MilkAMP: a comprehensive database of antimicrobial peptides of dairy origin

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ORIGINAL PAPER

MilkAMP: a comprehensive database of antimicrobial peptides of dairy origin

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Abstract The number of identified and characterized bioactive peptides derived from milk proteins is increasing. Although many antimicrobial peptides of dairy origin are now well known, important structural and functional information is still missing or unavailable to potential users. The compilation of such information in one centralized resource such as a database would facilitate the study of the potential of these peptides as natural alternatives for food preservation or to help thwart antibiotic resistance in pathogenic bacteria. To achieve this goal, we established MilkAMP, a new database that contains valuable information on antimicrobial peptides of dairy origin, including microbiological and physicochemical data. The current release of MilkAMP contains 371 entries, including 9 hydrolysates, 299 antimicrobial peptides, 23 peptides predicted as antimicrobial, and 40 non-active peptides. Freely available at http://milkampdb.org/, this database should be useful to help develop uses of biologically active peptides in both the pharmaceutical and food sectors. As more information about antimicrobial peptides becomes available, the database will be expanded and improved accordingly.

Keywords MilkAMP database · Dairy peptides · Antimicrobial peptides · Milk hydrolysates

1 Introduction

In addition to providing nitrogen in the form of amino acids, dairy proteins contain, encrypted within their primary structures, an array of bioactive peptides, which may be released upon hydrolysis. The number of such peptides identified in caseins and

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whey proteins continue to grow (Gobbetti et al. 2002). Some of these have been shown to possess opioid, immunomodulatory, antimicrobial, antithrombotic, growth stimulating or antihypertensive properties, as previously reviewed (Park 2009; Choi et al. 2012). Jones and Simms in 1930 reported the first dairy protein-derived antibacterial factor (lactenin), which slowed the growth of streptococci (Jones and Simms 1930). Antimicrobial peptides (AMPs) derived from casein were first reported by Hill et al. (1974) who isolated the antibacterial glycopeptides known as casecidins. Isracidin (α_{s1}-casein f[1–23]), a positively charged AMP, was shown to have a broad spectrum of activity against Gram-positive bacteria and to prevent mastitis in sheep and cows (Hill et al. 1974; Lahov and Regelson 1996). A minor fraction of whey, lactoferrin contains within its primary sequence several bioactive peptides that are released upon enzymatic hydrolysis (Vogel et al. 2002; Wakabayashi et al. 2003; Haney et al. 2009) and which appear in some cases to possess much more potent antimicrobial activity than the parent protein does (López-Expósito and Recio 2008).

One of the principal motivators of AMP research has been the observation that resistance to their broad-spectrum activity develops more slowly than in the case of conventional antibiotics (Zasloff 2002). The majority of AMPs display physicochemical similarities that appear to be essential for activity, including small molecular size (10 to 50 amino acids), and cationic and amphipathic characters. Most AMPs interact specifically with the bacterial membrane and kill the cell by causing leakage of its contents. Although membrane interaction might be essential, alternative mechanisms are increasingly considered as components of AMP action against microbes (Jenssen et al. 2006). To date, physicochemical features such as net charge, amphipathicity, hydrophobicity, polar angles and so on, have been shown to influence interaction with and insertion into membrane bilayers. Although several AMPs of dairy origin are well characterized, much information (e.g., amino acid sequence and antimicrobial spectra) is still missing, scattered in the scientific literature or otherwise unavailable to potential users. This deficiency could be improved by a central resource such as a database that could be analyzed to generate new useful information.

Several antimicrobial peptide databases have been created over the past several years. Some of these list peptides from various sources (Antimicrobial Peptide Database (APD) (Wang et al. 2009), Collection of Antimicrobial Peptides (CAMP) (Thomas et al. 2010), DAMPD (Seshadri Sundararajan et al. 2012), YADAMP (Piotto et al. 2012), etc.) while others are dedicated to a specific category of peptide (Defensins Knowledgebase (Seebah et al. 2007), Peptaibol Database (Whitmore and Wallace 2004), etc.) or phylogenetic origin (PhytAMP (Hammami et al. 2009), BACTIBASE (Hammami et al. 2010), PenBase (Gueguen et al. 2006), etc.), as previously reviewed (Hammami and Fliss 2010).

General databases provide an overview of the world of AMPs but have limitations, particularly for detailed searches. For example, APD and CAMP propose, respectively, one and four AMPs derived from lactoferrin. APD contains other lactoferrin-derived peptides, but from poorly specified origins, making it difficult to extract information. Lactoferricin B is noted as originating from "*Bos taurus*" while the peptide derived from its N-terminal portion (LFB0018) is listed as "cow." In addition, synthetic peptides mentioned in the same reference are nowhere to be found in APD (Ueta et al. 2001), which constitutes a significant loss of information

(provided as LFB0019, LFB0118, LFB0120 in MilkAMP). The deficiencies of the APD database could be alleviated at least partly by consistent use of unique and specific terminology.

BIOPEP is a database dedicated to bioactive peptides and is the most effective tool for studying AMPs of dairy origin, with 463 referenced antimicrobial peptides (Minkiewicz et al. 2008). However, most of these peptides are not specific to milk and are found elsewhere in the body. In addition, some are not supported by sufficient documentation, creating in the case of lactoferrampin the erroneous perception of a single peptide rather than a cluster, because of the absence of references and hence other peptides derived from this sequence. Finally, synthetic peptides and information relating to studies thereof, as well as references providing other useful information on the subject, are absent.

A new database designed specifically for milk antimicrobial peptides is therefore needed. The MilkAMP database lists natural and artificial (synthetic or modified) antimicrobial peptides derived from amino acid sequences of dairy proteins of different origins and provides the most complete information possible on peptide structure/function relationships, inhibitory activity, spectrum of action and minimal inhibitory concentration (MIC) determined for each tested microbial strain. It provides a nearly exhaustive list of references relating to each peptide. The information contained in this database will complement general databases by providing missing data and allows rapid prediction of structure/function relationships and target organisms and hence should lead to better use of the biological activities of peptides in both the pharmaceutical and food sectors.

2 Material and methods

2.1 Data sets

An exhaustive literature search was carried out to extract the relevant articles from databases such as PubMed (www.ncbi.nlm.nih.gov/pubmed/) and Google Scholar (www.scholar.google.com). The keywords used were milk peptide(s), antimicrobial peptide(s), and common milk protein names, aliases, and abbreviations (e.g., lactoferrin, LFCIN, etc.). All peer-reviewed papers thus found were screened for relevant content, such as MIC and sequence annotations. A physicochemical dataset was designed containing peptide mass, length, isoelectric point, net charge, number of basic, acidic, hydrophobic or polar amino acid residues, hydropathy index, protein-binding potential (Boman index) (Boman 2003), aliphatic index (Ikai 1980), instability index (Guruprasad et al. 1990), absent and most prevalent amino acids in peptide sequence, extinction coefficient, absorbance at 280 nm and tertiary structure (when available).

2.2 Implementation

The MilkAMP database was implemented on the Linux platform with MySQL (v5.1.62) and Apache (v2.2.2.1), while the Web interface was implemented with PHP (v5.2.17). Sequences were retrieved in SciDBMaker (Hammami et al. 2008),

compiled and the resulting tables exported to the MySQL server. MilkAMP includes numerous tools for sequence analysis, such as homology search (BLAST v2.2.15 (Altschul et al. 1997), FASTA v35.04 (Pearson and Lipman 1988) and Smith–Waterman v35.04 (Pearson and Lipman 1988)), multiple sequence alignment (ClustalW v2.10 (Larkin et al. 2007), MUSCLE v3.6 (Edgar 2004)), Hidden Markov Models (HMMER v2.3.2 (Durbin et al. 1998)), and physicochemical profile analyzer. Generated multiple sequence alignments are displayed graphically using the embedded Jalview applet (Waterhouse et al. 2009).

3 Results and discussion

3.1 General description of the graphical user interface

Information is easy to extract from the database using various tabs such as "Advanced search," "Explore data," "References," and "Statistics" (see Fig. 1). The "Explore data" interface allows querying of a peptide based on various parameters, including accession number, name, producer organism, native protein of origin, activity, and method of production. The physicochemical characteristics (length, mass, sequence, isoelectric point (pI), net charge, etc.) of each peptide are also listed and it is possible to retrieve and view them the same way as for the general information. Each record has specific characteristics and is unique. Multiple inputs may thus correspond to a given name. For example, lactoferricin H has five different records in this database. Two different sequences (LFH0005 and LFH0006) derived from recombinant bovine lactoferrin expressed in insect cells are proposed. A third entry corresponds to the human lactoferricin H sequence originally and most commonly cited in the literature (LFH0008). Another provides an alternative conformation proposed in subsequent works (LFH0009), while the remaining entry combines works lacking data on peptide origin (LFH0007).

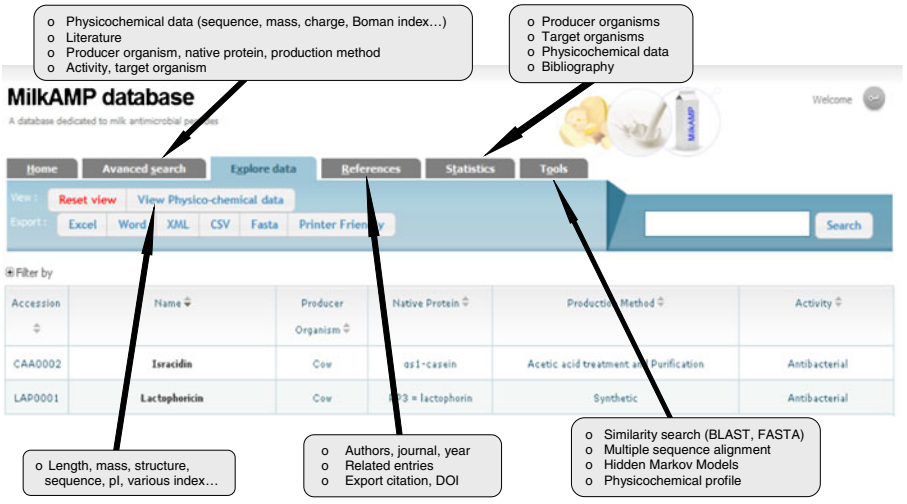

Fig. 1 Database contents and tools

Data can be extracted in various formats including Excel, Word, XML, CSV, FASTA, or printer-friendly. More detailed data, particularly on the inhibition of sensitive organisms, lists of non-sensitive organisms and experimental MIC can be obtained by clicking on the accession number of the entry or on its behalf. Any peptide can be found using the search bar by name or accession number. The ability to search for entries using a single keyword for general parameters (producer, native protein, method of production and antimicrobial activity) is also included in this search bar. The remaining displayed data allow quick searches of all related data simply by clicking on them. For example, clicking on "cow" in the column of producer organisms will automatically display all bovine entries. More detailed searches are requested using the "advanced search" tab, which offers additional possibilities by cross-checking data with a variety of both general and physicochemical settings as well as the opportunity to search for a peptide sequence appearing in different entries or to search the literature of reference (using author name, journal title, etc.). The "reference" tab provides tracing of all sources cited in the database and highlights the entries extracted from each cited source. Data accessibility will make possible to produce a variety of works by combining different tools such as BLAST, and to compare sequences by alignment with sequences imported by the user. The "Statistics" tab provides data on AMP producer organisms referenced previously and on target organisms, and compiles all species that have shown sensitivity to at least one related peptide or hydrolysate. These statistics therefore do not include resistant strains. Physicochemical statistics provide data on average peptide length (entire database or by producer organism), net charge, and pI. Statistics on amino acid presence and percentage are also provided, as well as acidic and basic amino acid content. Year of publication, related newspaper articles, and number of peptides discussed are provided among the statistics on references. All statistics are updated automatically as new sequences are added.

3.2 Data summary

3.2.1 Animal of origin

The database currently includes peptides derived from 10 animal species (Table 1). Although more than half of the peptides are of bovine origin, virtually all mammals are potential providers of AMP sequences. In the coming years, AMPs could be isolated from new origins such as domesticated mammals of which the milk is already part of the human diet (camel, horse, yak, donkey, etc.). Although only large animals provide enough milk for industrial-scale AMP production, peptides associated with smaller species could provide useful information for understanding and optimizing AMP action, as well as the possibility of activities for specific applications justifying production using recombinant technology.

3.2.2 Protein of origin

Since caseins are the major proteins of milk in general (82 % of cow milk protein and 40 % of human milk protein), many AMPs correspond to casein amino acid sequences. These have been identified in the four casein subunits (α_{s1}, α_{s2}, β, and κ) of various

Table 1 Origin of the antimicrobial peptides entered in the MilkAMP database (accessed 12 July 2012)

Producer organism	Current number of peptides
Buffalo	2
Camel	1
Cow	225
Goat	16
Human	93
Mouse	21
Pig	5
Rabbit	4
Rat	1
Sheep	6
Total	374

animals (cow, rabbit, human, and sheep). Their presence in caseins of animals such as goat or buffalo is presumed but has not yet been confirmed.

The composition of whey proteins varies widely across species. The most studied are lactoferrin, β-lactoglobulin, and α-lactalbumin, with mixed results. Several AMPs with broad-spectrum activity have been found in lactoferrin, while peptides derived from β-lactoglobulin and α-lactalbumin appear to be active only against Gram-positive bacteria. Lactoferrin of all origins accounts for 291 entries and is the most studied protein because of the presence of the two antimicrobial clusters named lactoferricin and lactoferrampin (see Fig. 2). Bovine lactoferrin alone accounts for 165 records in the database, of which, 97 are modified sequences by chemical means. Most of these peptides were synthesized for the purpose of studying the structure/function relationship of lactoferricin, the best-known antimicrobial peptide of dairy origin. Other proteins considered as minor components of milk (proteose peptone, lysozyme, etc.) also show potential as sources of antimicrobial peptides, but their low concentrations limit interest in them. Dairy substances such as whey acidic proteins are beginning to attract interest since they have characteristics suggesting potential antimicrobial activity. All referenced proteins are presented in Table 2. Finally, albumin has not been studied as an AMP source, and to the best of our knowledge, no albumin sequence with antimicrobial activity has been reported.

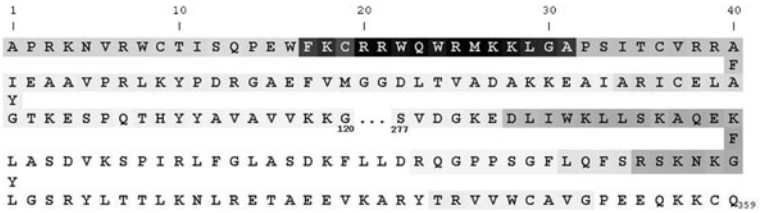

Fig. 2 Partial amino acid sequence of lactoferrin. The *shades of gray* represent the number of citation in the database. The darker the color, the more sequences are found in antimicrobial peptides

Table 2 Native proteins providing antimicrobial peptides listed in the MilkAMP database (accessed 12 July 2012)

	Native protein	Current number of peptides
Caseins	Casein hydrolysate	2
	αs1-casein	11
	αs2-casein	19
	β-casein	10
	κ-casein	19
Whey	α-lactalbumin	8
	β-lactoglobulin	6
	PP3 (lactophorin)	3
	Lactoferrin (LF)	291
	Recombinant lactoferrin (rLF)	3
	Whey acidic protein	1
	Whey protein hydrolysate	1
	Total	374

3.2.3 Peptide production

The identification of bioactive peptides encrypted within the amino acid sequence of a protein usually begins with acid or enzymatic hydrolysis followed by purification by liquid chromatography to separate and purify peptides and identify interesting sequences. These peptides are thus regarded as natural in the sense that they are likely to be found in food or to appear during digestion. In some cases, the use of two different enzymes may lead to the same peptide. This is in the case of lactoferricin, which can be obtained using pepsin (LFB0084) or chymosin (LFB0087), two enzymes from the aspartic acid proteinase family (Carginale et al. 2004). However, more than half (291) of the peptides listed in the database were obtained by synthesis. In many cases (122), this was in order to obtain larger amounts and determine the activity spectrum. Many (169) have been modified, usually in order to determine the importance of a specific amino acid in the sequence or to attempt to make the peptide more active. In rare cases, peptides were obtained in other ways, such as treatment with acetic acid or heat.

3.3 Antimicrobial activity

The current release of MilkAMP contains 371 records. Of the 272 supported by references, 56 concern antifungal activity and 2 concern anti-parasitic activity. Thirty concern antifungal activity only. Inclusion of antiviral peptides has also begun. The database also contains seven hydrolysates of interest due to the use of unusual dairy proteins or enzymes. Four of these exhibit antifungal activity. There are also 23 potentially antimicrobial but untested peptides, obtained primarily from studies predicting the antimicrobial activity of peptides derived from proteins. Finally, 39 peptides and one hydrolysate have not been found to inhibit any microbial species, but were added to the database as supplementary information (see Fig. 3).

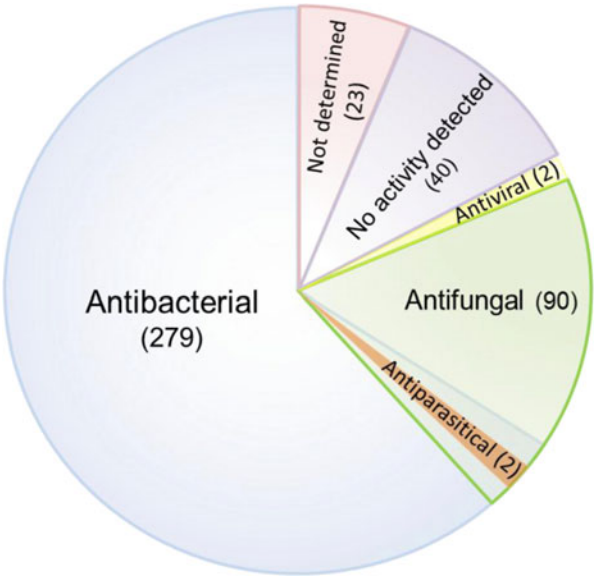

Fig. 3 Distribution of five principal biological activities among the peptides listed in the MilkAMP database (374 AMPs) (accessed 12 July 2012)

Bovine lactoferrin is the most cited dairy protein in the database, probably because lactoferricin is the most studied AMP derived from milk protein. The complete sequence of lactoferricin corresponds to lactoferrin fragment 17–41 (FKCRRWQWRM KKLGAPSITCVRRAF; LFB0084) and sequences from within this fragment are also antimicrobial. Fragment 20–30 (RRWQWRMKKLG; LFB0109) was the first of these to be studied and was produced in several mutant forms in order to characterize the structure/function relationships of lactoferricin (Kang et al. 1996), the authors examining the role of the α-helical structure in lactoferricin activity. Their results showed inhibition similar to lactoferricin for two tested strains and reduced hemolytic activity. Both N-terminal arginine residues appear to play a role as modulators of inhibitory activity. Subsequent study of fragment 17–31 (FKCRRWQWRMKKLGA; LFB0031) revealed the importance of hydrophobic amino acids, particularly, the two tryptophan residues, but also methionine to a lesser degree (Strøm et al. 2000). Fragments 20–28 (RRWQWRMKK; LFB0028) and 17–27 (RRWQWKMKKLG; LFB0020) have also been studied and modified in various forms. Using fragment 20–28, it was determined that N-terminal acylation could increase peptide antimicrobial activity as well as simplify synthesis (Wakabayashi et al. 1999). Study of fragment 17–27 suggested the importance of balance between aromatic and cationic amino acids (Strøm et al. 2001). The database contains a total of 84 peptides (natural, synthetic, and modified) comprising or derived from at least one of these four sequences, while other studies suggest that as many as 111 such sequences are of interest for the complete lactoferricin. In humans, lactoferricin corresponds to lactoferrin fragment 1–47 but consists of two subunits, namely fragments 1–11 and 12–47 (LFH0009), connected by a disulfide bridge. If this seems quite different from bovine lactoferricin, it is mainly because human lactoferricin does not correspond to the

homologous bovine sequence but rather to the peptide obtained under the same experimental conditions.

The second reason why lactoferrin is mostly studied is the presence of lactoferrampin, originally identified as fragment 268–284 (WKLLSKAQEKFGKNKSR; LFB0149) (van der Kraan et al. 2004). Its spectrum of action differs from that of lactoferricin, but the sequence has not yet been tested against filamentous fungi, parasites, or viruses. The various peptides derived just from the bovine version of this sequence account for 36 entries. In humans, the sequence corresponding to bovine lactoferrampin has not yielded any peptide with sufficient activity to arouse interest, and studies are therefore few (Haney et al. 2009). However, several other AMPs longer than the original sequence were later identified. Researchers now refer to an antimicrobial domain corresponding roughly to fragment 259–296 (Bolscher et al. 2006). In summary, lactoferricin and lactoferrampin account for 147 of the total of 165 entries for bovine lactoferrin. Hydrolysates, chimeras, and non-active or untested peptides aside, there remain 7 antimicrobial sequences derived from bovine lactoferrin, tested against only 10 different microbial strains. For humans, peptides derived in the course of studying human lactoferricin and lactoferrampin make up all entries for human lactoferrin, except for kaliocin-I (LFH0060), which is currently specific to human lactoferrin and much less potent than lactoferricin (human or bovine). Antimicrobial peptides from milk proteins are eclipsed by the success of lactoferricin, which accounts for the majority of studies, while other less-studied peptides might have strong potential for pharmacological use.

The bovine form of the caseins is the most studied. Of the 47 casein peptides that have demonstrated antimicrobial activity (two antifungal), 38 are bovine. The α and β subunits have provided peptides that show potential, but the number of microbial strains tested so far is insufficient to conclude pharmacological or food interest, except for fragment 183–207 (CAA0020), which looks promising with its low MIC. The κ subunit has not provided any peptides with low MIC, although many peptides with some antimicrobial activity have been identified in its sequence. Kappacin (CAK0013) might be of interest, but much information is still missing. On the β subunit, casecidins 15 (CAB0007) and 17 (CAB0008) appear inhibitory at low concentrations against a few microbial strains. In this case again, several AMPs of limited interest could become more interesting in light of additional information.

Studies of other animal species have been carried out almost exclusively on lactoferrin and specifically on the homologous region corresponding to lactoferricin. Other proteins including caseins have been examined and inhibitory peptides have been found, but none with interesting potency (Baranyi et al. 2003; López-Expósito et al. 2006).

The database also lists all of the microbial strains (sensitive and insensitive) with which the various sequences and hydrolysates have been tested. A general idea of the genetic diversity of organisms sensitive to at least one peptide in the database is thus provided. Sensitivity to peptides in the database has been observed among 23 genera of fungi including 8 yeasts, 23 genera of Gram-negative bacteria, 15 genera of Gram-positive bacteria, and one genus of parasite.

Among the bacteria, two species in particular have been used as reference strains and are tested almost systematically. These are *Escherichia coli* (representing Gram-negative) and *Staphylococcus aureus* (representing Gram-positive) (Table 3). Other Gram-negative genera frequently tested include *Pseudomonas*, *Salmonella*, and to a lesser degree, *Klebsiella* and *Serratia*. Frequently tested Gram-positive genera

Table 3 The ten most tested target organisms in the MilkAMP database

Genus	Number of entries	Main strains	Number of entries
Escherichia	363	*E. coli*	363
Staphylococcus	245	*S. aureus*	183
		S. epidermidis	24
		S. carnosus	21
Candida	140	*C. albicans*	114
Listeria	66	*L. innocua*	35
		L. monocytogenes	31
Streptococcus	65	*S. pneumoniae*	19
		S. mutans	17
Pseudomonas	57	*P. aeruginosa*	47
Bacillus	56	*B. subtilis*	42
Salmonella	41	*S. typhimurium*	16
Enterococcus	25	*E. faecalis*	18
Klebsiella	24	*K. pneumoniae*	22

Accessed 12 July 2012

include *Bacillus*, *Listeria*, *Streptococcus*, and to a lesser extent, *Enterococcus*, *Micrococcus*, and *Lactobacillus*. The overwhelming majority of the microbial organisms mentioned in the database are pathogenic or food spoilage bacteria. In view of the striking rarity of AMP testing on strains such as *Lactococcus lactis* or *Streptococcus thermophilus*, the possibility of interference with dairy product quality or processes should be studied.

Among the filamentous fungi, *Penicillium* and *Trichophyton* have been found to be sensitive to several peptides (4 and 11, respectively) and are the most frequently tested. In addition, the genus *Trichophyton*, of which some species are known to infect the skin, has been tested in two studies of susceptibility to lactoferricin B and derivatives. Filamentous fungi are not tested systematically, and information on antifungal activity is limited (Bellamy et al. 1994; Muñoz and Marcos 2006; Wakabayashi et al. 1996). The number of entries mentioning an effect on at least one filamentous fungus (dermatophyte or mold) is 14 (12 bovine-derived and two human-derived including one hydrolysate) and includes one hydrolysate. The most often tested yeast is *Candida*, which includes human pathogens. Other yeasts occasionally tested include *Cryptococcus*, *Dekkera*, and *Saccharomyces*. The database contains 91 entries that include data on yeasts.

4 Conclusion

The MilkAMP database is freely accessible for query at (http://milkampdb.org). It currently contains 371 entries, including 9 hydrolysates, 299 antimicrobial peptides, 23 peptides predicted as antimicrobial, as well as 40 non-active peptides, and is expected to grow quickly with the rapid development of genomic and proteomic

projects. As more information about dairy antimicrobial peptides becomes available, the database will be expanded and improved. Researchers in this field are invited to use the database, to make suggestions, and to submit their peptides.

MilkAMP allows all AMP sequence data and other information to be accessed via a user-friendly, web-based interface. Queries may be based on a variety of criteria to retrieve specific structural, physicochemical, or microbiological data. We expect this to allow better and more comprehensive structural and functional analysis and ultimately better understanding of milk AMPs. This will not only be useful in food preservation or food safety applications, but also has implications for the development of new drugs for medical use.

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