poorly integrated. There are few reasons for poor integration of relevant information regarding active peptides from food sources: first, their sequence information is to be gathered from the relevant published articles and databases; second, lack of professional classification in food sources of active peptide; and lastly, several connections between food-derived peptides, their origins, roles, and products are unknown. Due to the lack of a comprehensive database on food-derived peptides, researchers in laboratories and industry must scour the Internet for them [95]. Milk AMP database was designed specifically for milk antimicrobial peptides and lists natural and artificial antimicrobial peptides derived from amino acid sequences of dairy proteins of different origins. The database was created with an aim to provide comprehensive information on peptide structure/function relationships, inhibitory activity, spectrum of action, and minimal inhibitory concentration (MIC) determined for each tested microbial strain. It includes a fairly complete list of references for each peptide. The information in this database will supplement conventional databases by supplying missing data and allowing for rapid prediction of structure/function correlations and target organisms, resulting in improved usage of peptide biological activity in both the pharmaceutical and food industries. At the time of creation, it contained 371 entries, including 9 hydrolysates, 299 antimicrobial peptides, and 23 peptides predicted as antimicrobial, as well as 40 non-active peptides. This database also allows entries from users for expansion and improvement in data [89].

MBPDB is a comprehensive database of functional peptides in milk. This database was created to identify and analyze novel bioactive peptides and allows examination of patterns in the data of bioactive peptides. The database offered improvement over earlier databases in being specific and comprehensive to all milk bioactive peptides across species and proteins with several advanced search functions. This database helps in the creation of prediction models based

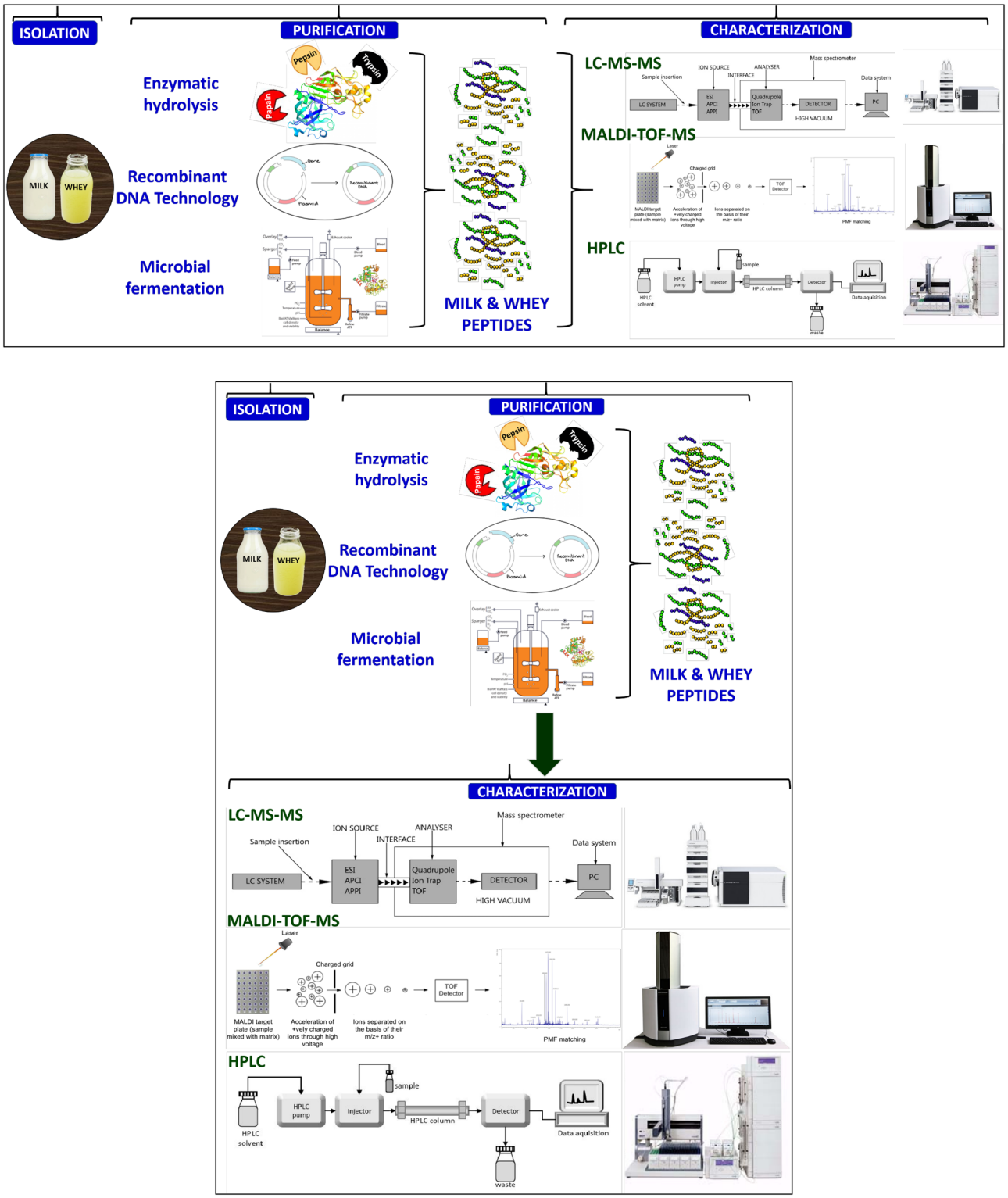

Fig. 1 Schematic representation of different antimicrobial peptides synthesis, purification, and identification strategies

on the relationship between peptide structure and activity to determine the likelihood of bioactive milk peptides identified in other types of biological samples. This database contains information for 177 AMPs only as some information was lacking in the rest of the AMPs from other databases such as the original research article, the full sequence

of amino acids in peptide, bioactive function, source species, and source proteins [93]. The BIOPEP-UWM database, a widely used database in food and nutrition science, is freely accessible. This database is continuously being updated and modified. The BIOPEP-UWM provides databases of proteins, allergenic proteins, and their epitopes in addition to bioactive peptides. The database enables users to help in update of the database by allowing them to submit a peptide sequence, which after verification gets updated. The information provided by the database consists of ID number, name, sequence, function, number of amino acid residues, activity, chemical mass, and bibliographic data [72]. FermFooDb is a consolidated database that maintains biologically active peptides obtained from fermented foods. As the food industry, especially dairy industry, is focusing mainly on the commercialization and development of novel fermented foods, this database can be of great use for the purpose. This database enables users to evaluate the medicinal potential of fermented foods using the wide range of bioactive peptide characteristics stored in this database, which are compiled from existing databases like AHTPDB, ACEPepDB, BIOPEP, and MBPDB [74]. This database maintains the different peptide properties like sequence, physicochemical properties, length, and IC50 value as well as fermentation process along with compiled details including experimental model, starter culture, and PubMed ID of research article.

Mechanisms of Action of Antimicrobial Peptides

Activity and specificity of AMPs precisely depend upon structural parameters, such as conformation, charge, hydrophobicity, amphipathicity, and polar angle. It is important to note that these molecular determinants are interdependent; hence, modification of one parameter often leads to compensatory alterations in others. Here, the article will discuss in general the major structural components of antimicrobial peptides that influence their mechanism of action. In spite of the structural conformational homology displayed during target membrane interaction, AMPs display immense diversity in their peptide sequences. The reason for this structural homology could lie in the presence of specific peptide sequences that are crucial for particular activity irrespective of the sequence of remaining peptide residue. One such important feature is the presence of glycine residue cap at N-terminal of peptide chain. Tossi and coworkers reported that glycine at the first position of N-terminus region of α-helical peptide is relatively conserved sequence [96]. Glycine residue cap prevents peptide cleavage by aminopeptidases. Likewise, presence of peptide amidation has been observed as another important post-translational modification for AMPs. The peptide amidation provides one additional H-atom which in turn transfers the energy to acquire helical structure as well as prevent the cleavage of peptide by carboxypeptidases [97]. In addition to these specificities, presence of long stretch of basic amino acids (lysine and arginine) also enhances the cationic nature of peptides [96]. The partition constant of AMP and membrane is an important factor that determines interaction between AMP and cell surface. Usually, AMPs have higher magnitude of partition constant as compared to the charged cell membranes. The aromatic amino acids are a major contributor towards partition constant and facilitate the anchoring of peptide to the head group of lipid bilayer [98]. Some amphipathic peptides acquire conformation in which hydrophobic residue resides on one side and hydrophilic on the other. The hydrophilic cationic domain initially interacts with the membrane surface and the hydrophobic portion leads to peptide insertion (mediated via Van der Waals interaction and hydrophobic interaction in hydrocarbon chain).

The degree of structure is another pivotal aspect of antimicrobial peptides. The peptides usually acquire α-helix or β-sheet conformation, which upon contact with the cell membrane helps peptide to combat with the differences in partition constants between AMP and cell membrane. Electrostatic charge is another driving factor that influences peptide attraction towards microbial cell and peptide folding at lipid peptide interface. Most of the antimicrobial peptides are cationic in nature with net positive charge ranging from + 2 to + 9. The presence of acidic phospholipids, phosphatidylserine, and cardiolipins imparts negative charge to the cell membrane. Additionally, presence of lipopolysaccharide in Gram-negative bacteria and teichoic/lipoteichoic acid in Gram-positive bacteria distributes the total anionic charge. Likewise, presence of phosphomannans, chitin, and β-1 → 3 glucan carries strong negative charge to the fungal cell wall. In contrast, mammalian cells have higher proportion of cationic components such as phosphatidyl choline, phosphatidylethanolamine, sphingomyelin, and no/low amount of anionic components (phosphatidylglycerol and cardiolipins) conferring net positive charge to the mammalian membrane [99]. Thus, the electrostatic charge is prime factor that regulates the initial interaction of peptide with cell surface. The difference in electrostatic potential and lipid composition of microbial cells plays a major role in the selectivity and specificity of antimicrobial peptides. For instance, Tossi and coworkers observed that increase in charge of magainin-2 peptide from + 2 to + 5, while keeping other factors stable, increased its antimicrobial activity [96]. Furthermore, it was also observed that the increase in cationic charge from + 6 to + 7 did not improve the antimicrobial activity. This may be due to the fact that increase in positive charge results in strong interaction between peptide and phospholipids, which hinders peptide translocation through

the microbial membrane. The addition of a formal net charge of + 8 to a peptide increased activity against yeast cells while decreasing activity against *Bacillus megaterium* and *Staph. aureus*. This could be explained by stearic impediment in peptide helix formation due to its near proximity and net repulsive contact between basic residues of packed peptides [96]. In addition to this, the electrostatic repulsion within the peptide decreases the lifespan of the pore, thus reducing its membranolytic activity.

Amphipathicity is the measure of relative ratio of hydrophobic and hydrophilic residues in the peptide. Quantitatively, the vector sum of all hydrophobic amino acid, normalized to ideal helix, gives the measure of hydrophobic moment and, hence, the amphipathicity. Alpha-helical conformation is the most favorable conformation acquired by amphipathic peptides having 3–4 amino acids per turn periodically. This is the crucial property of an AMP for its initial interaction with the cell membrane. In α-helix, polar phase is attracted towards the negatively charged membrane and non-polar phase causes insertion into membrane through Van der Waals forces and hydrophobic interactions leading to increased permeability. Hydrophobicity, the measure of hydrophobic residues within a peptide, determines partition constant of membrane hydrophobic core [100]. It is suggested that hydrophobicity has higher impact on toxicity towards host cell as compared to antimicrobial activity. Beyond some threshold value, the increase in hydrophobicity enhances peptide hemolytic activity and decreases its ability to discriminate between host and microbial cells [101]. For example, Chen and coworkers demonstrated that the increased hydrophobicity of L-V13K peptide enhanced its activity against RBCs by 62.5 times. Increasing hydrophobicity beyond a certain limit causes oligomerization or dimerization of peptide, resulting in the formation of energetically stable peptide aggregates [102]. The higher aqueous stability of peptide aggregates can prevent the partition into the membrane, displaying weaker interaction with membrane surface. A systematic study on gramicidin S exhibited that the balance between hydrophobicity and amphipathicity is the key factor that determine relative therapeutic ability of peptide (i.e., directly affects their hemolytic and antimicrobial activity) [103].

Polar angle of peptide represents the relative fraction of polar versus non-polar faces of an amphipathic helical peptide. Any aggregation or change in the polar or non-polar residue tends to change the polar angle. It is assumed that higher non-polar (hydrophobic) domain in peptide is directly related to smaller polar angles and increased membrane permeabilization [104], thus having a strong correlation with membrane stability and pore formation. Irrespective of the precise mechanism of action, all AMPs primarily act on the plasma membrane through the establishment of electrostatic bonding with plasma membrane components [105]. AMPs acquire α-helical or β-sheet conformation upon coming in contact with the membrane via electrostatic force of attraction. Anionic AMPs usually complex with zinc ion or highly cationic peptides. Cationic AMPs can easily bind to negatively charged cell surface. Nisin specifically binds with the lipid II component of cell wall. Mersacidin also interferes with transglycosylation and peptidoglycan synthesis of Gram-positive cell wall via targeting lipid II [106]. Following binding, a critical concentration of AMP is required to precede surface disruption. With multimerization, AMPs penetrate into the deeper layers of target cell surface. AMPs enter cells via membrane lytic or non-membrane lytic mode. Various models, viz., barrel stave model, toroidal model, and carpet models, have been identified for membrane lytic mechanism of AMPs (Fig. 2). In barrel stave model, AMPs adopt amphipathic conformation in the membrane to form a stave (spokes within barrel)-like structure which goes deep inside the membrane forming a stable pore-like structure that disrupts the membrane integrity [107]. Toroidal model is primarily shown by α-helical AMP molecules. The helical AMPs position parallel to the membrane, resulting in displacement of phospholipid groups. These cause break in hydrophobic regions and induce a strain on membrane. After attaining a threshold critical concentration, AMPs change their conformation perpendicular to the membrane and form toroidal pore complex [108]. In carpet model, AMPs initially bind to cell surface causing conformational change within them. After attaining threshold concentration, AMPs cover the surface of target cell in sheet/carpet-like manner, causing change in energy kinetics and fluidity of membrane which subsequently leads to membrane destabilization. In the final stage, AMPs saturate the cell membrane resulting in membrane collapse into micelles.

Several models (aggregate channel model, sinking raft model, electroporation model) have been proposed for explaining non-membrane lytic action mechanism of AMPs (Fig. 2). In "aggregate channel model," AMPs initially attract to the cell surface and consequently get inserted into the membrane. Following insertion, peptide conformed itself into unstructured aggregate that covers the membrane. These peptide aggregates associate with water molecules, thus leading to formation of channels through which ions and larger molecules get leaked. Some AMPs preferentially bind to specific lipid domains in the lipid membrane and thus causes imbalance in mass ratio. This imbalance increases membrane curvature of confined regions leading to peptide translocation. This phenomenon is referred to as sinking raft model, and is responsible for creating transient pores in the membrane through inducing mass imbalance in peptides of membrane leaflet [109]. Electroporation model proposes transient membrane pore formation under the influence of electric field. This only occurs when peptides have sufficient charge density to generate the electric potential of at least

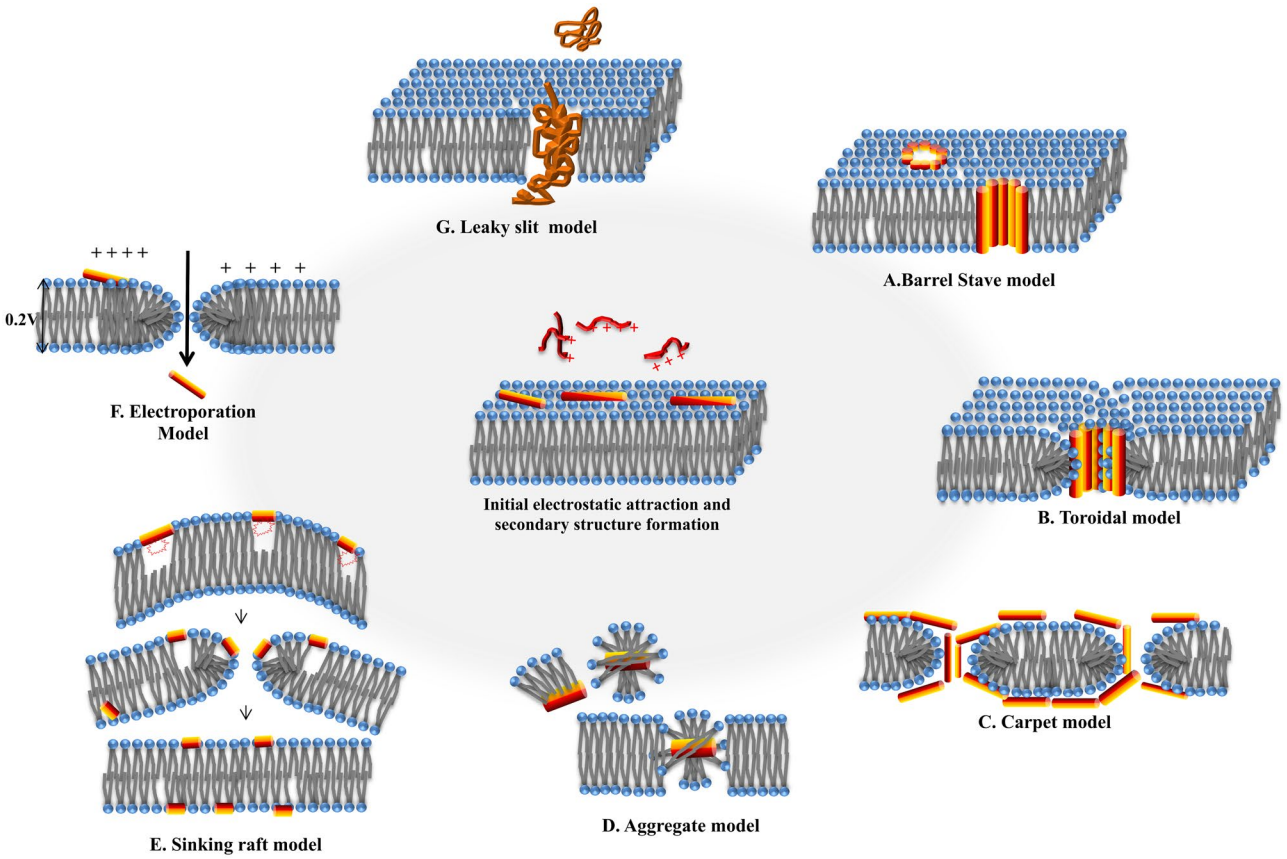

Fig. 2 Mechanism of action of the antimicrobial peptides by membrane lytic mechanism [**A** barrel stave model, **B** toroidal model, **C** carpet model] and non-membrane lytic mechanism, **D** aggregate model, **E** sinking raft model, **F** electroporation, and **G** leaky slit model]

0.2 V. This model explains the mechanism of entry opted by annexin V peptide [110]. Some peptides significantly disrupt cell membrane by forming lipid peptide domains. This phenomenon can be explained with the peptide-induced lipid segregation mechanism, where peptide induced the segregation of anionic components from zwitterionic lipids and can even cause the de-mixing of the anionic lipids in Gram-positive model membrane [111]. Arouri and coworkers proposed that peptides can induce lipid segregation in PG/PE (phosphoglycine/phosphoethanolamine) membranes, which could be the specific action of these peptides on bacterial membrane and hence their killing [112]. Zhao and coworkers suggested that AMPs can act similar to some bacterial cytotoxin proteins and cytolysin [113]. Peptide bound to lipids acquires linear amphipathic structure with hydrophobic portion facing towards lipid bilayer, which enhances the insertion of lipid-protein complex in the membrane. The hydrophilic face increases the hydrogen bond-assisted self-multimerization between the proteins leading to the formation of long fibrils that confer cytotoxicity. The insertion of lipid-protein aggregates increases the positive curvature in the membrane which causes transient leakage in the cell membrane (leaky slit) and thus enhances membrane permeabilization. Finally, these fibrils acquire conformation of amyloid-like structure that spans the complete cell membrane. This mechanism proved that the conformational flexibility, amphipathicity, and propensity to fold are the basic properties that affect the toxicity of AMPs.

AMPs cause microbial cell death via disruption of cell membrane. Membrane disruption causes the leakage of cell contents to the surrounding and thus killing the cells. AMPs also target several other microbial cell components. Various cationic AMPs can bind to nucleic acid (anionic) due to electrostatic force of attraction. Buforins isolated from *Bufo bufo gargarizans* can bind to DNA. Buforin II, a 21-amino acid peptide, is able to induce membrane permeability in Gram-negative bacteria. Similarly, indolicidin (13-amino acid peptide) derived from cytoplasmic granules of bovine neutrophils attacks at a basic site of DNA, thus inhibiting its biosynthesis. Furthermore, it also binds to DNA topoisomerases, thus preventing DNA relaxation of replication fork [114]. Another AMP microcin 25 (mcc25 or J25) isolated from the *E. coli* AY25 is a potent antibacterial peptide, particularly against Gram-negative bacteria. Microcin J25 binds to catalytic center of RNA polymerase and disrupts transcript elongation [115]. Lactoferricin B

hinders bacterial growth by suppressing phosphorylation of the two-component system [116].

Application of Milk-Derived Antimicrobial Peptides

AMPs with broad-spectrum activity against a wide range of pathogens are considered promising candidates for the development of new bio-preservatives. AMPs have been shown to have a number of biological actions, including antibacterial, antiviral, antifungal, and anti-mitogenic effects, as well as anticancer and anti-inflammatory capabilities and the potential to modulate the immune system. Their broad spectrum of activity, effective antibacterial action, less susceptibility to resistance development, and potent immunomodulatory effects make them suitable to be used as an alternative to a wide range of regularly used bio-preservatives and medications [117]. During the past decade, application of antimicrobial peptides in food and pharmaceutical industries for designing safer and functional food/pharmaceutical options has gained immense interest.

Application in Food and Dairy Sector

Milk-derived AMPs can serve as promising alternative to chemical preservatives. Introduction of milk-derived AMPs may provide a new arena to food industry, which can fulfill the consumers' demands without compromising industrial interests. Several AMPs have been evaluated for their potential to inhibit foodborne pathogens in a number of food matrices such as dairy, meat, beverage, and fruit-based products. Recently, Yang and coworkers reported an antimicrobial peptide from whey acidic protein (WAP) of large yellow croaker (*Larimichthys crocea*) [118]. The peptide LCWAP displayed MIC value of 15.6 µg/mL against *Staph. aureus*. The killing effect was due to the disruption of cell membrane integrity resulting in leakage of cell contents. The peptide had no cytotoxic effect on hepatocytic cells of human, but had strong inhibitory effect on the growth of *Staph. aureus* in milk. Similarly, a peptic hydrolysate of LF at a dose of ≤ 2 mg/mL under limiting conditions of temperature (4 °C) and pH 4.0 could restrict the growth of *E. coli* O157:H7 and *Listeria monocytogenes* in milk [119]. Earlier, Quinteri and coworkers established the potential of LFcin B in restricting the spoilage of mozzarella cheese from mesophilic bacteria [120]. The presence of isracidin and kappacin in Italian cheeses indicates the release of antimicrobial peptide by microbial proteases during cheese formation and ripening process [121]. Recently, a combination of LFcin B (0.5 mg/g) and high pressure (400–500 MPa) was reported to significantly control the population of *Pseudomonas fluorescens* ATCC948 [122]. On the other hand, lactoferrin has been incorporated in different food matrices such as in sausage batters [123], bologna [124], ground beef/meat fractions [125], and fennel [126]. However, rapid degradation in food matrix poses the major limitation with AMP application in food. Several milk-derived peptides such as casocidin and isracidin [127] and LFcin B were evaluated for their resistance against microbial proteolytic degradation using different starter cultures (*Streptococcus thermophilus* and *Lact. delbrueckii* subsp. *bulgaricus* strains). Long sequenced AMPs and chemical modification in sequences can help generate AMPs with reduce susceptibility to proteolysis.

The U.S. Food and Drug Administration (FDA) have conferred lactoferrin with Generally Recognized as Safe (GRAS) status since 2000. European Commission (EC) approved use of bovine Lf (bLf) in different food categories and documented its maximum levels under Regulation (EC) No. 258/97 [128]. Bovine lactoferrin has found its application in infant milk formula, fermented and skim milks, yoghurts, drinks, and nutritional supplements [128–130]. In oil industry, lactoferrin was reported to decrease oxidation of unsaturated fatty acids, thus enhancing shelf life of soybean oil powder. Lactoferrin also inhibit *Dekkera bruxellensis*, yeast responsible for deteriorating wine quality [131]. Enrique and coworkers reported that LF f(17–31) peptide-based approaches have potential to control the population of *Saccharomyces cerevisiae* and other spoilage wine yeasts (*Cryptococcus albidus*, *Dekkera bruxellensis*, *Pichia membranifaciens*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus*) and bacteria (*Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *Lactobacillus hilgardii*, *Pediococcus damnosus*, and *Oenococcus oeni*) without compromising wine attributes [132–134]. Functional coating with immobilized lactoferricin B controlled the microbial deterioration of cheese [120]. Del Olmo and coworkers showed that lactoferrin and its derivatives under hydrostatic pressure significantly control the bacterial (*E. coli* O157: H7 and *P. fluorescens*) contamination in chicken fillet [135]. Taylor and coworkers demonstrated that spray application of bovine milk-derived lactoferrin on raw beef reduces the microbial contamination [136]. A commercial LF-based spray for control of bacterial contamination in beef during carcasses processing was approved by the USDA–FSIS in 2008 (US Department of Agriculture Food Safety and Inspection Service). Barbiroli and coworkers used the combination of lysozyme and lactoferrin on carboxyl-methyl cellulose single use paper napkins, which effectively controlled growth of *Listeria* sp. [137]. In a similar study, cellulose film was coated with bLf and its antimicrobial activity was evaluated in packaging fresh sausage. The bLf-coated cellulose film could effectively reduce *E. coli* and *Staph. aureus* [138]. Nakamura reported the antimicrobial, iron binging, and emulsifying properties of glycosylated Lactoferrin (gLf) [139]. Glycosylated Lf showed 1.29 times higher iron

binding and emulsification property than native lactoferrin and repressed the growth of *E. coli* in cottage cheese at 15 °C for a week. Lactoferrin is stable at pasteurization conditions and significantly maintains its storage stability and iron binding activity. LFcin displays better bactericidal over fungicidal activity [134]. Milk-derived AMPs can also serve as potential candidate to control the growth of food spoilage microbes in agriculture produces such as fruits and vegetables. In this context, LFcinB and LF f(17–31) were tested on mandarins to control the population of *Penicillium digitatum*, thereby paving a way to use AMPs to replace the fungicides [134].

Pharmacological Applications

Antimicrobial peptides do have application as pharmaceutical agent as visible by several convincing reports. However, only a few AMPs have reached industry as drug options. Kappacin (milk peptides) along with Zn^{2+} ion has been shown to have antimicrobial effect by inhibiting biofilm formation by oral cariogenic microorganisms [140]. Kappacin and zinc ions are used as mouth wash solutions. Glycomacropeptide/kappacin and zinc at 1:15 produced comparable effects to chlorhexidine commercial preparations against plaque formation on teeth. Likewise, lactoferricin-derived peptides showed immense antibacterial potential against *Clostridium*. Teraguchi and coworkers demonstrated ability of bovine lactoferricin (intact or hydrolyzed with pepsin) to control growth of *Clostridium species* under in vivo conditions [141]. Mastitis, a mammary gland infection caused by *Staph. aureus* and *Streptococcus* species, is a big concern for dairy farming. Kawai and coworkers used infusion of LF hydrolysate in mastitis-infected cows [142]. They reported reduced somatic cell number on first infusion; however, the disease was eradicated in a total of 14 days. AMPs have good ability to cure systemic infection and subcutaneous infection caused by *Staphylococcus* spp., *Pseudomonas* spp., *E. coli*, and *Candida* spp. Intramuscular injection of isracidin showed protective action on mice subcutaneously infected with *Staph. aureus*, *L. monocytogenes*, and *Streptococcus pyogenes* (M3) and these protective effects lasted for 5 months. Recently, Elnagdy and Alkhazindar proposed that lactoferrin has the ability to enhance host immunity against viral infections, such as SARS-CoV [143]. Lactoferricin can serve as first line of defense against microbial infections as it is an important constituent of human and bovine milk. Lactoferricin hLF f(1–11) exhibited good antifungal activity against fluconazole resistant *Candida* species [144].

Recently, administration of hLF(1–11) at a rate of 40 µg/kg in MRSA-infected (20-h infection) neutropenic mice showed 15–60 fold cell reduction within 2 h irrespective of mode of administration (intravenous, intra-peritoneal, subcutaneous, or oral injections). However, dose-dependent effects were observed with increasing intravenous dosage. During the first phase of clinical trials, hLF(1–11) at a dose of up to 5 mg was found to be safe and well tolerated by health individuals [145]. The drug is in its second phase of clinical trial that includes testing on risk patients. Till now, LFcin B was known as potential peptide characterized with low MIC values and broad-spectrum activities in vitro. However, the claim is supported by limited in vivo studies [146]. The broad-spectrum activities such as anti-pathogenic, anticancer, and anti-inflammation characterized by LFcin B make it prime target for the development of drug molecules and functional foods. The antibacterial activity of milk sample supplemented with 1.5% whey protein concentrate and fermented with *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus* (NS4)) was attributed to antimicrobial peptide (ETVPYMFEN) which was identified as lactoferrin that blocks the entry of bacteria by binding to the surface receptors [147]. The analysis of ionic fraction of buttermilk peptide revealed that antimicrobial activity was key characteristic of cationic peptides; on the other hand, anionic and neutral peptides were inefficient against *Salmonella enterica* [148]. Any modification in the peptide structure can invariably alter the function of peptide. Alvarez-Ordonez and coworkers modified αS2-casein (183–207) peptide by c-terminal pentapeptide truncation followed by substitution of alanine at position 23 with arginine and loss of lysine, which significantly reduced the antibacterial activities against *L. monocytogenes* and *C. sakazakii*. On the other hand, modification of αs2-casein f(193–203) and αs2-casein f(197–207) peptides with hydrophobic end tagging statistically enhanced the antimicrobial activities against *L. monocytogenes* [149].

Moreover, due to its specific properties, lactoferrin has become the choice of ligand and component to drug delivery system. It is extensively used in targeted drug delivery system for intravenous administration for encephalopathy [150], as well as hepatic [151] and pulmonary tumors [152]. This phenomenon owes to the ability of lactoferrin to act as ligand that can cause modification in nano-carriers and can cross blood–brain barrier through lactoferrin receptor-mediated transcytosis [153]. Owing to proteolytically stable structure of lactoferrin, it can withstand the gastrointestinal environment and thus can be exploited for the development of oral delivery system drugs [154].

With respect to clinical application, there are a number of AMPs, which are not from milk origin but are well studied singly or in association with some antibiotics against multi-drug-resistant bacteria with positive outcomes. In a recent study, it was observed that the use of AMPs alone or in combination with conventional drugs is effective in combating different infectious agents, especially multi-drug-resistant bacteria. In one such study, a combined action of natural AMPs with different structures and modes of action with varied antibiotic agents including gentamicin,

Table 3 The antimicrobial peptide drugs approved by the Food and Drug Administration (FDA) (adapted from Lei et al. [159]

Drugs	Administration	In use
Bacitracin	Topical	Localized skin and eye infections, wound infections
Dalbavancin	Intravenous	Acute bacterial skin infections
Daptomycin	Daptomycin	Bacterial skin infections
Enfuvirtide	Subcutaneous	HIV-1 infection
Oritavancin	Intravenous	Bacterial skin infections
Teicoplanin	Intravenous and intramuscular	Bacterial skin infections
Telaprevir	Oral	Hepatitis C
Telavancin	Intravenous	Bacterial skin infections

ofloxacin, oxacillin, rifampicin, and polymyxin B toward selected bacteria was examined [155]. Akbari et al. [156] also studied the synergistic effect of antimicrobial peptides and antibiotics against multi-drug-resistant isolates of *Acinetobacter baumannii* and *P. aeruginosa* and reported a significant reduction in minimal inhibitory concentration of these organisms. Another study demonstrated SAAP-148 (synthetic antimicrobial peptide) as a promising compound against antibiotic-resistant bacteria. A single 4-h treatment with SAAP-148 containing ointment eradicated infections with methicillin-resistant *Staph. aureus* and MDR *A. baumannii* [157]. Conlon et al. [158] discovered that acyldepsipeptide (ADEP4) effectively activated the ClpP protease, having the ability to degrade more than 400 proteins, thereby forcing cells to digest themselves and killing persister cells. When combined with rifampicin, ADEP4 eliminated *Staph. aureus* biofilms in vitro and treated deep leg infection in mice. As an increasing body of research keeps on suggesting the development of drug resistance in microorganisms, it is becoming crucial and difficult at the same time to develop alternative antimicrobial compounds. The use of antimicrobial peptides provides a golden opportunity to develop the potential antimicrobial peptide drug candidates that can be used effectively in place of traditional antibiotics. The FDA has also approved the use of several AMPs in clinical applications (Table 3) [159]. AMPs derived from milk can also be explored further for their antimicrobial activity and can be considered to be as a potent alternative to antibiotics.

Conclusion

In modern world of functional and safe food options, milk serves as an important source for the production of antimicrobial bioactive peptides. The application of AMPs in food animals and food matrices is regarded as safe alternative to antibiotics and food preservatives. Addition of AMPs in food matrices can not only replace chemical preservatives but also enhance shelf life without compromising the quality and nutritional aspect of food. Unlike antibiotics and various food preservatives, AMPs do not pose risk of resistance development in pathogenic microbes, and thus can serve as resourceful alternative against the global threat of antibiotic resistance. The commercialization of AMP in food matrices and pharmacological products requires an elaborated assessment of safety measurements through human clinical trials, which can prove their efficacy and safety. Till now, only a few studies have been attempted to assess the safety aspects of antimicrobial peptides. Therefore, a detailed understanding of the mechanism and safety aspects of the magical milk-derived AMPs can pave the way towards the development of functional and safe options for food preservation and pharmaceutical formulations.

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Declarations

Conflict of Interest The authors declare no competing interests.

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