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Can cheese mites, maggots and molds enhance bioactivity? Peptidomic investigation of functional peptides in four traditional cheeses

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ABSTRACT

Aside from their amino acid content, dairy proteins are valuable for their ability to carry encrypted bioactive peptides whose activities are latent until released by digestive enzymes or endogenous enzymes within the food. Peptides can possess a wide variety of functionalities, such as antibacterial, antihypertensive, and antioxidative properties, as demonstrated by *in vitro* and *in vivo* studies. This phenomenon raises the question as to what impact various traditional cheese-making processes have on the formation of bioactive peptides in the resulting products. In this study, we have profiled the naturally-occurring peptides in two hard and two soft traditional cheeses and have identified their known bioactive sequences. While past studies have typically identified fewer than 100 peptide sequences in a single cheese, we have used modern instrumentation to identify between 2900 and 4700 sequences per cheese, an increase by a factor of about 50. We demonstrated substantial variations in proteolysis and peptide formation between the interior and rind of each cheese, which we ascribed to the differences in microbial composition between these regions. We identified a total of 111 bioactive sequences among the four cheeses, with the greatest number of sequences, 89, originating from Mimolette. The most

common bioactivities identified were antimicrobial and inhibition of the angiotensin-converting enzyme. This work revealed that cheese proteolysis and the resulting peptidomes are more complex than originally thought in terms of the number of peptides released, variation in peptidome across sites within a single cheese, and variation in bioactive peptides among cheese-making techniques.

INTRODUCTION

Although dairy proteins are normally viewed as a source of amino acids for anabolism, studies have revealed that specific milk protein-derived peptide sequences also possess bioactivities that could substantially impact consumer health. Dairy peptides and their bioactivities have been under investigation for nearly 40 years, starting with the discovery of casein-derived peptides with opioid activity.[1] Functional analyses have identified peptides in dairy products with a variety of bioactivities, including antibacterial,[2] hypocholesterolemic,[3] antihypertensive,[4–6] antioxidative[7] and opioid functions.[8] Though hundreds of bioactive peptides have been identified in dairy products, which peptides occur in each of the huge array of available dairy products remains underexamined, and whether these bioactive peptides can act *in vivo* in the consumer to enhance health remains unknown. Given the need for effective treatments for ailments such as hypertension, hypercholesterolemia and dysbiosis, the impact of dairy protein-derived bioactive peptides on consumers and their potential applications as novel therapeutics deserves to be examined.[9] Such research will require advanced mass spectrometry and bioinformatics-based peptidomics to profile the ensemble of peptides within each dairy product and their survival across digestion and absorption, and tests of *in vivo* efficacy.

Recently, studies have investigated the extent to which proteolysis occurs in human and animal milk prior to consumption. One hundred fifty-nine peptides have been identified in bovine milk[10] and over 1,100 peptides have been identified in human milk, many of which share homology with known bioactive sequences.[11] The measured proteolysis resulted from the action of naturally occurring milk proteases that function within the mammary gland.[12,13] Fermented dairy products, such as cheese and kefir,[14–16] also contain an array of peptides released during processing and ripening as demonstrated by peptidomic analyses.

The cheesemaking process exposes milk proteins to various enzymes and pH changes that may allow for a greater degree of proteolysis than in fluid milk, likely increasing the diversity of peptide sequences in the final product. Cheese production strategies vary substantially by cheese variety; however, typically milk proteins are coagulated using acid-producing bacterial starter cultures and/or the enzyme chymosin, the major enzyme in calf rennet.[17] Chymosin cleaves κ-casein between the phenylalanine-126 and methionine-127 residues (numbering includes signal sequence), releasing caseinomacropeptide from the casein micelle and causing protein aggregation.[17,18] This protease also cleaves at specific sites within α_{s1}-, α_{s2}-and β-casein and additional sites within κ-casein, releasing casein-derived peptides.[19] Rennet continues to act within the cheese during ripening, which can take as little as a few days or as long as several years, and can soften cheese structure.[19,20] Lactic acid-producing bacterial starter cultures are also used in cheesemaking to initiate protein coagulation by lowering the milk pH to the casein isoelectric point (approximately pH 4.6).[17,21] Starter cultures produce an array of proteases and peptidases[22] that can thoroughly digest milk proteins.[23] Native milk proteases can continue to function and release peptides during cheese processing and ripening as well. For example, plasmin, a protease present in bovine[24] and sheep milk,[25] contributes to proteolysis

during cheese ripening, increasing the presence of water-soluble peptides and altering texture of the cheese.[20] These effects have been observed experimentally with the addition of exogenous plasmin to bovine milk-based cheese.[26] Some strains of non-starter (adventitious of adjunct) lactic acid bacterial cultures also add to the proteolytic activity in a cheese during ripening.[27] Secondary microorganisms added to cheese or encouraged to grow in cheese based on environmental conditions (e.g., the mold *Penicillium roqueforti* in blue cheese) can also contribute to proteolysis during ripening.[28] A biofilm is formed on the surface of many aged cheeses, which consists of a variety of bacterial and fungal species other than those used as a starter culture.[29] These microbial communities are unique to the outer surface of the cheese and can, depending on the microbial composition, contribute to unique proteolysis in this region.[30]

The literature has shown that hundreds of casein-derived peptides are present in Cheddar,[31–36] Emmental,[14] and Manchego cheeses.[37] Furthermore, some cheese peptides are known to be bioactive. For example, peptide extracts from five different Italian cheeses have been characterized as antimicrobial.[38] However, many cheese varieties remain unexplored for their peptide profiles, especially those that originate from non-bovine milks. Moreover, whether the cheese peptidome varies between the interior and the rind of cheeses remains unknown. Therefore, the aim of this research was to characterize the peptidomes of several unique cheese varieties obtained with diverse cheesemaking techniques, determine how the peptidome differs between the interior and the rind and identify functional peptides which may benefit the consumer. We isolated the peptides present in the controversial cheese Casu Marzu, as well as in Taleggio, Stilton Blue, and Mimolette cheeses and used mass spectrometry (MS) and bioinformatics to identify their sequences. These cheeses were selected due to their unique production characteristics including different ripening stages and their inoculation with various

microorganisms that are likely to contribute to proteolysis: a variety of yeasts and molds in Taleggio,[39,40] *Piophila casei* maggots in Casu Marzu,[41] *Penicillium roqueforti* in Stilton blue[42] and *Acarus siro* mites in Mimolette.[43] Further details on each cheese are provided in Table 1. All the cheeses are produced solely in their specific regions and follow a particular traditional production process. Taleggio, Stilton Blue, and Mimolette have already been granted the European Union recognition as Protected Designation of Origin (PDO) products, whereas Casu Marzu has been listed among the 'Traditional Italian agricultural and food products" (N.0011264, 16/02/2018); however, some challenges related to the standardization of the production processes, and identification of the critical points of hygiene in the production have delayed the PDO status.

METHODS

Isolation of peptides for MS analysis. The rind and interior of each cheese were prepared and analyzed separately using the following protocol: 100 mg of cheese were cut into fine pieces and combined with 1 mL of Milli-Q water. The mixture was shaken at 40 °C for one hour with a Thermomixer C (Eppendorf, Hamburg, Germany), agitated via an ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA) for 15 minutes at 40 °C, and finally shaken at 40 °C for one more hour. To eliminate lipids and large particles, the solution was centrifuged at 3,080 × *g* at 4 °C for 30 min. The peptide-containing supernatant was collected (approx. 0.9 mL) and purified by Folch extraction and C18 solid phase extraction (SPE), essentially following the protocol of Dallas et. al.[44] Briefly, the aqueous samples were combined with 3.6 mL (4 volumes) 2:1 chloroform:methanol. The mixture was vortexed and then centrifuged at 4,200 × *g* and 4 °C for 10 min. The upper (methanol/water) layer was collected, dried by vacuum centrifugation and the

dried samples were re-dissolved in Milli-Q water. The peptides were further purified by microplate C18 SPE (Glygen, Columbia, MD, USA). The microplate wells were activated with 99.9% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) and equilibrated with 1% ACN/0.1% TFA. Samples were loaded, and the solid phase was washed with 1.2 mL 1% ACN/0.1% TFA. Peptides were eluted with 600 µL 65% ACN/0.1% TFA. The peptides were dried and re-dissolved in 100 µL Milli-Q water. Samples were filtered through 0.2-µm polyethersulfone syringe filters. Each extraction was performed in duplicate for each cheese rind and interior. For the purposes of this study, the rind was considered to be the outer cheese surface with a thickness of approximately 3 mm.

Liquid chromatographic separation and Orbitrap MS/MS analysis. Peptides were separated using a Waters Nano Acquity Ultra High Performance Liquid Chromatograph equipped with a 100 µm x 25 mm Magic C18 100Å 5U trapping column and a 75 µm x 150 mm Magic C18 200Å 3U analytical column, followed by ionization with a Proxeon nanospray source. Compounds were eluted from the analytical column using a gradient composed of 0.1% formic acid (FA) (A) and ACN (B) at a flow rate of 300 nL/min. The 60-min gradient was ramped from 5–35% B, 0–50 min; followed by 35–80% B, 50–53 min; 80% B, 53–54 min; 80–5% B, 54–55 min; and 5% B; 55–60 min.

Mass spectra were collected with an Orbitrap Q Exactive Plus mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using data-dependent acquisition, with 15 tandem-MS (MS/MS) scans following each MS scan. A dynamic exclusion time of 20 seconds was applied. MS spectra were collected at a resolution of 70,000 and a target of 1×10^6 ions or 30 ms maximum injection time. MS/MS spectra were collected with a resolution of 17,500 and an ion count target of 5×10^4, or a maximum injection time of 50 ms. Fragmentation was

performed on ions having a charge in the range of +1 to +4 using higher-energy collision dissociation, with a normalized collision energy value of 27.

Spectral analysis and peptide identification. The raw mass spectral files were exported in mgf file format and analyzed with X!Tandem peptide identification software,[45] as described previously,[46] with minor modifications. The peptide search was performed with the allowable mass error set to 20 ppm for both precursor and fragment ions, and with a log(e) < -2 threshold (corresponding to a 99% minimum match confidence). Isotope errors were allowed, allowing the software to recognize peptides from MS/MS spectra that resulted from the fragmentation of the M+1 isotope rather than the monoisotope. Oxidation of methionine was allowed as a potential modification. Deamidation of asparagine and glutamine and phosphorylation of serine and threonine were used as potential modifications during a secondary refinement search. No complete modifications were allowed. The data was searched against the entire animal proteome specific to each cheese (bovine for Stilton, Mimolette, and Taleggio; sheep for Casu Marzu, downloaded from www.uniprot.org) with a non-specific ([X]|[X]) proteolytic cleavage pattern. When calculating the number of unique peptides identified in each sample, amino acid sequence, number of modifications, and type of modifications were taken into consideration. Thus, two peptide sequences which appeared to have differing locations for the same type(s) of modification(s) were considered duplicates. Mass spectral data is available via ProteomeXChange Consortium via the PRIDE[47] partner repository with the dataset identifier PXD021426 and 10.6019/PXD021426. The location of each casein-derived peptide sequence within the parent protein was visualized with the PepExplorer module of the PatternLab for Proteomics software suite.[48]

Functional homology search. Peptides identified in the cheeses were searched for homology against the online Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/)[49] to identify sequences with known bioactivities. Only peptides with 100% sequence homology to known functional peptides were retained and included in the results.

Total protein analysis. The total protein content of each cheese was measured with the Dumas combustion method and a conversion factor of 6.38 (Vario MAX Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany).

O-phthaldialdehyde assay. The degree of hydrolysis of milk proteins in each cheese was estimated using the o-phthaldialdehyde (OPA) assay, which is based on the reaction of OPA with primary amines.[50] Peptides were extracted from each cheese as described by Pripp et. al.[51] Briefly, 5 g grated cheese was mixed with 25 mL water and incubated at 40 °C for 5 min. The mixtures were homogenized with an Ultra-Turrax T25 homogenizer (IKA Works, Inc. Staufen, Germany) for 5 min. The pH was adjusted to 4.6 using 2 M hydrochloric acid to precipitate intact proteins, and water was added to increase the total sample weight to 40 g. Samples were incubated at 40 °C for 1 hour and centrifuged at 3,000 × *g* for 30 min at 40 °C. The aqueous supernatant was filtered with a Whatman No. 1 filter and dried by centrifugal evaporation (Eppendorf Vacufuge Plus, Eppendorf, Hamburg, Germany). Samples were re-dissolved in 40 mL Milli-Q water for analysis.

The molar peptide concentration in each sample was measured with the OPA assay, essentially according to the method of Nielsen et. al.[52] The OPA reagent consisted of 6 mM OPA with 100 mM disodium tetraborate, 3.5 mM sodium dodecyl sulfate, and 5.7 mM dithiothreitol in water. Dilutions of a serine standard in water were prepared within the calibration range of 20 – 120 mg/L. Two hundred microliters of each standard and diluted sample were combined with 1.5

mL OPA reagent in duplicate. The mixtures were vortexed briefly, incubated for 2 min, and the absorbance values at 340 nm were read with a Shimadzu UV160U spectrophotometer (Shimadzu, Kyoto, Japan). Molar peptide concentrations were normalized by the protein content of each cheese and the results expressed as mmol Ser equivalents per gram of protein.

RESULTS AND DISCUSSION

Peptide profiles. Over 2,500 peptides were identified in each cheese, with most originating from caseins (Figure 1). Predictably, β-casein contributed the greatest number of sequences to the cheese peptidomes. β-casein is one of the most abundant proteins in both bovine[53,54] and sheep[55] milk, and is digested relatively easily.[56,57] The other three caseins (α_{s1}-, α_{s2}-, and κ-), were also major contributors of peptides in each cheese. The cheesemaking enzyme chymosin, present in each of these cheeses, is known to cleave each of the casein proteins in numerous locations.[19,58] Native milk enzymes, including plasmin, elastase, cathepsin B and cathepsin D,[20,59–62] as well as starter and secondary cultures in cheese,[20] can also degrade casein proteins. Therefore, it is not surprising that a multitude of casein fragments were identified in every cheese. The location of each casein-derived peptide in the sequence of the parent protein is depicted in Supplementary Figures S1-S4. As expected, the peptide maps show very little coverage in the protein signal sequence region, but substantial coverage in most other areas of the protein. Some notable exceptions were α_{s2}-casein, which showed relatively little coverage for amino acid positions 1-75 for all cheeses, as well as κ-casein, which had little coverage beyond amino acid position 110 for all cheeses. The region beyond amino acid position 126 in κ-casein (known as caseinomacropeptide) is highly glycosylated and is responsible for stabilizing casein micelles in solution. This segment of κ-casein is cleaved from the casein micelle during the enzymatic

coagulation that is used in all four cheeses examined. Therefore, it is likely retained in the whey fraction due to its hydrophilic nature and would therefore not often appear in the cheese peptidomes.

The large contribution to the overall peptidome by the casein proteins is consistent with past findings for other cheese types, including Cheddar,[31–36] Emmental,[14] and Manchego.[37] However, in contrast to these past studies, our dataset also identified numerous sequences derived from whey proteins and milk fat globule membrane proteins originating from each cheese. Among these, the most frequently-identified proteins were xanthine oxidase, β-lactoglobulin, lactoferrin, butyrophilin subfamily 1 member A1, and polymeric immunoglobulin receptor. Complete lists of these peptide sequences and their proteins of origin are provided as Supplementary Tables S1-S12.

Although the relatively high contribution of caseins to the cheese peptidome is consistent with past studies, this dataset is also unique for the vast number of total peptide sequences identified among the samples, as well as the relatively high number of whey protein-derived peptides. Past studies have used mass spectrometry to profile peptides in Parmigiano-Reggiano,[15] Valdeón,[63] Coalho,[64] Emmental,[14] and Manchego[37] cheeses, but have all identified fewer than 200 total sequences. By comparison, this experiment has identified as high as 4700 sequences per cheese. These results highlight the value in utilizing modern high-resolution mass analyzers, such as the Orbitrap, which are capable of fragmenting and analyzing an exponentially greater number of peptide sequences with high mass accuracy than what was possible with older instrumental analogs or amino acid sequencing devices. Our automated search of the tandem-MS spectra utilized a non-specific enzyme cleavage pattern ([X]|[X]), allowing peptides to be

identified regardless of the enzymatic pathways by which they were produced. These results indicate that cheese peptidomes are far more complex than originally thought.

The Milk Bioactive Peptide Database identified many bioactive casein-derived peptides that were present in more than one cheese (Figure 2, Supplementary Table S13). Furthermore, 18 bioactive sequences were identified in all four cheeses, despite the fact that Casu Marzu is produced from sheep milk, unlike the other three bovine milk-derived cheeses. These similarities highlight both the presence of conserved protein sequences among the milk types, as well as similarities in the enzymatic activities that cleave these peptides from the intact proteins. Among these universally-identified bioactive sequences, ten are inhibitors of the angiotensin-converting enzyme (ACE). ACE converts the peptide angiotensin I into angiotensin II, a vasoconstrictor believed to contribute to hypertension.[65] The remaining peptides possesses antimicrobial, antioxidant, and anxiolytic activities. The specific bioactive sequences identified in each cheese are discussed in more detail below.

Comparison of cheese interiors and rinds. All four cheeses showed substantial differences in peptide profiles between the cheese interior and rind (Figures 3 and 4). We selected cheeses that are known to have secondary microorganisms present on the rind or interior which could contribute to further proteolysis beyond that of rennet, the native milk proteases and the starter cultures. The peptide data indicates that differences in the microflora of the interior and rinds of these cheeses resulted in substantial differences in protein breakdown. For example, the rind of Casu Marzu contained nearly twice as many unique peptide sequences from β-casein and α_{s2}-casein as the interior. However, the rind of Stilton contained less than 20% of the number of sequences found on the cheese interior, and only 4.3% of the cheese's peptides were shared between the rind and interior. By comparison, the other three cheeses contained 37% (Casu

Marzu), 27% (Mimolette) and 14% (Taleggio) of their respective sequences in both the rind and interior. This finding could be explained by a relative lack of proteolysis on the rind of Stilton due to the absence of *P. roqueforti* on the outer surface.[66] However, it is also possible that peptides on the Stilton rind were further broken down to di- and tripeptides, which are typically not identified with proteomic database search algorithms and therefore require alternative data analysis strategies.[67] Development of more robust and accurate algorithms for *de novo* sequencing of these smaller peptides could help establish a more complete understanding of the proteolysis that occurs with cheese ripening.

The ability of each microbial genera to contribute to the unique peptide sequences identified in the cheese rinds and cheese interiors is relatively unexplored; however, based on the observed differences between the peptidome of the interior and rind for each cheese examined herein, we theorize that these microbial communities play a substantial role in shaping the peptide profile within the cheese rind and cheese interior. Indeed, studies of Taleggio and Stilton cheeses and have identified differences between the rind and interior microbial populations in each cheese.[40,68] In some specific cases, proteolytic capacity has been measured among members of these microbial species.[20,69–76] Cheese-specific details of peptide formation and microbial proteolysis are discussed in the following sections.

Taleggio. Taleggio is a soft Italian cheese produced with raw bovine milk, thermophilic starter lactic acid bacteria (1:1 *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) and rennet, and ripened in humid conditions that allow the growth of yeast (typically *Torulopsis*, *Saccharomyces*, *Debaryomyces* and *Hansenula*) and molds (typically *Mucor*, *Penicillium*, *Aspergillus* and *Geotrichum*) on the cheese exterior.[40,42] A total of 3,520 unique

peptides were identified in Taleggio. These peptides were likely produced by a combination of proteolysis from native milk enzymes, starter culture enzymes (both *S. thermophilus* and *L. bulgaricus* are proteolytic[69]), rennet and the exterior yeast and molds. Despite this cheese's overall lower total peptide count in the rind (1299 vs. 2730 in the interior), the rind showed an increase in β-lactoglobulin-derived sequences (44 in the rind vs. 4 in the interior). This finding is noteworthy for two reasons: first, it suggests that some intact β-lactoglobulin, or substantial portions of the protein, are present in the cheese despite its typical characterization as a whey component. Second, β-lactoglobulin is known for its resistance to digestive enzymes,[77,78] and the increase in unique sequences in the cheese rind indicates that the molds and yeast present on the cheese exterior possess different digestive specificities than the proteases in bovine milk, the starter cultures and rennet. The development of the rind microbial community and its corresponding proteases during ripening could therefore be a key contributor to β-lactoglobulin peptide formation. Taleggio is a washed-rind cheese, meaning that the exterior of the cheese is periodically washed with a salt solution during ripening. This washing may affect the peptide profile of the rind by eliminating some water-soluble peptide sequences, and it also likely shapes the rind's bacterial community,[29] which in turn could influence proteolysis. After 21 days of ripening, Gobbetti et. al. found proteolytic activity to be higher at the cheese surface than in the core.[40] Therefore, the lower number of peptides present in the rind observed herein could be due to the further degradation of peptides to lengths less than five amino acids, which are not detected by the database searching methodology used. Interestingly, Taleggio is unique among the four cheeses for having an edible rind,[79] which implies that this cheese provides the greatest diversity of peptide sequences to the consumer despite its short ripening time.

Numerous Taleggio peptides matched with known ACE-inhibitory sequences (35 peptides in total). Among other bioactive peptides, Taleggio also contained 23 antimicrobial peptides and the suspected anxiolytic peptide α_{s1}-casein(106-115), also known as α-casozepine.[80] α-casozepine has affinity for the γ-amino-butyric acid type A receptor[80] and may be responsible for the observed anxiolytic effects of oral consumption of α_{s1}-casein hydrolysates.[81–83] Further *in vivo* studies are needed to determine whether the bioactive peptides in Taleggio can survive across the consumer's digestive system to act in the gut (antimicrobial) or be absorbed into the bloodstream to act systemically (antihypertensive and anxiolytic). The complete list of functional peptides found in each cheese is provided as Supplementary Table S13.

Stilton blue. Stilton blue cheese, originating in the United Kingdom, is a semi-soft cheese produced with pasteurized cow's milk, *Lactococcus lactis* starter culture and rennet and ripened with added mold (*Penicillium roqueforti*), which is spread throughout the cheese interior with specialized rods.[42,66,84] Stilton contained 3,408 total peptides, with 2,998 in the interior and 557 in the rind. Stilton was unique for having both the greatest number of peptide sequences in the cheese interior and simultaneously the lowest number of sequences on the exterior. Only 147 peptides were identified in both the interior and rind, indicating substantial differences in the factors influencing proteolysis at each location. As with Taleggio, the peptide counts from specific whey proteins were elevated in the cheese rind, particularly from xanthine oxidase (26 sequences in the rind vs. 2 sequences in the interior). The highly distinct profiles observed in the interior and exterior could be due to the fact that *P. roqueforti* is encouraged, through processing, to move from the outside to the interior of the cheese by the repeated process of inserting and removing metal rods during aging, allowing the development of mold spores into hyphae.[85] Studies examining the bacterial and yeast profiles of Stilton have identified substantial

differences in microbial composition between the exterior and interior of the cheese, with the interior and veins of Stilton being dominated by *P. roqueforti, Lactococcus lactis* with some *Leuconostoc* and possibly some *Lactobacillus curvatus* and the rind containing very little *P. roqueforti* or *L. lactis* but a predominant amount of a variety of yeasts (including *Debaryomyces hansenii, Yarrowia lipolytica, Candida catenulata* and *Kluyveromyces lactis*)[66,68] and unidentified cocci with some *Lactobacillus plantarum* and *Leuconostoc* just under the crust.[68] The large differences in peptide profiles between the rind and interior aligns with the previous findings that these regions differed greatly in their microbial communities, which likely leads to differing proteolytic capacity. Past studies have established that Stilton blue, and blue cheeses in general, typically have quite high degrees of proteolysis,[86] likely owing to the summative effect of the multitude of microorganisms it contains, as summarized below. For example, the blue mold *P. roqueforti* alone can cleave α_{s1}- and β-casein each in over 90 locations.[70] *Lactococcus lactis,* a starter species present in Stilton's interior, produces cell wall proteases known as P_I- or P_{III}-type proteinases (PrtPs). These proteases can cleave β-casein (P_I-type) or α_{s1}-, β-, and κ-caseins (P_{III}-type) from bovine milk and therefore could contribute to peptide formation.[71,72] Most strains of *D. hansenii* have not shown proteolytic activity on milk proteins, but at least one has,[73] as have *Kluyveromyces lactis,*[74] *Candida catenulate,*[75] and 8 known strains of *Y. lipolytica*.[76]

The functional peptide profile of Stilton was similar to that of Taleggio, with the majority of homologous sequences possessing ACE-inhibitory (24 peptides) or antimicrobial (14 peptides) properties. This cheese also contained one immunomodulatory sequence, β-casein(208-224), which stimulates mouse macrophage phagocytosis *in vitro* and thus could help prevent infections.[87] Again, whether these bioactive peptides survive across the consumer gut to act

within it as antimicrobials or to modulate macrophage activity or are absorbed to act systemically as antihypertensives remains unknown.

Casu Marzu. Casu Marzu is a typical sheep's milk cheese produced by deliberate inoculation of Italian Pecorino cheese, produced with either raw or pasteurized milk, with larvae from the fly *Piophila casei*, a species that infests cheeses and meats.[41,88,89] The initial Pecorino cheese is produced from raw sheep's milk using a starter culture and rennet.[90] Sardinian Pecorino, the type of Pecorino often used to make Casu Marzu, contains a variety of yeasts[91] as well as enterococci[92] and numerous lactic acid bacterial species.[93] Furthermore, the use of lamb rennet for milk coagulation is a unique feature of Sardinian Pecorino. In comparison to calf rennet, lamb rennet has shown increased proteolytic activity[94] and therefore may have contributed to a relative increase in the peptide content of Casu Marzu. A total of 2,933 peptides were found in Casu Marzu. A unique feature of this cheese was the presence of serum amyloid A peptides (57 sequences total) in both regions of the cheese. Serum amyloid A is involved in a variety of immunological and inflammatory processes[95] and has been reported in past proteomic analyses of sheep milk and colostrum,[96,97] as well as bovine milk.[98] The presence of peptides from serum amyloid A in Casu Marzu but not in the other cheeses could be due to the unique proteolytic activity of *P. casei*. Other noteworthy findings included 23 sequences matched to a protein whose sequence is predicted to originate from the GLYCAM1 gene,[99] which codes for glycosylation-dependent cell adhesion molecule 1 in cows.[100,101] Although the current ovine Uniprot sequence is predicted from DNA rather than from identification of the protein, peptides originating from GLYCAM1 have been identified in past studies of the sheep milk proteome,[96,97,102] and identification of 23 peptide sequences in our own MS/MS data provides strong evidence that the protein, or its fragments, exists in Casu Marzu. The production of

peptides in Casu Marzu are likely due to a combination of the added rennet, native milk proteases, the proteases of microorganisms present in the original unheated milk and the proteolysis contributed by *P. casei*.[90] A past study observed that the amount of pH 4.6-soluble nitrogen more than doubled in Casu Marzu when compared to standard Pecorino cheese incubated without *P. casei,* indicating that the presence of these flies has a substantial impact on proteolysis.[89] The peptide profiles also differed considerably between the interior and rind of Casu Marzu, likely due to differences in the microbial composition at these sites. No research has compared the microbial composition of the rind and interior of Casu Marzu.

Casu Marzu contained 18 ACE-inhibitory peptides and 14 antimicrobial peptides, mainly derived from caseins and β-lactoglobulin. An interesting finding was the presence of β-casein(199-217), which possesses a unique anti-inflammatory function that operates independently of the ACE-inhibition mechanism often seen with other casein-derived peptides.[103] This peptide reduces the tumor necrosis factor-alpha-induced activation of the pro-inflammatory transcription factor NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) in human embryonic kidney cells.

Mimolette. Mimolette is a hard cheese produced from pasteurized bovine milk treated with lactic acid bacteria starter culture and rennet in France.[104] Its rind contains live cheese mites (*Acarus siro*), which we hypothesized would contribute to unique rind proteolysis. A total of 4,701 peptides were identified in the cheese, with substantially more sequences in the rind (3,336 peptides in the rind vs. 2,628 in the interior). This cheese had a higher number of peptides originating from the antimicrobial protein lactoferrin than the other cheeses examined. One hundred seventeen peptides from lactoferrin were identified, with all but one of those originating exclusively from the rind of the cheese. This enhanced proteolysis of lactoferrin may therefore

be the result of unique enzyme activity existing only on the rind of the cheese, originating either from the cheese mites or rind-specific microbial communities. As with Casu Marzu and Taleggio, Mimolette showed a greater number of peptide sequences from β-lactoglobulin in the rind than the interior (71 vs. 11, respectively). The microbial profile of Mimolette and whether this profile differs between the rind and the interior has not been examined.

A total of 110 bioactive peptide sequences were identified in Mimolette. As with the other cheeses, most of these peptides were ACE-inhibitory and antimicrobial. However, Mimolette contained a much greater number of peptides with these functionalities than the other cheeses. Casu Marzu, Stilton, and Taleggio contained similar numbers of ACE-inhibitory and antimicrobial peptides to that found in bovine milk by a previous study using similar techniques,[105] while Mimolette contained nearly double the number of sequences for each function. Mimolette also contained several antioxidant, opioid, and dipeptidyl-peptidase 4 inhibitory sequences. Dipeptidyl-peptidase 4 indirectly affects blood glucose levels by degrading hormones that promote insulin release: therefore dipeptidyl-peptidase 4 inhibitors can improve glucose tolerance.[106] A unique finding in this cheese was the presence of the hypocholesterolemic peptide β-lactoglobulin(57-76), which directly inhibits cholesterol absorption across Caco-2 cells and lowered serum cholesterol after feeding in rats.[3]

Degree of protein hydrolysis. To complement the peptide data obtained by mass spectrometry, an OPA assay was used to calculate the relative degree of protein hydrolysis in each cheese. Numerous strategies have been developed to measure cheese proteolysis, as reviewed previously.[28,107] In this case, we have measured the concentration of pH 4.6-soluble peptides and normalized the measurements to the total cheese nitrogen content to allow a comparison of the degree of proteolysis between different types of cheeses. As mentioned above, typical proteomic

database search algorithms suffer from limitations in their ability to identify peptides shorter than 5 amino acids. Furthermore, while proteomic data provides measures of relative abundance for each identified peptide, comparing total peptide abundance between samples is not always straightforward with proteomic data. In this study, we have measured the molar content of peptides per gram of total cheese protein in order to compare total peptide abundances more directly. As shown in Table 2, Stilton blue had the greatest degree of hydrolysis, with 1.93 ± 0.10 mmol Ser equivalents per g protein, closely followed by Casu Marzu at 1.74 ± 0.02 mmol per g protein. Interestingly, these two cheeses had lower peptide counts than Mimolette and Taleggio, indicating that there may be additional, smaller peptides in Stilton and Casu Marzu that are not accounted for by the peptidomic data, or that the sequences that were identified are present at relatively high concentration. Blue cheeses, such as the Stilton used in this study, have been similarly characterized by other research groups to have a high degree of proteolysis.[70,86] Although the OPA assay was conducted with the cheese interiors, the high degree of hydrolysis in Stilton may somewhat explain the relatively low number of peptides identified in the rind of this cheese, as the degree of hydrolysis may be the result of a high concentration of smaller peptides in this region. The total quantity of peptide sequences identified in Taleggio was initially surprising, considering that this cheese undergoes a much shorter ripening than the others (Table 1). However, the degree of hydrolysis data illustrates that the proteins in Taleggio are quantitatively less hydrolyzed than those of the other cheeses, consistent with what is expected of its shorter ripening time.

CONCLUSION

Modern peptidomics techniques and instrumentation are allowing food-derived peptides to be profiled at a much greater depth than was previously possible. We have determined that thousands of peptides are formed in cheeses during cheesemaking and ripening through proteolysis of milk proteins by a combination of rennet, native milk proteases, starter cultures, non-starter cultures and secondary cultures, and that many of these sequences are potentially bioactive. Furthermore, we showed that technological production methods and inoculation with various microorganisms influences protein digestion, as the interior and rind of each cheese differed in their peptidomic profile. Clearly, specific cheeses provide particular sets of functional peptides to the consumer. These findings raise obvious questions as to the therapeutic potential of cheese-derived peptides for enhancing gut health, reducing blood pressure and modulating inflammation. A key question remaining is whether these bioactive cheese peptides will survive intact upon exposure to the proteases of the digestive system to their site of action within the consumer (whether within the gut or systemically after absorption) and thus have potential to be biologically relevant. Although further research is needed to determine whether these released bioactive peptides are functional within the consumer, the current literature demonstrates that numerous bioactive peptides can be successfully used in therapeutic applications.[108,109]

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1. Total number of peptide sequences identified by LC-Orbitrap MS/MS. Casein-derived peptides are grouped by protein of origin. Although each cheese contained several hundred sequences from non-casein proteins, the caseins contributed the majority of peptide sequences.

Figure 2. Counts of ACE-inhibitory, antimicrobial, and antioxidant peptide sequences identified in each cheese by homology search against the Milk Bioactive Peptides Database.

Figure 3. Comparison of the number of peptide sequences identified in the rind and interior of each cheese. In each case, the substantial quantity of sequences that were unique to either the rind or interior suggests that different factors influence proteolysis in each cheese region.

Figure 4. Number of unique peptide sequences identified in the proteins that made the greatest contributions to the cheese peptidome. The interior and rind peptides are plotted separately for

each cheese. BT1A1, butyrophilin subfamily 1 member A1; PIGR, polymeric immunoglobulin receptor.

TABLES

Table 1. Information on the composition and traditional production methods of the cheeses used for peptide profiling. Cheese pictures are reproduced under the terms of their respective Creative Commons licenses.[110–113]

Cheese	Casu Marzu	Mimolette	Stilton	Taleggio
Type of milk used	Sheep	Cow	Cow	Cow
Raw or pasteurized milk	Raw or pasteurized	Pasteurized	Pasteurized	Raw
Area of production	Italy (Sardinia)	France	United Kingdom	Northern Italy
Type of coagulation	Rennet	Rennet	Rennet	Acid and rennet
Texture	Hard	Hard	Soft	Soft
Secondary microorganisms	*Piophila casei*	*Acarus siro*	*Penicillium roqueforti*	Yeasts and molds
Average ripening time	>8 months for Pecorino, plus 2-3 months after *P. casei* inoculation	6-12 months	6 months	1 month
Average wheel mass	2–4 kg	3.3 kg	4.5 kg	2.2 kg
European Union status	Traditional product of the Sardinia Region	Protected Designation of Origin	Protected Designation of Origin	Protected Designation of Origin
Representative photo				

Table 2. Degree of protein hydrolysis in each cheese. Values represent the average ± standard deviation of duplicate measurements.

Cheese	Degree of hydrolysis (mmol Ser equivalents/g cheese)
Mimolette	1.09 ± 0.03
Casu Marzu	1.74 ± 0.02
Stilton	1.93 ± 0.10
Taleggio	0.77 ± 0.02

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FIGURE LEGENDS

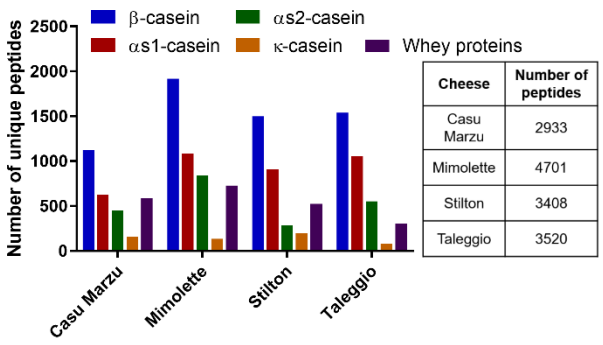
Figure 1. Total number of peptide sequences identified by LC-Orbitrap MS/MS. Casein-derived peptides are grouped by protein of origin. Although each cheese contained several hundred sequences from non-casein proteins, the caseins contributed the majority of peptide sequences.

Figure 2. Counts of ACE-inhibitory, antimicrobial, and antioxidant peptide sequences identified in each cheese by homology search against the Milk Bioactive Peptides Database.

Figure 3. Comparison of the number of peptide sequences identified in the rind and interior of each cheese. In each case, the substantial quantity of sequences that were unique to either the rind or interior suggests that different factors influence proteolysis in each cheese region.

Figure 4. Number of unique peptide sequences identified in the proteins that made the greatest contributions to the cheese peptidome. The interior and rind peptides are plotted separately for each cheese. BT1A1, butyrophilin subfamily 1 member A1; PIGR, polymeric immunoglobulin receptor.

FIGURES



Cheese	Number of peptides
Casu Marzu	2933
Mimolette	4701
Stilton	3408
Taleggio	3520

Figure 1

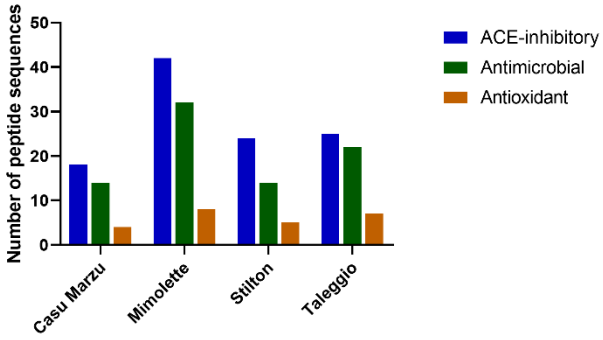


Figure 2

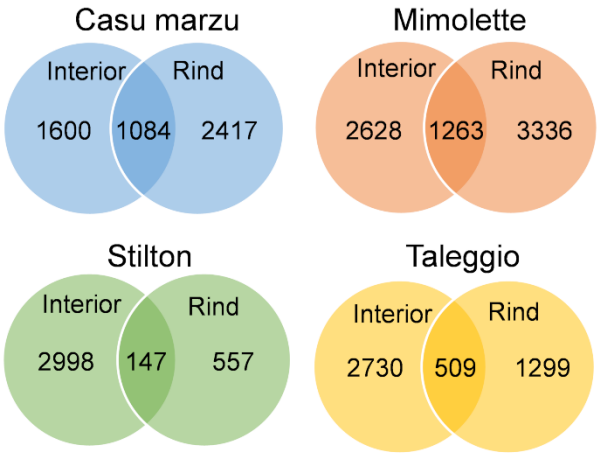


Figure 3

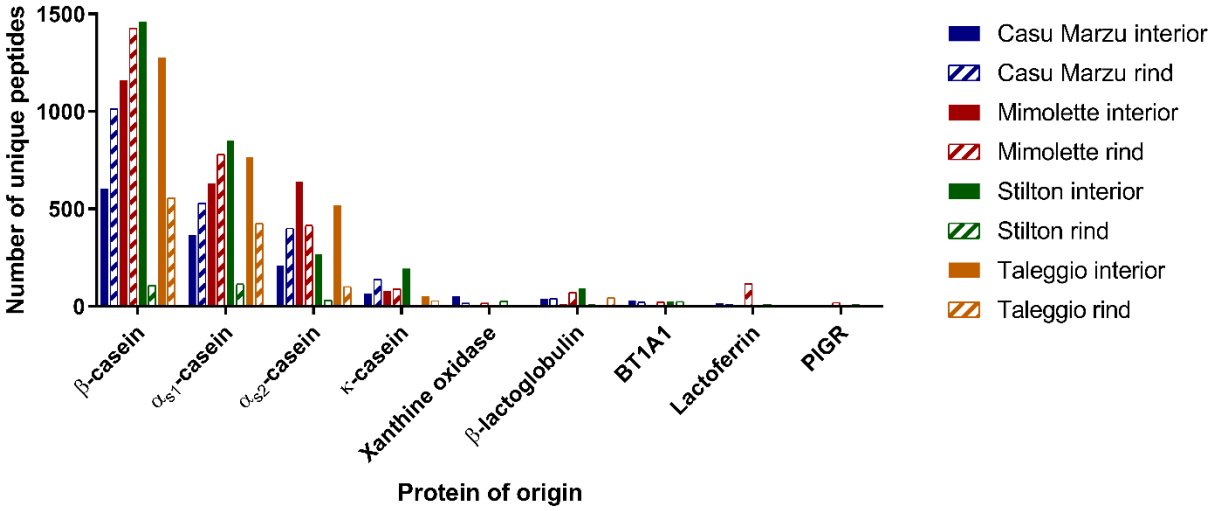
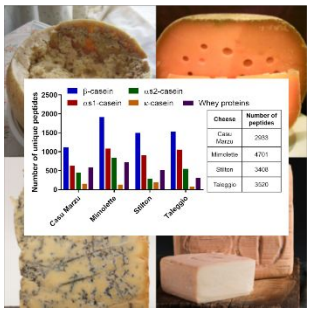


Figure 4



Peptide sequences identified in four cheeses by LC-Orbitrap MS/MS