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Bioinformatic Analysis of Changes in the Peptide Profile of Dairy Proteins During Storage

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ABSTRACT

Introduction: Enzymatic processes occurring in dairy products during storage can lead to changes in protein composition, affecting products' quality. Key players in these changes include endogenous enzymes, such as plasmin, and bacterial proteases like the heat stable protease from *Pseudomonas* LBSA1. The application of bioinformatic methods enables the modeling of protein hydrolysis and prediction of peptide formation with specific properties (e.g., organoleptic characteristics, bioactivity, molecular weight, amino acid sequence).

Purpose: To evaluate changes in the peptide profiles of β-CN, αs1-CN, αs2-CN, and κ-CN caseins during simulated hydrolysis by plasmin and the heat stable bacterial protease *Pseudomonas* LBSA1.

Materials and Methods: Casein sequences were analyzed using the UniProt database. Hydrolysis was modeled using BIOPEP-UWM (for plasmin) and regular expressions in RStudio (for *Pseudomonas* LBSA1). The degree of hydrolysis (DH) was calculated as the ratio of cleaved peptide bonds to the total possible bonds in the protein. Peptide sequences were analyzed using the "stringr" library in RStudio. Bitter and antioxidant peptides were identified using the BIOPEP-UWM database. Molecular weight and isoelectric point data were obtained via the "Peptides" library in RStudio.

Results: 2D diagrams revealed distinct distributions of peptides based on molecular weight and isoelectric point, dependent on enzyme specificity. In the combined hydrolysis model, 4 bitter peptides, 3 types of bitter amino acids, and 6 antioxidant peptides were identified.

Conclusion: Bioinformatic modeling enables the prediction of enzymatic changes in milk proteins during storage, their impact on quality, and enhances the efficiency of related experiments. These findings may support the development of approaches for assessing dairy product storage conditions and identifying quality markers.

Keywords: bioinformatics; milk proteins; peptide profile; storage of dairy products; enzymatic spoilage

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ОРИГИНАЛЬНОЕ ЭМПИРИЧЕСКОЕ ИССЛЕДОВАНИЕ

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Биоинформатический анализ изменений пептидного профиля молочных белков при хранении

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АННОТАЦИЯ

Введение: Ферментативные процессы, происходящие в молочных продуктах при хранении, могут приводить к изменениям белкового состава, что влияет на их качество. Ключевую роль в этих изменениях играют как эндогенные ферменты, такие как плазмин, так и бактериальные протеазы. Применение биоинформатических методов позволяет моделировать гидролиз белков и прогнозировать образование пептидов со специфическими свойствами (с конкретными органолептическими характеристиками, биологической активностью, молекулярной массой, аминокислотной последовательностью и др.).

Цель: Оценить изменения пептидного профиля казеинов β-CN, αs1-CN, αs2-CN и κ-CN при моделировании их гидролиза плазмином и термостабильной бактериальной протеазой *Pseudomonas* LBSA1.

Материалы и методы: Анализ последовательностей казеинов проводили с использованием базы данных UniProt. Гидролиз моделировали в BIOPEP-UWM (для плазмина) и с помощью регулярных выражений в RStudio (для *Pseudomonas* LBSA1). Степень гидролиза (DH) рассчитывали на основе количества разорванных пептидных связей в отношении к общему числу возможных связей в белковой молекуле. Для анализа пептидных последовательностей применяли библиотеку "stringr" в RStudio. Горькие и антиоксидантные пептиды выявляли с использованием базы данных BIOPEP-UWM. Данные о молекулярной массе и изоэлектрической точке полученных пептидов извлекали с помощью библиотеки "Peptides" в RStudio.

Результаты: Результаты 2D-диаграмм показали различия в распределении пептидов по молекулярной массе и изоэлектрической точке в зависимости от специфичности ферментов. В комбинированной модели гидролиза идентифицировано 4 горьких пептида и 3 вида горьких аминокислот, а также 6 антиоксидантных пептидов.

Выводы: Биоинформатическое моделирование позволяет прогнозировать ферментативные изменения белков в молочных продуктах, их влияние на качество, а также повышать эффективность проводимых в этом поле экспериментов. Полученные данные могут использоваться для разработки подходов к оценке хранения молочных продуктов и идентификации маркеров качества.

Ключевые слова: биоинформатика; молочные белки; пептидный профиль; хранение молочных продуктов; ферментативная порча

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INTRODUCTION

Ensuring the quality of dairy products over a long period of time remains a relevant challenge for both science and industry (Muir, 2011; Fan et al., 2023). Various chemical, biochemical and microbiological processes can degrade the components of dairy products and cause defects in flavor, consistency, etc. For example, Crudden et al. (2005) highlighted that plasmin activity in raw milk is responsible for influencing subsequent coagulation with rennet during the production of cheese and cottage cheese, as well as affecting cheese yield and the thickening of UHT milk. This finding is supported by Chavan et al. (2011). Chemical and biochemical spoilage is typically activated at elevated storage temperatures, accelerating processes such as lipid oxidation, the Maillard reaction, and enzymatic activity (Fox et al., 2015; Lu & Wang, 2017; Fan et al., 2023). Conversely, microbiological spoilage can begin at low temperatures, as psychrotrophic bacteria sustain activity and produce extracellular heat stable enzymes. Moreover, these enzymes, like plasmin, remain active even after pasteurization and ultrahigh temperature processing, making them key factors in milk spoilage during storage due to protein degradation, primarily caseins (Muir, 2011; Fox et al., 2015). Endogenous enzymes such as plasmin are initially present in milk. Their activity is regulated by a balance of activators (serine proteases such as tissue and urokinase plasminogen activators) and inhibitors (plasma proteins, such as plasminogen activator inhibitors PAI-1 and PAI-2 and α2-antiplasmin, which binds plasmin explicitly). In contrast, exogenous enzymes produced by microorganisms are more resistant to heat treatment and often have a broad specificity (Fox et al., 2015). Regarding plasmin's negative impact on milk quality, Chavan et al. (2011) also emphasized that heat processing alters the balance of enzyme's activators and inhibitors in favor of increased activators, thereby initiating degradation processes.

Modern proteomics and bioinformatics methodologies facilitate the efficient modeling of proteolysis and the comprehensive analysis of its effects on protein properties. Tools such as BIOPEP UWM, PeptideRanker, AllerTOP, and ToxinPred are utilized to model protein hydrolysis by different enzymes, predict the formation of bioactive peptides, and evaluate their sensory properties, allergenicity, and toxicity (Pooja et al., 2017; Zhang et al., 2020; Kruchinin & Bolshakova, 2022). These methods optimize experimental design and reduce labor costs, making hypothesis testing more efficient. For example, Kruchinin et al. (2023) utilized *in silico* approaches to optimize bioactive peptides production from whey, minimizing the need for extensive laboratory work. Similar approaches have been implemented by other researchers for a wide range of bioactive peptides substrates (Panjaitan et al., 2018; Kartal et al., 2020; Iram et al., 2022). A review by Barati et al. (2024) highlighted the use of *in silico* methods for enzyme immobilization, and specific bioinformatics applications including software for molecular dynamics simulation, metal ion binding site prediction servers, and methods for enzyme structure and surface analysis. However, *in silico* methods rarely applied to predict food spoilage and assess safety. Recent studies in this area have examined a limited range of food safety and spoilage aspects. Some research focused on bacterial communities and their metabolic activities through genomic and metagenomic analyses (Remenant et al., 2015). Other studies have investigated enzymes that can degrade aflatoxins in food, a significant safety concern (Dellafiora et al., 2017). Additionally, the migration of packaging substances in milk and their interaction with digestive enzymes was assessed (Xiong et al., 2024). Finally, *Pseudomonas* spp. were studied for their role in milk spoilage, with predictions made about their mechanisms and strategies for prevention using quorum-sensing inhibitors via *in silico* methods (Quintieri et al., 2021).

The implementation of *in silico* approaches to study dairy products spoilage is complex, largely due to the need for the identification of specific enzymes involved, especially the heat stable enzymes produced by psychrotrophic bacteria. Accurate inclusion of these enzymes in bioinformatics databases is challenging because of the limited data on their specificity, which complicates their classification and modeling. However, some studies have attempted to characterize these enzymes. For instance, Yan et al. (1985) classified extracellular proteases from *Bacillus coagulans, Bacillus* sp., *Bacillus subtilis*, and *Pseudomonas fluorescens* as metalloproteinases. Notably, the protease from *Pseudomonas fluorescens* also demonstrated trypsin-like activity. Baur et al. (2015) highlighted the role of *Pseudomonas* spp. as significant producers of heat stable proteases through the analysis of 231 strains isolated from raw milk. Additionally, Matéos et al. (2015) identified an extracellular protease from *Pseudomonas* LBSA1, also classified as a metalloproteinase from the serralysin family, which specifically targeted caseins (αs1, β-, and κ-CN). This protease showed a preference for cleaving at the N-terminus after amino acids such as arginine and lysine and at the C-terminus before amino acids like valine and methionine.

Thus, this study aimed to develop an approach that combines *in silico* modeling with experimental data to study the spoilage processes of dairy products. In this research, the hydrolysis of caseins (αs1-, αs2-, β-, and κ-CN) by one of the identified proteases with the described specificity (from *Pseudomonas* LBSA1) as well as plasmin was carried out. The modeling of casein hydrolysis by plasmin was performed using BIOPEP-UWM. In contrast, the hydrolysis by the heat stable protease from *Pseudomonas* LBSA1 was modeled by automated identification of suitable amino acid sites in RStudio. The molecular masses, isoelectric points, and the bitterness and antioxidant activity of the resulting hydrolysis products were determined in this study.

MATERIALS AND METHODS

Proteins Under Study

The protein sequences were obtained from the UniProt database. The signal peptides were excluded from the analyzed sequences. The UniProt accession numbers for the proteins under study are αs1-CN — P02662, αs2-CN — P02663, β-CN — P02666, and κ-CN — P02668.

Hydrolysis Modeling

Hydrolysis of β-CN and αs2-CN by plasmin (EC 3.4.21.7) was modeled using the analytical tool "enzyme(s) action" from the BIOPEP-UWM database. Hydrolysis of β-CN, αs1-CN, and κ-CN by the bacterial protease from *Pseudomonas* LBSA1 (Matéos et al., 2015) was modeled in RStudio using the "stringr" library to account for the enzyme's specificity to the P1- and P1'-positions.

Degree of Hydrolysis (DH) Calculation

The degree of hydrolysis (DH) was determined based on the number of peptides formed and the primary protein sequence. The formula used for the calculation was:

$$DH = \frac{d}{N} \times 100\,\%, \quad (1)$$

where d — the number of peptide bonds cleaved by the enzyme; N — the total number of amino acid residues in the primary sequence.

DH analysis was performed in RStudio, using regular expressions to identify the peptide bond cleavages and compute their count. Data processing and DH calculations were carried out separately for each enzyme and protein.

Analysis of Peptides' Properties

The identification of bitter and antioxidant peptides in the hydrolysis model's peptide list was done using the BIOPEP-UWM database. Molecular mass and isoelectric point data for the peptides were extracted using the "Peptides" library in RStudio. A complete list of hydrolysis products, including their molecular masses and isoelectric points, was compiled into a separate table and is presented in the supplementary materials of the paper (Appendix 1).

Data Visualization

2D histograms (heatmaps) and scatter plots were generated in RStudio using the ggplot2 library to represent the hydrolysis results and peptide characteristics visually. The 2D histogram illustrates the distribution of peptides by molecular mass and isoelectric point. It is based on counting the number of peptides within each histogram cell and was implemented using the "geom_hex" function.

RESULTS

Hydrolysis Modeling by Plasmin

Mostly, hydrolysis products have a molecular weight of up to 2000 Da; however, there are also fragments in the range of 2000 to 3000 Da, as well as a few products with molecular weights between 5000 and 6500 Da (Figure 1).

In the combined hydrolysis model of β-CN and αs2-CN, 8 bitter peptides and 2 types of amino acids were released, along with 5 peptides with annotated antioxidant activity (Table 1). The degree of hydrolysis (DH) for αs2-CN was 14.6 %, while for β-CN, it was 7.2 %.

Hydrolysis Modeling by Bacterial Protease

Unlike the hydrolysis products in the model with plasmin, which are mainly concentrated in the pI range of 9–11, those produced by bacterial protease show a broader distribution across the isoelectric point (pI) spectrum, spanning from 5 to 12. Additionally, most of the hydrolysis products are predominantly found within the molecular

Figure 1

Distribution of Hydrolysis Products Generated from αs2-CN and β-CN by Plasmin in the Model

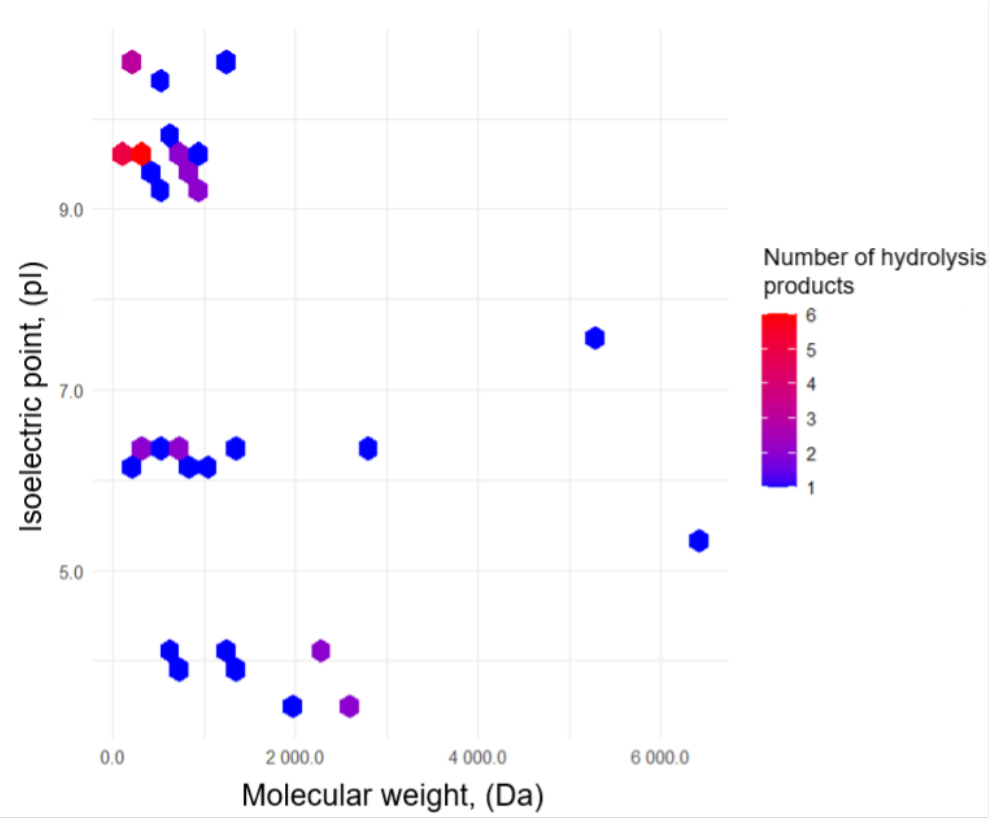

Table 1

Properties of hydrolysis products generated from αs2-CN and β-CN by plasmin in the model

Protein	Peptides and amino acids	Bitterness	Antioxidant activity	Number of hydrolysis products
αs2-CN, β-CN	K	+		5
	R	+		2
αs2-CN	FALPQYLK	+	+	1
	YL	+	+	1
	YQK		+	1
β-CN	AVPYPQR		+	1
	GPFPIIV	+		1
	VLPVPQK		+	1

weight range of up to 2000 Da, with a small number of fragments in the 2000–3000 Da range and a few peptides exceeding 5000 Da (Figure 2).

In the hydrolysis model of αs1-CN, β-CN, and κ-CN, 1 bitter peptide and 1 type of amino acid with a bitter taste were released, along with 3 peptides with annotated antioxidant activity (Table 2). The degree of hydrolysis (DH) for αs1-CN was 10.1 %, for β-CN, it was 4.8 %, and for κ-CN, it was 6.6 %.

Figure 2

Distribution of Hydrolysis Products Generated from αs1-CN, β-CN, and κ-CN by Bacterial Protease in the Model

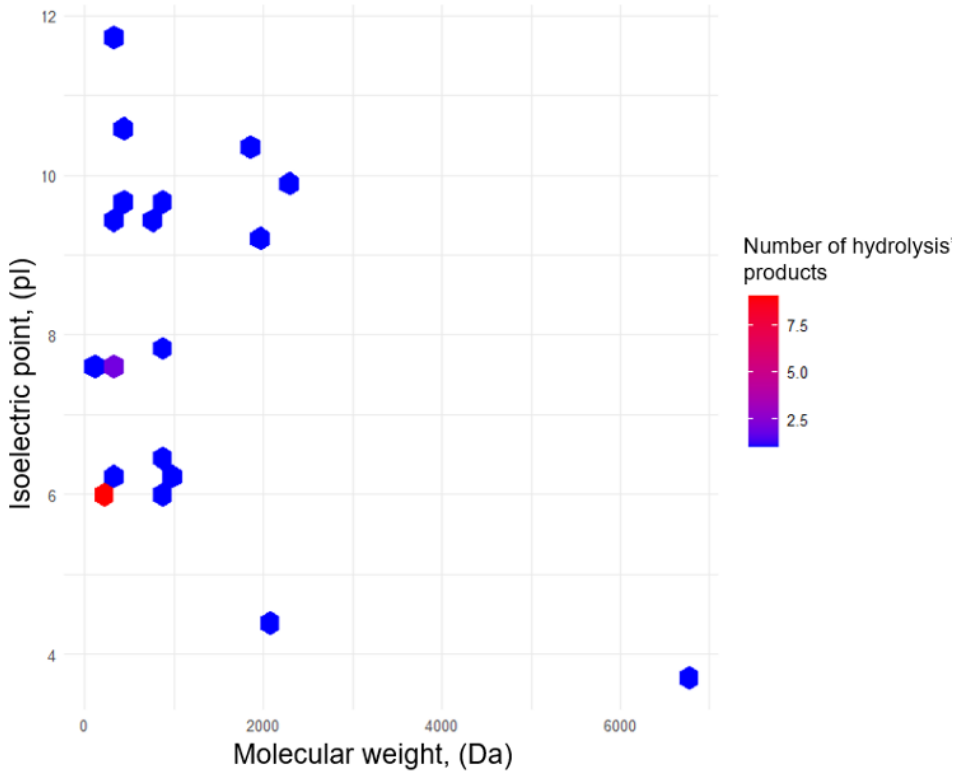

Table 2

Properties of Hydrolysis Products Generated from αs1-CN, β-CN, and κ-CN by Bacterial Protease in the Model

Protein	Peptides and amino acids	Bitterness	Antioxidant activity	Number of hydrolysis products
αs1-CN, β-CN, κ-CN	F	+		5
αs1-CN	HIQKEDVPSER		+	1
β-CN	VKEAMAPK	+	+	1
κ-CN	HPHPHLSF		+	1
	YIPIQY		+	1

Combined Hydrolysis Modeling

The specificity of plasmin partially overlaps with the specificity of the bacterial protease from *Pseudomonas* LBSA1, as both enzymes cleave peptide bonds after arginine (R) and lysine (K) residues. However, the bacterial protease imposes additional restrictions, requiring specific amino acids in the P1'-position. These differences are reflected in the distribution of peptides by isoelectric point and molecular weight (Figure 3).

Combined hydrolysis with plasmin and bacterial protease leads the formation of the maximum number of hydrolysis products with the widest range of isoelectric points (pI

Figure 3

Distribution of Hydrolysis Products from Combined Action of Plasmin on αs2-CN and β-CN, and Bacterial Protease from *Pseudomonas* LBSA1 on αs1-CN, β-CN, and κ-CN

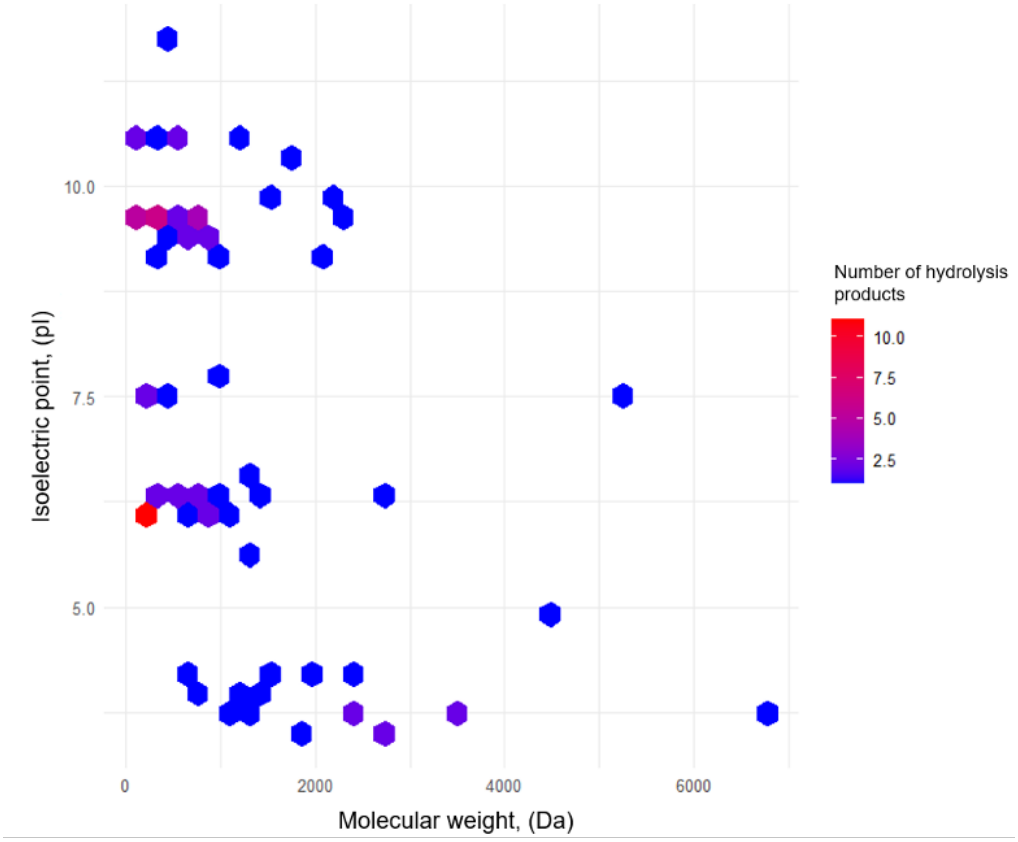

Table 3

Properties of Hydrolysis Products Generated by Combined Plasmin and Bacterial Protease Action

Protein	Peptides and amino acids	Bitterness	Antioxidant activity	Number of hydrolysis products
αs1-CN, β-CN, κ-CN	F	+		5
αs2-CN, β-CN	K	+		5
	R	+		2
αs1-CN	HIQKEDVPSER		+	1
αs2-CN	FALPQYLK	+	+	1
	YL	+	+	1
β-CN	VKEAMAPK	+	+	1
	GPFPIIV	+		1
κ-CN	HPHPHLSF		+	1
	YIPIQY		+	1

from 2.5 to 12.5). This modeling also results in the most variable distribution of hydrolysis products by molecular weight, covering the ranges up to 2000 Da, 2000–4000 Da, and 5000–6500 Da. The obtained results demonstrate that the synergistic action of the enzymes promotes the formation of a more complex and diverse peptide profile, driven by differences in enzyme substrate specificity and a higher degree of hydrolysis.

In the combined hydrolysis model of all four proteins — which includes the cleavage of αs2-CN and β-CN by plasmin, as well as αs1-casein, β-CN, and κ-CN by the bacterial protease from *Pseudomonas* LBSA1 — three types of amino acids and four peptides with a bitter taste were released, along with six peptides annotated with antioxidant activity (Table 3).

DISCUSSION

Predicting changes in milk proteins during storage is an important challenge in food science, as their degradation affects the texture, taste, and nutritional value of products. Early studies by Chavan et al. (2011), Dalabasmaz et al. (2019), and Class et al. (2024) have noted that plasmin and bacterial proteases play a key role in these processes. However, most research is based on experimental data, while bioinformatic methods remain less explored in this context.

The results of the modeling conducted in this study correlate with previously reported empirical findings, supporting the feasibility of applying a bioinformatic approach to the prediction and analysis of enzymatic spoilage in food products. For instance, the peptides FALPQYLK and AVPYPQR identified in the hydrolysis model of αs2-CN and β-CN by plasmin were also described in the study by Nath et al. (2022) in the context of casein cleavage by trypsin, which has similar specificity. The authors also highlighted the bitterness and antioxidant properties of these peptides, which were likewise detected through modeling in the present study. According to Nath et al. (2022), the bitterness and antioxidant activity of peptides are mainly associated with the hydrophobicity of their terminal amino acids.

Other peptides (EAMAPK, EMPFPK) and the antioxidant peptide AVPYPQR identified in the plasmin hydrolysis model were also reported by Sedaghati et al. (2016). For AVPYPQR, the authors determined antibacterial activity with a minimum inhibitory concentration (MIC) value of 40 mg/mL against *Escherichia coli*, which was not assessed in the present study.

The hydrolysis products identified as a result of hydrolysis modeling with the bacterial protease from *Pseudomonas* LBSA1 — specifically, HIQKEDVPSER and HPIK — also overlap with data from other studies (Gupta et al., 2010; Stuknytė et al., 2016; Nath et al., 2022). Notably, Stuknytė et al. (2016) investigated the proteolytic activity of heat stable enzymes from *Pseudomonas fluorescens* PS19, while Gupta et al. (2010) analyzed antioxidant peptides in cheese. Verhegghe et al. (2021) studied the hydrolysis of milk proteins by six bacterial strains — *Pseudomonas fluorescens* MB4999, *Pseudomonas* sp. MB4988, *Pseudomonas fluorescens* MB5000, *Pseudomonas* sp. MB4996, *Pseudomonas lundensis* MB4984, and *Pseudomonas fragi* MB4972 — and identified peptides such as EPVLGPVR, VSKVKEAM, and GPFPIIV.

At the same time, although the protease from *Pseudomonas* LBSA1 has shown high amino acid sequence similarity (93 %) to the protease from *P. fluorescens* F as reported by Mateos et al. (2015), the marker peptides mentioned above (EPVLGPVR, VSKVKEAM, and GPFPIIV) were not detected in our hydrolysis model with *Pseudomonas* LBSA1 protease. This finding may indicate differences in substrate specificity or proteolytic mechanisms between closely related strains, highlighting the need for further research to better understand the proteolytic potential of individual *Pseudomonas* strains.

In the study by Class et al. (2024), the peptides PLW and FSDIPNPIGSENSEK derived from αs1-CN were identified as markers of milk spoilage; however, these peptides were not detected in the present study. Since the authors refer to the possible combined action of endogenous enzymes (plasmin, cathepsin D) and bacterial proteases, the discrepancy between their findings and the hydrolysis products observed in this work is likely due to the greater enzymatic diversity present in milk, which contains not only plasmin and the *Pseudomonas* LBSA1 protease. In the study by Verhegghe et al. (2021), which focused on developing a rapid method for detecting bacterial proteolytic activity in raw milk, a β-casein-derived peptide with the sequence GPFPIIV was identified among six peptide markers. This peptide was also detected in our model, though it was generated by plasmin. The authors reported that in samples with high levels of heat-resistant bacterial proteases, the concentrations of this and other peptides significantly increased (Verhegghe et al., 2021). In contrast, during the modeling of hydrolysis by the *Pseudomonas* LBSA1 protease in the present study, this peptide was not detected. This may be attributed to differences in the substrate specificity of proteases from

various *Pseudomonas* strains, in particular, a preference for glycine in the P1′ position (C-terminal) or arginine in the P1 position (N-terminal), as well as the possible subsequent activation of plasminogen.

Although Verhegghe et al. (2021) applied heat treatment (95°C for 8 minutes and 45 seconds) to eliminate the influence of plasmin in their experiment, they did not conduct additional studies to confirm the complete inactivation of the plasmin system. Meanwhile, Van Asselt et al. (2008) reported that even extreme heat treatment conditions (180°C for 0.2 seconds), followed by heating at 80°C for up to 4 minutes, do not guarantee full inactivation of plasmin activity in milk. Furthermore, France et al. (2021) noted that plasminogen activators may remain active after heat treatment and can initiate protein hydrolysis during subsequent milk storage. The authors also reported a D-value of 16 seconds at 140°C for the inactivation of such activators. Taken together, these findings highlight the complexity of studying the effects of individual factors on the milk peptide profile. They underscore the need to consider the intricate interactions and known specificities of proteolytic systems. Therefore, in studies of milk protein proteolysis, it is essential to assess the cumulative nature of the resulting changes and conduct additional experiments to quantitatively determine the contribution of secondary factors, such as plasmin system activity.

The 2D diagrams generated through modeling in the present study can serve as a tool for comparing results from empirical experiments, enabling faster identification of patterns, improving the efficiency of data analysis, and facilitating the creation of 'fingerprints' that characterize the condition and quality of dairy products. The fingerprint-based approach to peptide and protein analysis has already been applied in several studies, including investigations into the effects of heat treatment and storage on the milk peptide profile (Meltretter et al., 2008), detection of milk adulteration by the addition of milk powder (Du et al., 2020), and analysis of dynamics of protein changes in breast milk (Thesbjerg et al., 2023). The use of such visual tools can support the interpretation of proteomic data and accelerate the identification of key peptides, thereby strengthening the synergy between *in silico* modeling and laboratory research.

Limitations

The modeling performed in this study does not account for all storage-related factors, including temperature fluctuations, protein–protein and protein–lipid interactions. It is also important to consider the influence of the plasmin system and other endogenous enzymes, whose concentration and activity are affected by zootechnical factors (such as breed, age of the cow, and stage of lactation), as well as by processing conditions. The presented models are limited by the selection of proteases; expanding the dataset to include the specificities of a broader range of bacterial enzymes responsible for spoilage in milk would enhance the effectiveness of bioinformatic analysis and improve the predictability of dairy product spoilage.

CONCLUSION

The application of bioinformatic modeling enabled the prediction of casein hydrolysis (specifically β-CN, αs1-CN, αs2-CN, and κ-CN) under the action of plasmin and the heat stable bacterial protease from *Pseudomonas* LBSA1. This made it possible to assess potential changes in the milk peptide profile during storage. The results confirmed that these proteins are sources of peptides with bitter taste and antioxidant activity, which may serve as a basis to develop new approaches to dairy product quality assessment. In particular, this could involve the analysis of peptide bitterness and antioxidant properties as indicators of changes occurring in milk during storage. Further studies are needed to evaluate the practical applicability of these approaches, including the development and adaptation of sensitive analytical methods to objectively assess the impact of enzymatic processes on the sensory and functional properties of milk proteins.

The partial agreement between the *in silico* results and experimental findings supports the potential of this approach to predict key peptides. However, differences in enzyme specificity highlight the need for additional validation.

Future research may focus on the identification of microbial enzyme systems present in raw milk, expanding bioinformatic databases, and verifying predictions through mass spectrometry. Integrating bioinformatic modeling with laboratory studies will enable more accurate predictions of protein composition changes and help optimize dairy quality control. Conducting a scoping review on the use of bioinformatics in food spoilage assessment would further systematize existing approaches and define key directions for future development.

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APPENDIX 1

Complete list of hydrolysis products

Protein	Hydrolysis product	Molecular weight, Da	Isoelectric point (pI)
	Plasmin hydrolysis model		
αs2-CN	ALNEINQFYQK	1367.52	6.40
αs2-CN	AMK	348.46	9.70
αs2-CN	ENLCSTFCK	1044.21	6.14
αs2-CN	EQLSTSEENSK	1251.27	3.98
αs2-CN	EVVR	501.58	6.41
αs2-CN	FALPQYLK	979.19	9.30
αs2-CN	FPQYLQYLYQGPIVLNPWDQVK	2710.13	6.32
αs2-CN	HYQK	574.64	9.30
αs2-CN	ISQR	502.57	10.55
αs2-CN	ITVDDK	689.76	4.11
αs2-CN	K	146.19	9.70
αs2-CN	K	146.19	9.70
αs2-CN	K	146.19	9.70
αs2-CN	K	146.19	9.70
αs2-CN	LNFLK	633.79	9.70
αs2-CN	LTEEEK	747.80	3.98
αs2-CN	NANEEEYSIGSSSEESAEVATEEVK	2688.71	3.49
αs2-CN	NAVPITPTLNR	1195.38	10.55
αs2-CN	NMAINPSK	874.02	9.70
αs2-CN	NR	288.31	10.55
αs2-CN	NTMEHVSSSEESIISQETYK	2299.45	4.19
αs2-CN	PWIQPK	767.93	9.70
αs2-CN	QEK	403.44	6.41
αs2-CN	R	174.20	10.55
αs2-CN	TK	247.29	9.70
αs2-CN	TK	247.29	9.70
αs2-CN	TVDMESTEVFTK	1386.54	3.93
αs2-CN	TVYQHQK	903.01	9.30
αs2-CN	VIPYVR	745.92	9.35
αs2-CN	YL	294.35	6.09
αs2-CN	YQK	437.50	9.30
β-CN	AVPYPQR	829.95	9.35
β-CN	DMPIQAFLLYQEPVLGPVR	2186.60	4.18

Protein	Hydrolysis product	Molecular weight, Da	Isoelectric point (pI)
β-CN	EAMAPK	645.77	6.41
β-CN	ELEELNVPGEIVESLSSSEESITR	2646.84	3.55
β-CN	EMPFPK	747.91	6.41
β-CN	FQSEEQQQTEDELQDK	1982.00	3.56
β-CN	GPFPIIV	741.93	6.10
β-CN	HK	283.33	9.70
β-CN	IEK	388.46	6.41
β-CN	IHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK	5319.25	7.54
β-CN	INK	373.45	9.70
β-CN	K	146.19	9.70
β-CN	R	174.20	10.55
β-CN	VK	245.32	9.70
β-CN	VLPVPQK	779.98	9.70
β-CN	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK	6362.32	5.36
	Bacterial protease hydrolysis model		
αs1-CN	F	165.19	6.10
αs1-CN	F	165.19	6.10
αs1-CN	F	165.19	6.10
αs1-CN	H	155.16	7.55
αs1-CN	HIQKEDVPSER	1 337.46	5.55
αs1-CN	HPIK	493.61	9.70
αs1-CN	QF	293.32	6.10
αs1-CN	QGLPQEVLNENLLR	1 622.84	4.26
αs1-CN	QLDAYPSGAW	1 107.19	3.75
αs1-CN	QMEAESISSSEEIVPNSVEQK	2 321.50	3.71
αs1-CN	RPK	399.49	11.65
αs1-CN	VAPFPEVFGKEK	1 347.58	6.53
αs1-CN	VNELSKDIGSESTEDQAMEDIK	2 438.60	3.73
αs1-CN	VPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	3 593.96	3.74
αs1-CN	VPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAY	4 415.02	4.81
αs1-CN	Y	181.19	6.09
αs1-CN	Y	181.19	6.09
αs1-CN	Y	181.19	6.09
αs1-CN	YK	309.37	9.30
αs1-CN	YLGYLEQLLRLKK	1 637.00	9.93
αs1-CN	YPELFR	823.95	6.40
β-CN	F	165.19	6.10

Protein	Hydrolysis product	Molecular weight, Da	Isoelectric point (pI)
β-CN	HKEMPFPK	1 013.22	9.54
β-CN	MH	286.35	7.55
β-CN	QEPVLGPVRGPFPIIV	1 718.07	6.41
β-CN	QPH	380.40	7.55
β-CN	QPLPPTVMFPPQSVLSLSQSK	2 281.70	9.70
β-CN	QSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK	7 136.06	3.99
β-CN	RELEELNVPGEIVESLSSSEESITRINKKIEK	3 657.09	4.36
β-CN	VKEAMAPK	873.08	9.54
β-CN	VLPVPQKAVPYPQRDMPIQAFLLY	2 784.36	9.15
β-CN	YPVEPFTESQSLTLTDVENLHLPLPLLQSW	3 467.92	3.78
κ-CN	F	165.19	6.10
κ-CN	FSDKIAK	807.95	9.54
κ-CN	HPHPHLSF	971.09	7.81
κ-CN	MAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV	6707.43	3.77
κ-CN	QEQNQEQPIRCEKDER	2030.16	4.30
κ-CN	QQKPVALINNQFLPYPY	2033.36	9.15
κ-CN	QVLSNTVPAKSCQAQPTTMAR	2231.57	9.83
κ-CN	VLSR	473.57	10.55
κ-CN	Y	181.19	6.09
κ-CN	YAKPAAVRSPAQILQW	1799.11	10.45
κ-CN	YIPIQY	795.93	6.09
κ-CN	YPSYGLNY	976.05	6.08
	Combined hydrolysis model		
αs1-CN	F	165.19	6.10
αs1-CN	F	165.19	6.10
αs1-CN	F	165.19	6.10
αs1-CN	H	155.16	7.55
αs1-CN	HIQKEDVPSER	1337.46	5.55
αs1-CN	HPIK	493.61	9.70
αs1-CN	QF	293.32	6.10
αs1-CN	QGLPQEVLNENLLR	1622.84	4.26
αs1-CN	QLDAYPSGAW	1107.19	3.75
αs1-CN	QMEAESISSSEEIVPNSVEQK	2321.50	3.71
αs1-CN	RPK	399.49	11.65
αs1-CN	VAPFPEVFGKEK	1347.58	6.53
αs1-CN	VNELSKDIGSESTEDQAMEDIK	2438.60	3.73
αs1-CN	VPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	3593.96	3.74

Protein	Hydrolysis product	Molecular weight, Da	Isoelectric point (pI)
αs1-CN	VPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAY	4415.02	4.81
αs1-CN	Y	181.19	6.09
αs1-CN	Y	181.19	6.09
αs1-CN	Y	181.19	6.09
αs1-CN	YK	309.37	9.30
αs1-CN	YLGYLEQLLRLKK	1637.00	9.93
αs1-CN	YPELFR	823.95	6.40
αs2-CN	ALNEINQFYQK	1367.52	6.40
αs2-CN	AMK	348.46	9.70
αs2-CN	ENLCSTFCK	1044.21	6.14
αs2-CN	EQLSTSEENSK	1251.27	3.98
αs2-CN	EVVR	501.58	6.41
αs2-CN	FALPQYLK	979.19	9.30
αs2-CN	FPQYLQYLYQGPIVLNPWDQVK	2710.13	6.32
αs2-CN	HYQK	574.64	9.30
αs2-CN	ISQR	502.57	10.55
αs2-CN	ITVDDK	689.76	4.11
αs2-CN	K	146.19	9.70
αs2-CN	K	146.19	9.70
αs2-CN	K	146.19	9.70
αs2-CN	K	146.19	9.70
αs2-CN	LNFLK	633.79	9.70
αs2-CN	LTEEEK	747.80	3.98
αs2-CN	NANEEEYSIGSSSEESAEVATEEVK	2688.71	3.49
αs2-CN	NAVPITPTLNR	1195.38	10.55
αs2-CN	NMAINPSK	874.02	9.70
αs2-CN	NR	288.31	10.55
αs2-CN	NTMEHVSSSEESIISQETYK	2299.45	4.19
αs2-CN	PWIQPK	767.93	9.70
αs2-CN	QEK	403.44	6.41
αs2-CN	R	174.20	10.55
αs2-CN	TK	247.29	9.70
αs2-CN	TK	247.29	9.70
αs2-CN	TVDMESTEVFTK	1386.54	3.93
αs2-CN	TVYQHQK	903.01	9.30
αs2-CN	VIPYVR	745.92	9.35
αs2-CN	YL	294.35	6.09

Protein	Hydrolysis product	Molecular weight, Da	Isoelectric point (pI)
αs2-CN	YQK	437.50	9.30
κ-CN	F	165.19	6.10
κ-CN	FSDKIAK	807.95	9.54
κ-CN	HPHPHLSF	971.09	7.81
κ-CN	MAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV	6707.43	3.77
κ-CN	QEQNQEQPIRCEKDER	2030.16	4.30
κ-CN	QQKPVALINNQFLPYPY	2033.36	9.15
κ-CN	QVLSNTVPAKSCQAQPTTMAR	2231.57	9.83
κ-CN	VLSR	473.57	10.55
κ-CN	Y	181.19	6.09
κ-CN	YAKPAAVRSPAQILQW	1799.11	10.45
κ-CN	YIPIQY	795.93	6.09
κ-CN	YPSYGLNY	976.05	6.08
β-CN	AVPYPQR	829.95	9.35
β-CN	DMPIQAFLLY	1210.45	3.75
β-CN	EAMAPK	645.77	6.41
β-CN	ELEELNVPGEIVESLSSSEESITR	2646.84	3.55
β-CN	EMPFPK	747.91	6.41
β-CN	F	165.19	6.10
β-CN	GPFPIIV	741.93	6.10
β-CN	HK	283.33	9.70
β-CN	IEK	388.46	6.41
β-CN	IHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK	5319.25	7.54
β-CN	INK	373.45	9.70
β-CN	K	146.19	9.70
β-CN	MH	286.35	7.55
β-CN	QEPVLGPVR	994.16	6.41
β-CN	QPH	380.40	7.55
β-CN	QPLPPTVMFPPQSVLSLSQSK	2281.70	9.70
β-CN	QSEEQQQTEDELQDK	1834.82	3.56
β-CN	R	174.20	10.55
β-CN	VK	245.32	9.70
β-CN	VLPVPQK	779.98	9.70
β-CN	YPVEPFTESQSLTLTDVENLHLPLPLLQSW	3467.92	3.78