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Bioactive peptides preliminary profile assessment in milk which comes from the indigenous Greek goat breed "Skopelos"

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Abstract: The "Skopelos" goat, a native Greek breed, located in the Northern Sporades islands, stands out for its exceptional milk productivity and unique milk characteristics that gives to it. This study presents preliminary data on the biopeptide profile of "Skopelos" goat milk from four producers in Magnesia, Greece. Using High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS), we identified bioactive peptides with potential health benefits, including antibacterial, anti-diabetic, anti-hypertensive and antioxidant activities. These preliminary results show the nutritional value and potential health-promoting properties of "Skopelos" goat milk. Further research will take place to fully characterize the biopeptides and assess the identification of functionality. The "Skopelos" goat breed, indigenous to the Northern Sporades islands, holds significant promise for the Greek economy through its unique milk production profile and bioactive peptide content.

Keywords: Bioactive peptides, Goat breed Skopelos, potential health, antibacterial peptides ,anti-hypertensive peptides,

• Introduction

Goat milk offers nutritional benefits and health-promoting properties due to its biologically active constituents, including peptides from proteins, oligosaccharides, fatty acids, and phospholipids. These components contribute to its functional qualities and potential health advantages[1], [2], [3]. Also, goat milk is of high biological value, due to its bioavailability and hypoalleginicity due to the very low α1-casein content [4]. Bioactive peptides can be formed from goat milk proteins, during the fermentation process by the proteolytic action of the starter cultures or by endogenous proteolytic enzymes. Bioactive peptides are short amino acid sequences, that upon release from the parent protein may play different physiological roles, including antioxidant, antihypertensive, antimicrobial, and other bioactivities [5], [6].The release of bioactive peptides is significantly influenced by the action of indigenous milk enzymes, playing a primary role in proteolysis during the storage of goat milk, even after heat treatment [7] and this process produce of large variety and quantity of peptides. Thermal processes used in the food industry for safety reasons can affect protease activities in milk, but according to *Leite. et al* [7] mild heat treatments preserve certain protease activities in goat milk. More intense heat treatments can lead to significant reductions in these activities. Greece primarily raises local goat breeds, *Capra prisca* and *Skopelos* goat, alongside some international breeds [8] and the climate and the biodiversity of the Greek land enhance the production of unique agricultural products of these breeds. The aim of this study was to conduct an initial investigation into the release of peptides by endogenous peptidases in raw and pasteurized goat milk from *Skopelos* breed and the in silico detection of their bioactivity.

• Material and method

2.1 Sampling

Fresh raw goat milk from *Skopelos* breed goats was provided by four farmers from the Magnesia region in Greece. Milk samples were collected during two different seasonal periods: the first sampling at the beginning of the lactation period in December and the second in mid-season in February. The eight raw goat milk samples were analyzed in duplicate using a *Lactoscan Milkanalyser (Farm Eco)* to determine their protein, fat, lactose, solids-not-fat, total solids, and salt content.All milk samples were defatted by centrifugation at 4000g for 20 minutes at 4°C, with the upper fat layer subsequently removed. The defatted raw milk samples were then divided in half. One portion was kept as raw milk and frozen at −20°C until further analysis, while the other half was pasteurized under 85°C for 5 minutes in a thermostatically controlled, shaking water bath. Also pasteurized milk was kept frozen at -20ºC until further analysis.

2.3 Peptide Analysis

2.3.1Peptide Extraction

Extraction of the endogenous peptides from the milk samples was performed as described by *Leite et al*[7] with minor modifications. Briefly, 500 μL aliquots of all skimmed raw and heat-treated goat milk samples were acidified with 5% (v/v) acetic acid (Fluka, Steinheim, Germany) solution to pH 4.6, and precipitated casein was removed from samples by centrifugation at 14,000 g for 30 min at 4ºC. The remaining supernatant was filtrated through a 0.45μm syringe filter. It was then transferred into vials and stored in the refrigerator until the analysis**.**

2.3.2 Peptide Characterization via LC-MS

All samples analyzed with an Agilent 6130 single Quadrupole LC/MS spectrometer (Agilent Technologies, Inc), equipped with Agilent 1200 HPLC, consisting of a binary pump, an automatic sampler, a degasser, a column oven and a DAD detector performed at 230nm. Working conditions for ESI were as follows: the gas flow was 10 L/min with a temperature of 300 ◦C, a nebulizer of 50 psi, and the capillary voltage of 3200 V. Analysis was done in positive electrospray ionization mode. Chromatographic separation was achieved with an ACME (Phase Analytical Technology, LLC) C18 column (4.6 mm × 250 mm, 5.0 μm). 5 μL of the sample was injected into the HPLC system and to LC-MS system. The two eluent solutions used were A: TFA (Trifluoroacetic acid, HPLC grade, Chem-Lab NV) in ultra-pure 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 ultra-pure water, Human Corporation's New P. Nix Power I was used. The total duration of the method was 50 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 for the next 10 min 25% A and 75% eluent B. Mass data collection was performed automatically with qualitative analysis software. Mass spectra were recorded in full scan ion mode (100–2000 m/z).

2.3.3 Biopeptide Identification

In order to identify the peptides based on the masses, the tool Expacy Find Pept (http://web.expasy.org/findpept/, accessed on 10 May2024) was used and the parameters were selected: [M+H]+ , Average, Mass Tolerance ± 0.5. The used milk protein sequences were αs1-casein (UniProtKB-P18626), αs2-casein (UniProtKB-P33049), β-casein (UniProtKB-P33048), k-casein (UniProtKB-P02670), β-lactoglobulin (UniProtKB-P02756) and α-lactalbumin (UniProtKB-P00712) obtained from UniProt Knowledgebase (UniProtKB) (https://www.uniprot.org).

2.3.4 Biopeptide characterization

The peptide sequence of the skimmed raw and pasteurized goat milk samples was used to search against the Milk Bioactive Peptide Database (MBPD) (http://mbpdb.nws.oregonstate.edu, accessed on 10 May 2024) [9] to identify potential bioactive peptides.

The search type was sequence, and a similarity threshold of 100% was used to identify peptides with the same sequence to known functional milk peptides in the database. Moreover, Peptide Ranker (https://distilldeep.ucd.ie/PeptideRanker/) was used to assess the potential bioactivity of all the identified peptides, with a limit of 0.8, which indicates stronger bioactivity and fewer false positive rates

• Results and discussions

In the 16 samples of goat milk analyzed, a total of 25 different biopeptides were detected. On the 8 samples of raw milk 9 biopeptides were detected **(Table 1**), while on the 8 samples of the pasteurized milk were detected the same 9 biopeptides and 7 more (**Table 2**).Endogenous proteases that we can find in goat milk, such as plasmin, elastase, and cathepsin D, are enzymes that produce peptides from the breakage of proteins. These enzymes are naturally present in goat milk and their activity can be influenced by thermal treatments[7] The peptides that produced from this process were mainly derived from β-casein[7].This is like our results, as most of the new peptides found in the pasteurized milk samples originate from β-casein. Out of the 7 new extra peptides detected, 6 came from β-casein. The enzymatic hydrolysis during this mild heat treatment resulted in the formation of new potential bioactive peptides, which were different from those found in raw goat milk[7]. This finding aligns with the bioactivity analysis conducted using Peptide Ranker, which revealed that peptides from pasteurized milk have a higher probability of bioactivity compared to those from raw milk. Among the 11 peptides analyzed, only 4 were produced from raw milk, while the remaining 7 were from pasteurized milk. Notably, the peptide PFPI had the highest bioactivity probability, with a value of 0.91.Out of 25 biopeptides, 9 were inhibitors of the ACE enzyme (ACE-inhibitory), 3 were inhibitors of dipeptidyl peptidase-4 (DPP-IV-inhibitory), 11 of them were related to antimicrobial/antibacterial activity and 2 to antioxidant activity

Table 1. Bioactive peptides found in raw goat milk.

Peptide sequence	Protein	Fragment	Peptide/Fragment mass (Da)	Bioactivity	Reference
KIHPFAQAQ	Beta-casein	63-71	1040,21	ACE-inhibitory	Quiros, A. et al.
TPEVDKEALE	Beta-lactoglobulin	143-152	1131,224	Antimicrobial	Almaas, H. et al.
PTVHSTPTTE	Kappa-casein	151-160	1070,144	Antimicrobial	Almaas, H. et al.
ASAEPTVH	Kappa-casein	147-154	811,869	Antimicrobial	Almaas, H. et al.
SLPQ	Beta-casein	84-87	445,508	ACE-inhibitory	Geerlings, A. et al.
YQKFPQY	Alpha-S2-casein	105-111	1653,877	ACE-inhibitory	1) Silva, SV. et al., 2) Contreras, M. del Mar. et al.
YQKFPQY	Alpha-S2-casein	105-111	974,103	Antioxidant	1) Silva, SV. et al., 2) Contreras, M. del Mar. et al.
SLAMAASDISLL	Beta-lactoglobulin	39-50	1192,412	Antimicrobial	Almaas, H. et al.
LQKW	Beta-lactoglobulin	76-79	574,700	ACE-inhibitory	Hernandez-Ledesma, B. et al.

Table 2. Bioactive peptides found in pasteurized goat milk.

Peptide sequence	Protein	Fragment	Peptide/Fragment mass (Da)	Bioactivity	Reference
KIHPFAQAQ	Beta-casein	63-71	1040,21	ACE-inhibitory	Quiros, A. et al.
TPEVDKEALE	Beta-lactoglobulin	143-152	1131,224	Antimicrobial	Almaas, H. et al.
PTVHSTPTTE	Kappa-casein	151-160	1070,144	Antimicrobial	Almaas, H. et al.
ASAEPTVH	Kappa-casein	147-154	811,869	Antimicrobial	Almaas, H. et al.
SLPQ	Beta-casein	84-87	445,508	ACE-inhibitory	Geerlings, A. et al.
YQKFPQY	Alpha-S2-casein	105-111	1653,877	ACE-inhibitory	1) Silva, SV. et al., 2) Contreras, M. del Mar. et al.
YQKFPQY	Alpha-S2-casein	105-111	974,103	Antioxidant	1) Silva, SV. et al., 2) Contreras, M. del Mar. et al.
SLAMAASDISLL	Beta-lactoglobulin	39-50	1192,412	Antimicrobial	Almaas, H. et al.
LQKW	Beta-lactoglobulin	76-79	574,700	ACE-inhibitory	Hernandez-Ledesma, B. et al.
MHQPPQPL	Beta-casein	159-166	948,127	Antimicrobial	Almaas, H. et al.
MHQPPQPL	Beta-casein	159-166	948,127	DPP-IV Inhibitory	Zhang, Y. et al.
REQEELNV	Beta-casein	16-23	1017,083	Antimicrobial	Almaas, H. et al.
GPFPILV	Beta-casein	216-222	742,936	ACE-inhibitory	Quiros, A. et al.
GPFPILV	Beta-casein	216-222	742,936	DPP-IV Inhibitory	Zhang, Y. et al.
PFTGPIPNSLPQ	Beta-casein	76-87	1268,455	Antimicrobial	Almaas, H. et al.
INNQFLPYPY	Kappa-casein	72-81	1268,442	DPP-IV Inhibitory	Zhang, Y. et al.

Table 3. Bioinformatic analysis

Peptide sequence	Protein	Peptide Ranker	Type of Milk
PF	Kappa-casein	0.800416	R
IC	Kappa-casein	0.832898	P
FL	Kappa-casein	0.829203	P
PFPI	Beta-casein	0.912809	P
PFLQ	Beta-casein	0.828219	P
VLGPVRGPFPILV	Beta-casein	0.884977	P
PPQPLSPTVM	Beta-casein	0.811871	R
PVLGPVRGPFP	Beta-casein	0.804607	R
AYPSGAWYY	Alpha-S1-casein	0.833705	P
PF	Alpha-S1-casein	0.861654	P
CLLLALGLALA	Beta-lactoglobulin	0.886395	R

• Conclusions

In this paper, 16 samples of goat milk were analyzed, identifying 25 biopeptides, with pasteurized milk showing an increased number of bioactive peptides compared to raw milk.

Bioactive peptides with potential health benefits, including antibacterial, anti-diabetic, anti-hypertensive and antioxidant activities were identified. These preliminary results show the potential health-promoting properties of goat milk. Also these preliminary results suggest that optimizing thermal treatments could further enhance the functional properties of goat milk.

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