dietetics

Article

Profile Assessment of Bioactive Peptides in the Greek Traditional Cheese "Tsalafouti"

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Abstract: In the Greek regions of Agrafa and Tzoumerka, Tsalafouti, a traditional spreadable cheese made from goat's and sheep's milk is produced. This product has emerged in recent years as a result of the campaign to acquire Geographical Indication. This study aimed to assess the biopeptide profile of Tsalafouti cheese in order to highlight its nutritional value. Using HPLC-MS, bioactive peptides in Tsalafouti cheese samples were identified and classified according to their bioactivity. The biopeptides detected are known to present antibacterial, anti-diabetic, anti-hypertensive, anti-thrombotic, antioxidant, and immunomodulatory activities, while ACE enzyme and dipeptidyl-4 (DPP-IV) inhibitors were also identified. Based on these results, Tsalafouti cheese presents an interesting bioactive peptides profile that may act as special motivation for consumers to choose this specific cheese.

Keywords: Tsalafouti; cheese; Greek; traditional; biopeptides; HPLC-MS

Citation: Meleti, E.; Alexandraki, M.; Samara, A.; Loffi, C.; Tedeshi, T.; Galaverna, G.; Manouras, A.; Koureas, M.; Malissiova, E. Profile Assessment of Bioactive Peptides in the Greek Traditional Cheese "Tsalafouti". *Dietetics* **2024**, *3*, 16–29. https://doi.org/10.3390/dietetics3010002

Academic Editor: Carol Johnston

Received: 27 November 2023
Revised: 8 January 2024
Accepted: 10 January 2024
Published: 16 January 2024

1. Introduction

Worldwide, the most popular fermented dairy product is cheese in all its variants. Cheese is one of the basic everyday food products, and in some cases is a product of high gastronomical and cultural value. In different regions of the world, people's identity, history, culture, and way of life are reflected in their eating habits and the goods they produce. A set of factors such as soil, topography, climate, and flora directly contribute to the special characteristics of cheeses and express a valuable gastronomic heritage [1,2]. In Greece, different types of cheeses are traditionally produced, mostly with small ruminants' milk. The vast majority of Greek cheeses are mainly made from sheep or goat's milk (approximately 15 million small ruminants are reared in Greece) [3]. Different types of Greek cheeses have evolved into a very diverse range as a result of alterations in production methods over time. So far, 22 Greek cheeses have been granted a Protected Designation of Origin (PDO) indication, which helps in their protection and secures economic benefits for their producers [1,3]. Tsalafouti is a traditional Greek spreadable cheese made from ewe's milk, or in some areas a mixture of ewe's and goat's milk, produced in the mountainous and semi-mountainous regions of Agrafa and Tzoumerka that are located in the Pindos mountain range in Greece. Its production relies heavily on the spontaneous milk fermentation by native microbiota, particularly lactic acid bacteria (LAB), and is historically manufactured using ewe's milk throughout the summer (at the end of the lactation period) [4]. Tsalafouti is recently gaining

more and more recognition from consumers, which makes research on the nutritional value of this traditional product greatly important. In our previous work, [5] we highlighted the cheese's history, the cultural importance of the product, the cheesemaking technology, and the nutritional value of traditional Tsalafouti cheese. Based on the special attributes that Tsalafouti may hold, it is considered valuable to be further investigated in terms of its protein and peptides profile, fatty acids profile, and other possible important nutritional characteristics.

Fermented dairy products, and especially cheeses, are considered to be a food category rich in bioactive peptides [6,7]. The physiological activity of some peptides that positively affect human health have attracted the interest of researchers and food industries [8]. Biopeptides display a wide range of biological activity, including antimicrobial, antiviral, angiotensin-converting enzyme (ACE) inhibition, dipeptidyl peptidase IV (DPP-IV) inhibition, antihypertensive, opioid activities, immunomodulation, mineral binding, antioxidative functions, and cytomodulation [7,9]. Biopeptides are described as certain protein fragments that improve body functions or conditions and might have beneficial effects on health [10–12]. Although milk is regarded as a major source of biopeptides, with distinct nutritional and functional characteristics, biopeptides originate from an array of food proteins during gastrointestinal digestion and fermentation. Dairy products such as milk, cheese, and yoghurt have been widely studied in terms of their bioactivity due to the biopeptides that exist in these products [9,11,13–16]. According to Nielsen's et al. [6] database research, most biopeptides are derived from these main milk proteins: 36% β-casein, 13% αs1-casein, 11% β-lactoglobulin, 10% κ-casein, 8% αs2-casein and 5% α-lactalbumin, while the secondary milk proteins lactoferrin constituted 15%, and less than 1% comes from other secondary proteins such as serum albumin.

However, most of the already known biological properties of peptides were demonstrated through in vitro digestion, which tends to hold poor translatability to physiological conditions in vivo [16]. This discrepancy is mainly due to the structural instability and the effective digestion of bioactive peptides in the gastrointestinal (GI) environment [16]. The functionality of bioactive peptides can be directly affected by various factors such as process conditions, protein sources, sequence and amino acid composition, molecular weight and charge distribution, pH, and certain chemical treatments [17].

The benefits that milk and cheese biopeptides offer to people's health, as well as the increasing consumer demand for traditional products, played an important role in conducting this research. Additionally, the recent characterization of Tsalafouti cheese as a PDO product, can offer Tsalafouti the recognition it deserves from consumers. All of this may empower the added commercial value of Tsalafouti cheese, which is likely to be a great advantage for the producers, as well as the societies of the marginal regions where it is produced.

The aim of this study was to identify the profile of biopeptides in traditional Tsalafouti cheese and highlight this unique product as one of high nutritional value, which can benefit the consumer's health through valuable bioactivity.

2. Materials and Methods

2.1. Sampling

Twelve samples were obtained from eight of the thirteen Tsalafouti manufacturers in Greece and were freeze-stored at −20 °C. Water-soluble extracts were prepared according to the method used by Gomez-Ruiz, Ramos, and Recio [18] with some modification. For the samples' preparation, cheese was weighted (5 g) and centrifuged (4000 rpm, 25 min, at 20 °C). Following centrifugation, the supernatant was filtered through paper after the fat layer on top was removed and then filtered through a 0.45 μm syringe filter. It was then transferred into vials and stored under refrigeration until the analysis was conducted.

2.2. Biopeptide Separation

In order to analyze the water-soluble extracts, an Agilent 6130 single Quadrupole LC/MS spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) was used, equipped with Agilent 1200 HPLC, consisting of an automatic sampler, a degasser, a binary pump, a column oven, and a DAD (Diode Array Detector) performed at 230 nm. The following were the ESI's (Electrospray ionization) working conditions: the capillary voltage was 3200 V, the gas flow was 10 L/min, the temperature was 300 °C, and the nebulizer pressure was set at 50 Psi. Positive electrospray ionization was used for the analysis. Chromatographic separation was accomplished (Phase Analytical Technology, LLC, State College, PA, USA), using an ACME^{TM} C18 column (4.6 mm × 250 mm, 5.0 µm). A total of 5 µL of the sample was injected into the HPLC system and the LC-MS system after the samples were filtered using a 0.45 µm membrane filter. The two eluent solutions used were A: TFA (Trifluoroacetic acid, HPLC grade, Chem-Lab NV) in ultrapure water (0.1% v/v) and B: 0.09% v/v TFA in Acetonitrile (Reag. Ph. Eur.) for UHPLC Supergradient, ACS, PanReac AppliChem ITW Reagents) and ultrapure water in a ratio of 90:10. For ultrapure water, Human Corporation's New P. Nix Power I was used.

The total duration of the method was 60 min, and the gradient elution profile was as follows: 100% eluent A for 10 min, from 10th to 20th min 75% eluent A and 25% eluent B, for the next 10 min 50% A and 50% eluent B, and vice versa for the next 30 min up to 100% eluent B. With the aid of qualitative analysis software, mass data were automatically collected. A full scan ion mode was used to record the mass spectra (100–2000 m/z).

2.3. Biopeptide Identification

The resulting peptide sequences' functional characteristics were identified using online databases like BIOPEP, milk Bioactive Peptide Database, EROP-Moscow, and BioDADPep, that are available online at: https://biochemia.uwm.edu.pl/biopep-uwm/ (accessed on 20 May 2022) [19], http://mbpdb.nws.oregonstate.edu (20 May 2022), http://erop.inbi.ras.ru (accessed on 20 May 2022), and http://omicsbase.com/BioDADPep/biodadpep-search/ (accessed on 20 May 2022), respectively.

The milk protein sequences that were used came from the UniProt Knowledgebase (UniProtKB) (https://www.uniprot.org (accessed on 20 May 2022)), including αs1-casein (UniProtKB—P04653, P18626), αs2-casein (UniProtKB—P04654, P33049), β-casein (UniProtKB—P11839, P33048), k-casein (UniProtKB—P02669, P02670), β-lactoglobulin (UniProtKB—P67976, P02756), and α-lactalbumin (UniProtKB—P09462, P00712). The mass of the mature proteins was obtained by subtracting the signal peptides from the total mass of the pre-proteins. The Expasy peptide mass calculator (https://web.expasy.org/peptide_mass/ (accessed on 20 May 2022)) was used to calculate the mass of the signal peptide.

2.4. Biopeptide Characterization pre and after In Silico Digestion

The possible bioactivity of the peptides identified was further determined, and in order to evaluate the end effect of the biopeptides following digestion by humans, simulated gastro-pancreatic digestion was used to assess the effect of in silico digestion on the biopeptides identified in Tsalafouti. The ExPASY Peptide Cutter application (http://web.expasy.org/peptide_cutter/ (accessed on 15 June 2022)) was used to simulate gastro-pancreatic digestion for the detected peptides. Specifically, the generated protein fragments were put through simulated digestion with pepsin, trypsin, and chymotrypsin under the assumption that 100% of cleavage probability occurred at each possible location. Again, based on the assumption that there is a 100% chance of cleavage at each probable site, we were able to compile a list of short peptides that are produced following protein digestion and are resistant to the major gastrointestinal proteases. Then, utilizing the BIOPEP (https://biochemia.uwm.edu.pl/biopep-uwm/ (accessed on 20 June 2022)) database, the lists of peptides were checked for the presence of any new or similar bioactive peptides.

Additionally, we used the PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/ (accessed on 20 December 2023)) [20], a server for the prediction of bioactive peptides based

on a novel N-to-1 neural network. A list of small chain (2–5 amino acids) biopeptides was submitted to the PeptideRanker in order to evaluate the probability that these peptides will be bioactive. This evaluation focused on small-chain peptides because according to multiple bibliographic references, peptides resistant to in vitro GI digestion have a shorter chain length and a smaller molecular weight [16,21,22].

3. Results

By identifying the peptides in Tsalafouti cheese, describing their bioactivity, and projecting the outcome of these peptides after in silico digestion, this study evaluated the peptide profile of Tsalafouti cheese. In the Supplementary Materials section, the HPLC chromatograms of all twelve samples, where peaks relate to specific retention times of peptide fragments for the entire duration of the established method, are presented.

Peptides and their fragments were detected in the analyzed cheese samples. The identified peptides or peptide fragments were reviewed for their potential biological activity at MBPDB, BIOPEP-UWM, and BioDADPep applications. Also, the peptides found in cheese samples were checked for their bioactivity in literature sources. Table 1 presents the biopeptides identified in Tsalafouti cheese samples and correlates them to their known bioactivity.

In the twelve Tsalafouti cheese samples analyzed, a total of 39 different biopeptides were detected. Out of these 39 biopeptides, 12 were inhibitors of the ACE enzyme (ACE-inhibitory), 7 were inhibitors of dipeptidyl peptidase-4 (DPP-IV-inhibitory), 10 of them were related to antimicrobial/antibacterial activity, 4 to antidiabetic activity and 2 were antithrombotic, while 3 were related to multiple biological activities (ACE-inhibitory, Antidiabetic, Antioxidant, Opioid, etc.), and finally, 1 of them belonged to the category of immunomodulatory biopeptides. The largest percentages of biopeptides detected were inhibitors of the ACE enzyme (30.7%) and biopeptides with antimicrobial activity (25.5%), (Figure 1).

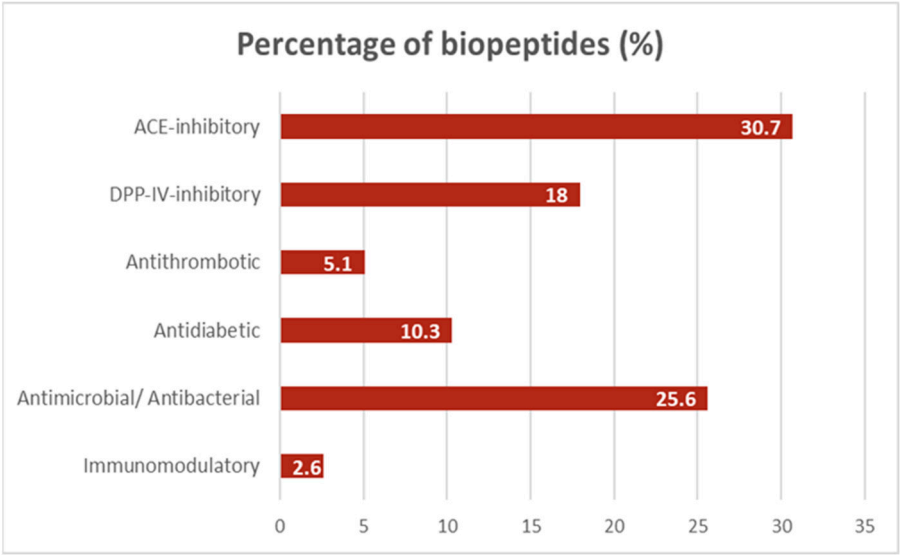

Figure 1. Percentage of biopeptides according to the type of bioactivity.

Also, the analysis results indicate that the vast majority of the biopeptides are casein-originated as 79% of the biopeptides found in Tsalafouti cheese come from caseins (28% β-CN, 23% κ-CN, 18% α_{s1}-CN, 10% α_{s2}-CN) and only a relatively small percentage of 21% originate from serum proteins like α-lactalbumin (5%) and β-lactoglobulin (16%) (Figure 2).

Table 1. Bioactive peptides found in the samples of Tsalafouti cheese.

Peptide Sequence	Protein	Fragment	Peptide/Fragment Mass (Da)	Type of Milk	Bioactivity	Reference
			SAMPLE 1			
MHQPPQPL	β-CN	159–166	947.106/341.300 (QPP)	Sheep	DPP-IV inhibitory	[23]
SPTVMFPPQSVL	β-CN	165–178	1302.536/430.450 (PQSV)	Sheep	DPP-IV inhibitory	[23]
QEPVLGPVRGPFP	β-CN	207–219	1392.601/475.500 (VRGPF)	Sheep	Antidiabetic	[24]
KVLILA	β-CN	2–7	656.700	Sheep	ACE inhibitory	[25]
TAQVTSTEV	κ-CN	184–192	934.981/707.700 (TAQVTST)	Sheep	Antithrombotic	[26]
SDIPNPIGSE	αs1-CN	195–204	1028.066/502.500 (PIGSE)	Sheep/Goat	Antidiabetic	[23]
VPSERY	αs1-CN	101–106	750.900	Sheep	ACE inhibitory	[27]
VRYL	αs2-CN	220–223	550.800	Sheep	ACE-inhibitory	[27]
KFAWPQ	αs2-CN	189–194	776.900	Sheep	ACE-inhibitory	[28]
LKGYGGVSLPE	a-La	34–44	1119.265/232.200 (GGV)	Sheep/Goat	DPP-IV inhibitory	[29]
			SAMPLE 2			
QTPVVVPPF	β-CN	94–102	983.200	Sheep	Immunomodulatory	[30]
NAGPFTPT	αs2-CN	131–138	803.800	Sheep	Antibacterial	[31]
YAKPVA	κ-CN	82–87	647.600	Sheep	ACE-inhibitory	[32]
LKKISQ	αs2-CN	180–185	716.600	Sheep/Goat	Antimicrobial	[31]
NPWDQVKR	αs2-CN	123–130	1042.144/659.350 (NPWDQ)	Goat	Antidiabetic	[24]
LKGYGGVSLPE	a-La	34–44	1119.265/658.100 (GGVSLPE)	Sheep/Goat	DPP-IV inhibitory	[29]
IPIQY	κ-CN	47–51	633.600	Goat	DPP-IV inhibitory	[33]
GPFPILV	β-CN	216–222	741.911/342.400 (PIL)	Goat	DPP-IV inhibitory	[23]
			SAMPLE 3			
TGPIPN	β-CN	78–83	597.652/383.200 (GPIP)	Sheep	ACE-inhibitory	[15]
GPFPILV	β-CN	216–222	741.911/342.400 (PIL)	Sheep	DPP-IV inhibitory	[23]
SPTVMFPPQSVL	β-CN	167–178	1302.536/430.400 (PQSV)	Sheep	DPP-IV inhibitory	[23]
MHQPPQPL	β-CN	159–166	947.106/454.350 (PQPL)	Sheep	DPP-IV inhibitory	[23]
VPSERY	αs1-CN	101–106	750.750	Sheep	ACE inhibitory	[27]
VDQHQKAMKPWTQ-PKTNAIPYVRYL	αs2-CN	184–208	3013.491/628.500 (PWTQP)	Sheep	Antibacterial	[31]
VLVLDTDYK	B-Lg	110–118	1065.213/659.600 (VLVLDT)	Sheep	DPP-IV inhibitory/ACE inhibitory	[34]
			SAMPLE 4			
PPKKDQDKTEVPA	κ-CN	130–142	1452.608/341.300 (PPK)	Goat	Antimicrobial	[35]
ASAEPTVH	κ-CN	147–154	810.844/474.250 (ASAEP)	Goat	Antimicrobial	[35]
PTVHSTPTTE	κ-CN	151–160	1069.118/635.400 (STPTTE)	Goat	Antimicrobial	[35]

Table 1. *Cont.*

Peptide Sequence	Protein	Fragment	Peptide/Fragment Mass (Da)	Type of Milk	Bioactivity	Reference
ENLLRF	αs1-CN	33–38	790.900/662.600 (NLLRF)	Goat	ACE-inhibitory	[28]
NPWDQVKR	αs2-CN	123–130	1042.144/830.800 (WDQVKR)	Goat	Antidiabetic	[24]
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
SDIPNPIGSENSEK	αs1-CN	195–208	1486.536/668.600 (DIPNPI)	Goat	Antibacterial	[30]
			SAMPLE 5			
SPTVMFPPQSVL	β-CN	167–178	1302.536/304.200 (STP)	Sheep	DPP-IV inhibitory	[23]
YQEPVLGP	β-CN	206–213	1302.536/739.600 (QEPVLGP)	Goat	Antioxidant/Antidiabetic	[24]
SDIPNPIGSE	αs1-CN	195–204	1028.066/668.700 (DIPNP)	Goat	Antidiabetic	[24]
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
DAQSAPLR	B-Lg	51–58	858.000	Goat	Antimicrobial	[35]
TGPIPN	β-CN	78–83	597.652/383.200 (GPIP)	Sheep	ACE-inhibitory	[36]
NPWDQVKR	αs2-CN	123–130	1042.144/659.600 (NPWDQ)	Goat	Antidiabetic	[24]
YPYY	κ-CN	79–82	605.500	Sheep	DPP-IV inhibitory/ACE-inhibitory/Opioid	[33,37]
YLAHK	a-La	104–108	630.600	Goat	ACE-inhibitory	[15]
LKPTPEGD	B-Lg	46–53	969.085/314.350 (PTP)	Goat	ACE-inhibitory	[38]
			SAMPLE 6			
MHQPPQPL	β-CN	159–166	947.106/341.300 (QPP)	Sheep	DPP-IV inhibitory	[23]
ASAEPTVH	κ-CN	147–154	810.844/473.400 (ASAEP)	Goat	Antimicrobial	[35]
SDIPNPIGSENSEK	αs1-CN	195–208	1028.066/502.450 (PIGSE)	Goat	Antibacterial	[30]
SPTVMFPPQSVL	β-CN	167–178	1302.536/778.400 (SPTVMFP)	Sheep	DPP-IV inhibitory	[23]
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
NPWDQVKR	αs2-CN	123–130	659.500/ (NPWDQ) 402.400 (VKR)	Goat	Antidiabetic	[24]
LKGYGGVSLPE	a-La	34–44	1119.265/1.046.300 (GGVSLPEWVC)	Sheep/Goat	DPP-IV inhibitory	[29]
YLAHK	a-La	104–108	630.600	Goat	ACE-inhibitory	[15]
LKPTPEGD	B-Lg	46–53	855.926/628.600 (KPTPEG)	Goat	ACE-inhibitory	[38]
ALPMHIR	B-Lg	142–148	837.037/360.350 (LPM)	Sheep	ACE-inhibitory	[39]
			SAMPLE 7			
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
VPSERY	αs1-CN	101–106	749.804/863.000 (VPSERYL)	Sheep	ACE inhibitory	[27]
SPTVMFPPQSVL	β-CN	167–178	1302.536/341.300 (PPQ) 360.350 (FPP)	Sheep	DPP-IV inhibitory	[23]
NPWDQVKR	αs2-CN	123–127	1042.144/659.550 (NPWDQ)	Sheep	Antidiabetic	[24]

Table 1. *Cont.*

Peptide Sequence	Protein	Fragment	Peptide/Fragment Mass (Da)	Type of Milk	Bioactivity	Reference
IPAVF	B-Lg	96–100	545.662/286.350 (PAV)	Sheep	DPP-IV inhibitory	[40]
TGPIPN	β-CN	78–83	597.652/286.350 (GPI)	Sheep	ACE-inhibitory	[36]
			SAMPLE 8			
SLPQ	β-CN	84–87	443.484/558.600 (NSLPQ)	Sheep/Goat	ACE-inhibitory	[36]
MHQPPQPL	β-CN	159–166	947.106/284.350 (HQ)	Sheep/Goat	DPP-IV inhibitory	[23]
SPTVMFPPQSVL	β-CN	167–178	1302.536/360.400 (FPP)	Sheep/Goat	DPP-IV inhibitory	[23]
NPWDQVKR	αs2-CN	123–130	1302.536/230.300 (NP)	Goat	Antidiabetic	[24]
INNQFLPYPY	κ-CN	72–81	1268.415/360.400 (INN)	Goat	DPP-IV inhibitory	[23]
ALNEINQF	αs2-CN	96–104	948.026/878.000 (LNEINQF)	Goat	Antimicrobial	[15]
TAQVTSTEV	κ-CN	184–192	934.981/418.500 (TAQV)	Sheep/Goat	Antithrombotic	[26]
LKPTPEGD	B-Lg	64–72	855.926/314.400 (PTP)	Sheep/Goat	DPP-IV inhibitory	[29]
			SAMPLE 9			
KFAWPQ	αs2-CN	174–179	775.887/550.500 (KFAW)	Goat	ACE-inhibitory	[28]
YPFTGPIPN	β-CN	60–68	1005.12/286.400 (GPI)	Goat	Antimicrobial	[16]
SLSSSEESITH	β-CN	30–40	1176.184/477.300 (EESI)	Goat	Antidiabetic	[24]
SDIPNPIGSE	αs1-CN	195–204	1028.066/286.400 (PIG)	Goat	Antidiabetic	[24]
NPWDQVKR	αs2-CN	123–130	1042.144/402.400 (VKR)	Goat	Antidiabetic	[24]
MHQPPQPL	β-CN	159–166	947.106/454.400 (PQPL)	Sheep	DPP-IV inhibitory	[23]
ALPMHIR	B-Lg	142–148	837.037/384.200 (LPM)	Sheep	ACE-inhibitory	[39]
LKGYGGVSLPE	a-La	34–44	1119.265/536.600 (LKGYG)	Sheep/Goat	DPP-IV inhibitory	[29]
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
			SAMPLE 10			
MHQPPQPL	β-CN	159–166	947.106/ 381.200 (HQP)	Sheep	DPP-IV inhibitory	[23]
SDIPNPIGSE	αs1-CN	195–204	1028.066/1544.500 (APSFSDIPNPIGSEN)	Goat	Antidiabetic	[24]
NPWDQVKR	αs2-CN	123–130	1042.144/1854.200 (IVLNPWDQVKRNAGPF)	Goat	Antidiabetic	[24]
GPFPILV	β-CN	216–222	741.911/342.400 (PIL)	Goat	DPP-IV inhibitory	[23]
LKGYGGVSLPE	a-La	34–44	1119.265/431.300 (GGVSL)	Sheep/Goat	DPP-IV inhibitory	[29]
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
YQEPVLGP	β-CN	206–213	901.997/649.600 (LYQEP)	Goat	Antioxidant/Antidiabetic	[24]
			SAMPLE 11			
KDQDK	κ-CN	133–137	633.600	Sheep	Antithrombotic	[26]
NPWDQVKR	αs2-CN	123–130	1042.144/545.400 (PWDQ) 402.400 (VKR)	Goat	Antidiabetic	[24]
ASAEPTVH	κ-CN	147–154	810.844/376.200 (ASAE)	Goat	Antimicrobial	[35]

Table 1. *Cont.*

Peptide Sequence	Protein	Fragment	Peptide/Fragment Mass (Da)	Type of Milk	Bioactivity	Reference
PTVHSTPTTE	κ-CN	151–160	1069.118/541.600 (HSTPT)	Goat	Antimicrobial	[35]
LKGYGGVSLPE	a-La	34–44	1119.265/231.300 (GGV)	Sheep/Goat	DPP-IV inhibitory	[29]
ENLLRF	αs1-CN	33–38	791.00	Goat	ACE-inhibitory	[28]
YAKPVA	κ-CN	82–87	647.600	Sheep	ACE-inhibitory	[32]
LKKISQ	αs2-CN	180–185	715.874/ 446.500 (KIS)	Sheep/Goat	Antimicrobial	[31]
YLAHK	a-La	104–108	630.727/468.450 (LAHK)	Goat	ACE-inhibitory	[15]
			SAMPLE 12			
SDIPNPIGSE	αs1-CN	195–204	1028.066/555.700 (DIPNP)	Goat	Antidiabetic	[24]
KFAWPQ	αs2-CN	189–194	775.887/429.550 (WPQ)	Sheep	ACE-inhibitory	[28]
KDQDK	κ-CN	133–137	633.600/261.800 (KD)	Sheep	Antithrombotic	[26]
LKGYGGVSLPE	a-La	34–44	1119.265/261.800 (GVS)	Sheep/Goat	DPP-IV inhibitory	[29]
YLAHK	a-La	104–108	630.700	Goat	ACE-inhibitory	[15]
LLF	B-Lg	103–105	391.494/555.700 (YLLF)	Goat	ACE-inhibitory	[38]
YAKPVA	κ-CN	82–87	647.600	Sheep	ACE-inhibitory	[32]
ASAEPTVH	κ-CN	147–154	810.844/218.100 (AE)	Goat	Antimicrobial	[35]

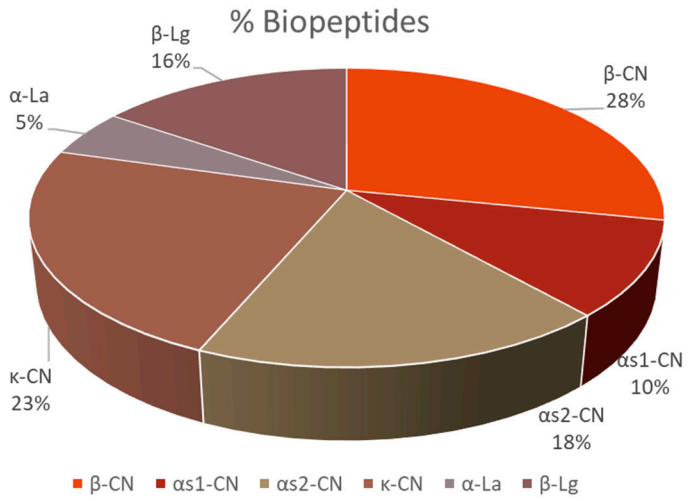

Figure 2. Percentage of biopeptides according to their protein origin.

Nevertheless, in this study an in-silico digestion tool was used for the identification of the changes in existing biopeptides. The results showed that some bioactive sequences have not been affected/cut from the digestion process and some others showed new released biopeptides (Table 2).

Table 2. Released bioactive peptides found in the samples of Tsalafouti cheese after a simulated digestion.

		Biopeptides after Digestion			
Original Peptide	**Peptide after Digestion**	**Protein**	**Type of Milk**	**Bioactivity**	**Reference**
LKGYGGVSLPE	KGYGGVSL	a-La	Sheep/Goat	ACE inhibitor	[40]
IPIQY	IPI	κ-CN	Goat	DPP IV inhibitor	[41]
IPIQY	QY	κ-CN	Goat	DPP IV inhibitor	[42]
GPFPILV	LV	β-CN	Goat	DPP IV inhibitor	[43]
TGPIPN	TGPIPN	β-CN	Sheep	ACE inhibitor	[15]
SPTVMFPPQSVL	VL	β-CN	Sheep	DPP IV inhibitor	[43]
VPSERY	RY	αs1-CN	Sheep	Antioxidative	[44]
VDQHQKAMKPWTQPKTNAIPYVRYL	RYL	αs2-CN	Sheep	Antioxidative	[44]
VDQHQKAMKPWTQPKTNAIPYVRYL	PYVRYL	αs2-CN	Sheep	Antioxidative	[31]
VLVLDTDYK	VLDTDYK	B-Lg	Sheep	ACE inhibitor	[45]
PPKKDQDKTEVPA	PPK	κ-CN	Goat	Antithrombotic	[30]
ASAEPTVH	ASAEPTVH	κ-CN	Goat	Antimicrobial	[35]
SDIPNPIGSENSEK	SDIPNPIGSENSEK	αs1-CN	Goat	Antibacterial	[30]
YQEPVLGP	YQEPVL	β-CN	Goat	ACE inhibitor	[46]
YPYY	YPY	κ-CN	Sheep	DPP IV inhibitor	[33]
YPYY	YY	κ-CN	Sheep	DPP IV inhibitor	[43]
ALPMHIR	ALPMH	B-Lg	Sheep	ACE inhibitor	[47]
SLPQ	PQ	β-CN	Sheep/Goat	ACE inhibitor	[48]
LLF	LF	B-Lg	Goat	ACE inhibitor	[49]

Finally, after the simulated digestion of the bioactive peptides, the PeptideRanker tool [20] was used in order to rank the bioactivity of small-chain biopeptides, found pre or post the simulated digestion (Table 3). Small-chain peptides were ranked based on a N-to-1 neural network in the PeptideRanker (https://distilldeep.ucd.ie/PeptideRanker/ (accessed on 20 December 2023)) and a threshold of small-chain peptides higher than 0.5 can be deemed as bioactive.

Table 3. Bioinformatics analysis for SCPs (short-chain peptides) in Tsalafouti.

Short Chain Peptides	Confirmed Bioactivity	Peptide Ranker [c]
VRYL	ACE-inhibitory [b]	0.3127
IPIQY	DPP-IV Inhibitory [b]	0.3607
KDQDK	Antithrombotic [a]	0.0764
YPYY	ACE-inhibitory [b], DPP-IV Inhibitory [a,b], Opioid [b]	0.7586
YLAHK	ACE-inhibitory [a]	0.1832
IPAVF	ACE-inhibitory [b], DPP-IV Inhibitory [a,b]	0.6821
SLPQ	ACE-inhibitory [b]	0.3658
LLF	ACE-inhibitory [b]	0.9389
Short chainPeptides after simulated digestion	Confirmed Bioactivity	Peptide Ranker [c]
PPK	Antithrombotic [a]	0.6062
YPY	DPP-IV Inhibitory [a,b]	0.7358
LF	ACE-inhibitory [b], Immunomodulatory [b], Anti-inflammatory [b]	0.9869

[a] The bioactivity for corresponding SCP was retrieved in BIOPEP-UWM (https://biochemia.uwm.edu.pl/ (accessed on 20 December 2023)) [19]. [b] The bioactivity for corresponding SCP was retrieved from the Milk Bioactive Peptide Database (MBPDB, https://mbpdb.nws.oregonstate.edu/ (accessed on 20 December 2023)) [9]. [c] The probability of SCPs as bioactive peptides was calculated in the PeptideRanker (https://distilldeep.ucd.ie/PeptideRanker/ (accessed on 20 December 2023)) [9].

4. Discussion

Based on the abovementioned results, Tsalafouti cheese contains peptides that may possess a variety of biological activities and offer benefits to human health in various ways. The biopeptides contained in Tsalafouti cheese reveal the inhibitory action of the ACE enzyme (ACE-inhibitory) and of dipeptidyl peptidase-4 (DPP-IV-inhibitory), while other biopeptides are related to antimicrobial/antibacterial, antidiabetic, immunomodulatory, and antithrombotic activity. However, there were peptides present in the cheese samples that displayed multiple biological activities (ACE-inhibitory, antidiabetic, antioxidant, opioid, etc.).

Several types of sheep or goat's milk cheeses have been analyzed for their biopeptide content [3,14,27,50], however spreadable cheeses such as Tsalafouti, or similar cheeses such as Katiki and Galotiri (traditional Greek PDO cheeses), have not been studied before in relation to their biopeptide profile.

The existence of antimicrobial biopeptides in sheep and goat's milk cheeses is also recorded by Tomazou et al. [50] for feta cheese, where they identified 63 and 64 antimicrobial peptides from sheep and goat's milk, respectively, used for feta's preparation, however there is no data available for the biopeptides' fate after the digestion process.

From the results, it is also evident that the great majority of biopeptides originate from caseins (β-Casein, κ-Casein, αS_1-Casein, αS_2-Casein), while the percentage of biopeptides that originate from basic serum proteins (β-lactoglobulin, α-lactalbumin) is significantly smaller, as seen in Figure 2. This is constantly in line with the outcomes of the research by Nielsen et al. [9] mentioned earlier in the introduction. Furthermore, this confirms that sheep and goat's milk were discovered to be abundant in bioactive peptides mainly derived from α-, β- and κ-caseins [13]. Additionally, investigating the relationship between proteolysis of cheeses made from raw and pasteurized sheep's milk and bioactivity, Pisanu et al. [14] detected 187 biopeptides derived from αs1-casein, β-casein, and αs2-casein. Nine of the detected peptides exhibited strong antioxidant activity and were proteolysis products of β-casein, and more specifically originated from the f207–221 fragment (QEPVL-GPVRGPFPIL). We also detected smaller fragments from the above fragment in Tsalafouti cheese samples.

It is noteworthy that the same biopeptides appeared in several of the cheese samples (Figure 3), which may result from the local breeds of animals, from the feeding of the animals, as well as the similar production methods followed for Tsalafouti cheese. There are peptides that appear in the majority of samples, such as NPWDQVKR, KDQDK,

LKGYGGVSLPE, which show antidiabetic activity, antithrombotic activity and inhibitory activity against dipeptidyl peptidase 4, respectively.

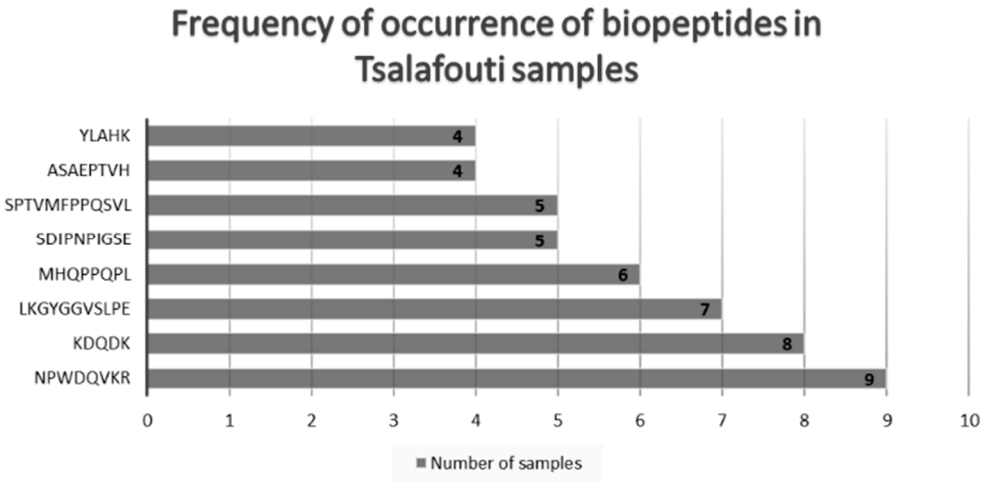

Figure 3. Frequency of occurrence of biopeptides in Tsalafouti cheese samples.

The presence of biopeptides in food in general does not assure the consequential health effects, as the digestion process may alter the peptides' sequence. In order to acquire a better awareness of the possible fate of Tsalafouti cheese's biopeptides, ideally in vitro or in vivo gastrointestinal digestion would be an asset. In that sense, supplementary quantitative studies are required to validate that these biopeptides exist in ample quantities to have their biological effect after digestion.

More specifically, the following sequences TAQVTSTEV, SDIPNPIGSE, TGPIPN, ASAEPTVH, SDIPNPIGSENSEK, and TGPIPN have not been affected by the digestive enzymes. New bioactive peptides were released via simulated digestion as shown in Table 2. The bioactive peptides found in Tsalafouti were of various amino acid chain sizes, from dipeptides (LF) and tripeptides (PPK, LLF, YPY) after simulated digestion to much bigger chain peptides such as VDQHQKAMKPWTQPKTNAIPYVRYL. SCPs (short-chain peptides) have been demonstrated as health-promoting substances that can be directly absorbed by the human organism, therefore the abundance of endogenous SCPs in foods can reflect their nutritional value [51]. Short peptides show a more remarkable resemblance to polar metabolites than longer peptides for their low molecular weights and wide range of physicochemical properties (acid–base properties and polarity) [52]. Pepsin, pancreatic trypsin, elastase, chymotrypsin, carboxypeptidase, and brush border peptidases are among the digestive proteases and peptidases found in the digestive tract [53], which would inevitably result in the generation of massive amounts of small-chain peptides during macromolecular protein digestion, moreover, these SCPs with multiple bioactivities have a higher likelihood of surviving these digestive enzymatic actions and permeating intestinal epithelial cells [54]. Most peptides of more than three amino acids are extracellularly hydrolyzed by enzymes in the brush border membrane of the intestinal epithelium [22]. In their study, Du and Jia [54] identified that goat's milk bioactive SCPs, after in vitro digestion, have a very high chance of exerting their biological activity in vivo due to their low molecular weight and fewer protease recognition and cleavage sites.

Di- and tripeptides, in contrast, can be absorbed intact and hydrolyzed later [22]. However, this is not always the rule, as the biopeptide KFAWPQ, found in the Tsalafouti sample, according to the review of Ahmed, Sun and Udenigwe [16], seems to be one of the stable peptides after in vitro gastrointestinal digestion. In addition, for the confirmation of the obtained results, in vitro and in vivo studies should be carried out.

Possible limitations of the present study might be the representativeness. Nevertheless, as 8 out of the 13 factories producing Tsalafouti cheese participated in this study, we believe that this result can act as a steppingstone for future research, giving some indications

and trends of its bioactivity profile. A national registry of establishments producing specific types of cheeses would be helpful to map the current number of Tsalafouti cheese producers. Another point for consideration might be the method selected to evaluate the peptide profile of Tsalafouti cheese. Tomazou et al. [50] used 1-D nano LC-MS/MS to identify the proteome in feta cheese, while Pappa et al. [55], used 2-dimensional electrophoresis and MALDI/TOF-MS for the separation of proteins and peptides, and liquid chromatography for separation. The authors also used Edman degradation for amino acid sequence identification, where peptides were identified based on the most abundant amino acid released after each degradation cycle, and identified by MS. It would be interesting to cross-check the peptide profile in Tsalafouti and other cheeses, with different analytical approaches in order to identify possible assets that could be exploited to increase the accuracy of the final results.

5. Conclusions

In this attempt to characterize the bioactive profile of Tsalafouti cheese, it was found that Tsalafouti possesses biopeptides with antimicrobial/antibacterial activity, as well as antidiabetic, antithrombotic, and antihypertensive biopeptides. Moreover, in the sampled Tsalafouti, biopeptides with antioxidant and immunomodulatory activity were identified, as well as biopeptides that inhibit the angiotensin-converting enzyme I (ACE, angiotensin converting enzyme, EC 3.4.15.1) and dipeptidyl peptidase-4 (DPP-IV) inhibitors. Based on an in-silico digestion approach, their bioactivity seems to be maintained. The bioactivity of the peptides contained in Tsalafouti, and the quantitative determination of proteins and peptides, should be further evidenced in order to empower its nutritional value.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dietetics3010002/s1, supplementary figures present the chromatograms of the analysis for the peptide identification in the Tsalafouti cheese's samples.

Author Contributions: Conceptualization, E.M. (Eleni Malissiova); methodology, T.T., G.G. and C.L.; validation, M.A. and A.M.; formal analysis, E.M. (Ermioni Meleti) and A.S.; data curation, A.M., M.A., E.M. (Ermioni Meleti) and M.K.; writing—original draft preparation, E.M. (Ermioni Meleti), A.S., M.A., T.T. and C.L.; writing—review and editing, E.M. (Eleni Malissiova), G.G., T.T. and A.M.; visualization, E.M. (Ermioni Meleti) and M.A.; supervision, E.M. (Eleni Malissiova). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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