The effect of starter cultures on peptide profiles identified in camel milk cheese

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Abstract

Cheese contains a lot of biologically active compounds; among them, peptides with functional properties are important assembles. In this study, camel milk cheese samples are studied. The camel milk was fermented by mesophilic and thermophilic cultures. Cheese samples were ripened for 30 days, and the peptides released from casein were analyzed using liquid chromatography–tandem mass spectrometry. Identified peptides were analyzed by applying chemometrics. Furthermore, peptides from camel milk cheeses (β-CN- and α-CN-derived) were identified using the milk bioactive peptides database (MBPDB) and Peptide Ranker. A total of 52 peptides were identified. In all, 21 peptides were identified from β-CN in cheeses using mesophilic culture and five peptides from α-CN besides 17 peptides from β-CN and nine peptides from α-CN in cheeses using thermophilic culture. From a biological point of view, the peptides identified in camel milk cheese were homologous to sequences that were previously reported in the literature to exhibit angiotensin-converting enzyme inhibitory, antioxidant, antimicrobial, and dipeptidyl-peptidase IV inhibitory activities based on peptide sequence databases.

Keywords: camel milk cheese; starter cultures; biologically active peptides; chemometric analysis; peptide identification

Introduction

Cheese is a traditional fermented food with high nutritional value and functional potential. Proteolysis is one of the most important processes of cheese, where the enzymatic hydrolysis of milk proteins during fermentation and ripening produces bioactive peptides. During ripening, proteolysis leads to the release of bioactive peptides, which may exhibit antihypertensive, antioxidant, immunomodulatory, and antimicrobial activities (Chourasia *et al.*, 2021; Daliri *et al.*, 2017; Öztürk and Akın, 2021).

The proteolytic system of lactic acid bacteria (LAB) used as starter cultures play a key role in the formation of bioactive peptides primarily through the production of proteolytic enzymes, proteinases, and, notably, peptidases, which actively hydrolyze milk proteins (McSweeney, 2017). Mesophilic species, such as *Lactococcus lactis*, are active in the initial phase of proteolysis. In contrast, thermophilic strains, such as *Streptococcus thermophilus*, provide deeper cleavage of casein fractions, particularly β- and αS1-caseins. This promotes the formation of long-chain peptides with potential physiological activity. In particular, strains of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* are reported to enhance the formation of peptides, such as Val-Pro-Pro (VPP) and Ile-Pro-Pro-Pro (IPP), which are well known for their angiotensin-converting

enzyme (ACE) inhibitory and cardiovascular protective effects (Fan, *et al.*, 2019; Helal *et al*., 2023; Baptista *et al*., 2020).

The novelty of this study dwells in the investigation of the effect of mesophilic and thermophilic cultures on the capability of bioactive peptides from camel milk cheese. Camel milk has traditionally been consumed in arid regions because of its nutritional and therapeutic properties. Owing to its unique casein profile and rich content of bioactive molecules, camel milk is a promising substrate for the development of functional dairy products (Konuspayeva *et al.*, 2023). Despite its potential, the application of camel milk in making cheese historically has been limited by its low coagulation capacity and unique protein composition (Faye *et al.*, 2020). However, recent advances in enzymatic coagulation systems and starter culture technology have facilitated the successful production of soft, semi-hard, and hard cheeses from camel milk (Faye *et al.*, 2020; Mohamed and El Zubeir, 2021). Previous studies have investigated the fermentation of camel milk in fermented milk drinks using *Lactobacillus rhamnosus*, and reported ACE inhibitory and antioxidant activities of the released peptide fractions (Moslehishad *et al.*, 2013). Yet, no specific bioactive peptides have been identified in camel milk cheese, which has served to fill this scientific gap.

The present study aimed to assess the effect of different starter cultures on the formation of biologically active peptides in camel milk cheese throughout the entire ripening period. This was achieved using a database of milk bioactive peptides and chemometrics, particularly principal component analysis (PCA), which not only simplifies data analysis but also enhances the presentation of results by making complex datasets more accessible and highlighting key insights (Tarapoulouzi *et al.*, 2020).

Materials and Methods

Cheese-making

Raw milk of camel (*Camelus dromedarius*) was collected from a private herd in the Turkestan region (Kazakhstan). Two trials for producing cheese were conducted. In the first trial, cheese was prepared using 1.5% of commercial liquid mesophilic culture (*Lactococcus lactis*, *subsp. Lactis*; *Lactococcus cremoris*; *Lactococcus lactis*, *subsp. lactis biovar diacetylactis* [Biochem srl, Italy]), and in the second trial, 1.5% of commercial liquid thermophilic culture (*Streptococcus thermophiulus*; *Lactobacillus debrueckii*, *subsp. lactis*; and *Lactobacillus helveticus* [Biochem srl]) was used. Cheese samples were produced with the addition of rennet Chy-Max M 1000 (Ch. Hansen, Denmark). In all, 6 L of camel milk were divided into two portions of 3 L each. Protein, fat, total solids, lactose, pH, and non-fat milk solids of camel milk were analysed by Milkoscan FT120 (Foss, Denmark). Raw camel milk was clarified and 40% $CaCl_2$ (w/v) was added to it in a dose 0.1% to 1 L of milk to improve its rennet ability. The trial camel milk was inoculated with mesophilic culture and acidified to a pH of 5.8–6, and rennet Chy-Max M 1000 was added at 50 international milk clotting units per litre (IMCU/L) of milk. Fermentation of camel milk was realised at 32–34°C. After coagulum formation, it was cut into 4–6-mm cubes. The whey was separated and the cheese was self-pressed and moulded. The cheese samples were treated with hot whey (85–95°C) for 5 min and then cooled on a cheese table (for up to 30 min) and salted in abrine (10% NaCl, w/v) at 10°C. The cheese samples were ripened in moulds filled with brine at 10–12°C for 30 days. The samples were studied on the 1st, 10th, 20th, and 30th day of ripening. The same approach was used for producing the second trial, except for adding thermophilic starter culture, and fermentation was realised at 38–40°C.

Preparation of water-soluble extracts

The method described by Théolier *et al.* (2014), with some modifications, was used to prepare water-soluble extracts. In this method, 0.2 g of cheese was thoroughly rinsed with Milli-Q water (Millipore Corp., Bedford, MA, USA). The sample was homogenised by rigorous vortexing in 5× volume of Milli-Q water for 5 min at room temperature. The homogenate was incubated for 1 h at 40°C, with stirring at 300 rpm. The homogenate was centrifuged at 3,800 revolutions per minute (rpm) for 30 min and the pellet was discarded. The supernatant was stored at –20°C for mass spectrometry analysis.

Mass spectrometry analysis

The tandem mass spectrometry (MS/MS) parameters were based on Makhammajanov *et al.* (2024). The samples were reduced, alkylated, and then digested by trypsin (20 ng/μL; Sigma-Aldrich, Italy) at 37°C overnight. The obtained peptides were purified and concentrated using ZipTip-C18 (Millipore, Cork, Ireland). The eluted peptides were dried using a rotation-type evaporator (Eppendorf, Hamburg, Germany) and re-suspended in 10 μL of 0.1% (w/v) trifluoroacetic acid. Peptides were analysed using online nanoflow reversed-phase C18 liquid chromatography–tandem mass spectrometry (LC-MS/MS). A trapping column (Acclaim PepMap 100 C18 pre-column) and a Dionex HPLC pump were used for chromatography. Peptides were separated on an Acclaim Pep-Map RSLC column (Thermo, MA, USA) using

a 75-min multistep acetonitrile gradient at a flow rate of 0.3 mL/min. An unmodified captive spray ion source (capillary 1,300 V, dry gas 3.0 L/min, dry temperature 150°C) was used to interface the LC system to the Impact II ESI-QUAD-TOF mass spectrometer (Bruker Daltonics, Germany). Full-scan MS spectra were acquired at a spectral rate of 2.0 Hz, followed by the acquisition of one MS/MS spectrum. The MS/MS peak lists in Mascot generic format were searched on a local server using the Mascot 2.6.1 software (Matrix Science, UK) against the Swiss-Prot protein database (release 2022_03 and NCBI). The studied parameters were oxidation of methionine, and carbamidomethylation of cysteine residues as variable modifications. Peptide and protein data were extracted using high peptide confidence. A mass error window of 100 ppm and 0.05 dalton (Da) was allowed for MS and MS/MS, respectively.

Chemometric analysis

Owing to potentially large diversity and unidentified origin of bioactive peptides and their precursors, chemometrics was first proposed as a method for an objective approach to assess peptide formation in camel milk cheese using different starter cultures during ripening, although this is still insufficiently studied. Chemometric analysis was carried out by the SIMCA software (version 17; Umetrics, Sweden). The PCA was applied. Ellipse in the plots defines Hotelling's T2 confidence region, which is a multivariate generalisation of Student's *t*-test and provides a 95% confidence interval (95% CI) for observations. The number of studied components chosen is given with the symbol A. R2X and R2Y represent the cumulative modelled variation, explaining the quality of the model, and Q2 is an estimate of the model's predictive ability. R2X, R2Y, and Q2 values (not less than 0.5) show robust models with predictive reliability (Tarapoulouzi *et al.*, 2020).

Identification of bioactive peptides

Peptide sequences identified by LC-MS/MS were screened for known biological activities using the milk bioactive peptide database (MBPDB; Nielsen *et al.*, 2017). Each peptide was manually entered into the database's sequence search tool (http://mbpdb.nws.oregonstate.edu) to determine its sequence identity to previously characterised bioactive peptides. The search results were filtered based on exact matches, and peptides with full homology to document functional sequences were considered. For each matched peptide, the reported biological activity (e.g., ACE inhibitory, antioxidant, antimicrobial, and immunomodulatory) was recorded according to the annotation provided in the database.

Additionally, Peptide Ranker (http://distilldeep.ucd.ie/PeptideRanker/ [accessed: 14 March 2024]) server for the prediction of bioactive peptides was used. For every peptide, Peptide Ranker predicts the bioactive probability (between 0 and 1) of that peptide. The closer the predicted probability of peptide to 1, the more confident is Peptide Ranker for the peptide to be bioactive (Mooney *et al.*, 2012).

Data processing

To examine differences in chemical composition, ANOVA was applied. If variations were detected, Tukey test was used to compare mean values, with a significance level set at 5%. For each sample, three replicates were measured.

Results

The physicochemical characteristics of raw camel milk were as follows: protein content, 3.18 ± 0.20%; fat content, 4.03 ± 0.20%; and lactose content, 4.66 ± 0.10%. Total solids were measured at 12.73 ± 0.20%, while non-fat solids accounted for 8.44 ± 0.10%. The pH value of the milk samples was 6.42 ± 0.05. The relatively low standard deviations (SD) across samples indicated a homogeneous composition of milk, which was favourable for reproducible cheese production. These values were consistent with previously reported compositional ranges for raw camel milk and confirmed its technological suitability for further fermentation and curdling (Alhaj *et al.*, 2023).

Mass spectrometry measurements

Proteins of camel milk cheese, produced using mesophilic and thermophilic cultures, had a complex peptide profile, which was analysed by LC-MS/MS. Peptides in camel milk cheeses found were β-CN- and α-CN-derived peptides (established in the current study).

Figure 1 shows the β-CN-derived peptide with the sequence GLHPVPQPLVPVIA. The observed molecular mass of the peptide was 1435.8551 Da. The MS/MS spectrum provided various b- and y-ions from the fragmentation of peptide GLHPVPQPLVPVIA. Ion b5 with a mass-to-charge ratio (m/z) of 583.2745 corresponds to the fragment GLHPV; ion b6 with m/z 698.3413 corresponds to the fragment GLHPVP; ion b7 with m/z 813.4079 corresponds to the fragment GLHPVPQ; ion b8 with m/z 928.4745 corresponds to the fragment GLHPVPQP; ion b9 with m/z 1043.5411 corresponds to the fragment GLHPVPQPL; and ion y4 with m/z 503.2785 corresponds to the fragment VPIA.

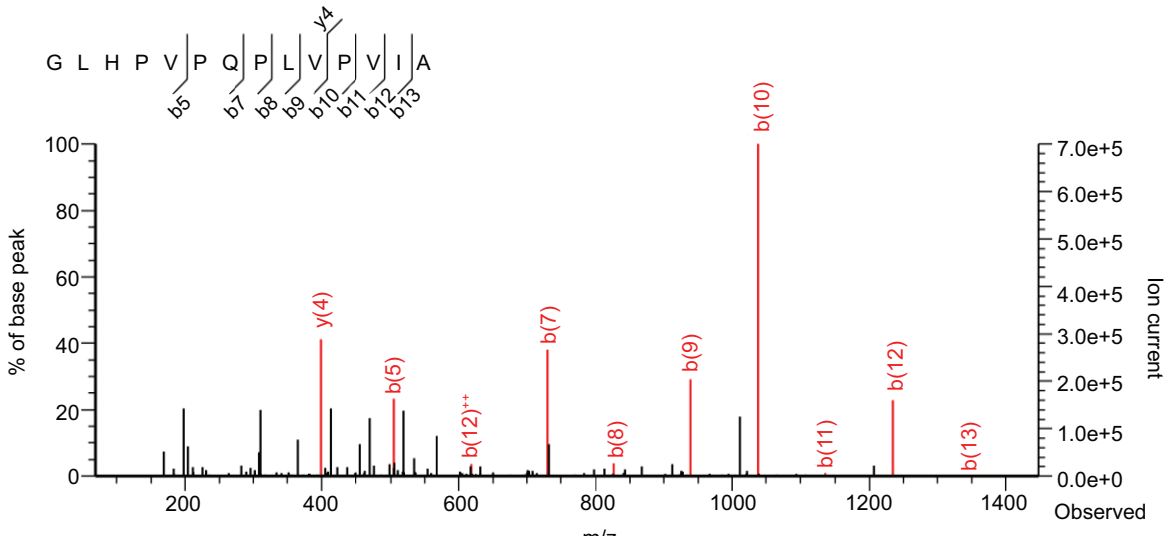


Figure 1. MS/MS spectrum of sequence GLHPVPQPLVPVIA. Following sequence interpretation and database searching (NCBI), the peptide was identified from β-CN casein at 20 days of cheese ripening. Observed mass: 1435.8551 Da; ions score: 73.

A Mascot score of 73 indicated a high-confidence match; however, it could not be directly referred from Figure 1. The Mascot software obtained Mascot score from the quality and several matched fragment ions of MS/MS spectrum. By analysing the peaks corresponding to b- and y-ions in the spectrum and their intensities, the score provided a quantitative measure of confidence in peptide identification. GLHPVPQPLVPVIA was identified as a high-confidence peptide, and the observed masses of the fragment ions matched well with theoretical values. The experimental mass (1435.8597 Da) was very close to the calculated mass (1435.8551 Da), with a deviation within acceptable limits (24.4 ppm), indicating high mass accuracy (Table 1). The b- and y-ions provided strong evidence supporting the peptide sequence. The detailed LC-MS/MS spectrum analysis confirmed the presence of the peptide GLHPVPQPLVPVIA by matching observed fragment ions (b5, b6, b7, b8, b9, and y4) to their theoretical m/z values. This high-confidence identification, supported by the Mascot search results, demonstrated the reliability of this particular analysis of β-CN and α-CN peptides. In this study, it was revealed that most of the identified peptides had common sequences. It was also observed that the largest number of peptides was detected by β-CN casein origin.

Chemometric analysis of peptides during cheese ripening

Changes in the peptide dynamics of camel milk cheese with mesophilic and thermophilic cultures during the 30 days of ripening are presented in Figure 2. The results obtained by chemometric analysis using the SIMCA software show that the PCA chemometric method successfully classified samples into two groups.

In addition, the two groups, mesophilic and thermophilic, were well separated, as shown in Figure 2a. Within these groups, clusters were obtained for the observations based on the ripening period of sample groups. In addition, as seen in Figure 2A, all thermophilic samples were grouped together; however, two mesophilic samples, both of 30 days of ripening, were significant outliers above Hotelling's ellipse. Figure 2B presents mesophilic and thermophilic samples on the 1st, 10th, 20th, and 30th day of ripening for each category. Only one sample, particularly from the mesophilic group, on the 1st day of ripening was closer to mesophilic samples on the 10th day of ripening.

Figure 3 presents PCA score plots of the ripening results for 30 days. In Figure 3A, for mesophilic group samples, the 20- and 30-day measuring intervals had good separation and repeatability among the replicates of each interval (cluster). Besides, two mesophilic samples were significant outliers on the 1st and 10th day of ripening. In addition, there was only one sample from the 1st day of ripening which emerged in the group of 10 days of ripening, and this outcome was in agreement with the data given in Figure 2A. Furthermore, Figure 3B shows, for thermophilic samples, an excellent separation of samples in four time intervals; however, two outliers appeared from the 1st day and the 20th day of ripening, and this was not observed in Figure 2B. Overall, good separation and repeatability among the replicates of each group (mesophilic and thermophilic) were observed for the 30-day ripening interval.

Table 1. Identified peptides, and their bioactivity from camel milk cheeses using mesophilic culture during 30 days of ripening.

	Mass observed (Da)	Mr (expt)[a] (Da)	Mr (calc)[b] (Da)	Score	Sequence	Bioactive propertiesc	Potential bioactivity[d]	0[e]	10	20	30
β-CN-derived peptides	465.2915	928.5685	928.5593	22	TKETI**IP**K	DPP-IV inhibitory; ACE inhibitory	0.0750801			+	
	718.9386	1435.8626	1435.8551	35	GLHPVPQPL**VP**VIA	ACE inhibitory	0.556007			+	
	826.4536	1650.8927	1650.8916	26	V**LP**VPQQM**VPYPQ**R	DPP-IV inhibitory; antioxidant	0.350489	+			
	1045.5595	2089.1044	2089.1030	43	A**MPVQA**VLPFQEP**VP**DP**VR**	DPP-IV inhibitory; ACE inhibitory	0.359886			+	
	494.7530	987.4914	987.4873	44	QENID**EL**K	Antioxidant	0.114525			+	
	1669.3455	6673.3530	6673.3771	53	IEEQQQTEDEQQDKIYTFPQPQSL**VY**SHTEP**IP**YPI	ACE inhibitory; DPP-IV inhibitory	0.104117		+		
	1649.2135	4944.6188	4944.6332	67	IYT**FP**QPQSL**VY**SHTEP**IPYP**ILPQN**FL**PPLQPAVM	DPP-IV inhibitory; ACE inhibitory	0.0588597		+		
	459.2658	916.5170	916.5052	37	VMD**VP**KTK	ACE inhibitory	0.131996		+		
	799.9666	1597.9187	1597.9113	22	VMD**VP**KTKETI**IP**K	ACE inhibitory; DPP-IV inhibitory	0.151499				+
	826.4569	1650.8993	1650.8916	75	V**LP**VPQQMVP**YP**QR	DPP-IV inhibitory; ACE inhibitory	0.350489				+
	1045.5602	2089.1059	2089.1030	37	A**MPVQA**VLPF**QEPV**PDP**VR**	DPP-IV inhibitory; immunomodulatory; ACE inhibitory	0.359886				+
	718.9392	1435.8639	1435.8551	38	GLHPVPQPL**VP**VIA	ACE inhibitory	0.556007		+		
	1669.3518	6673.3782	6673.3771	39	IEEQQQTEDEQQDKIYT**FP**QPQSLVYSHTEP**IPYP**I	ACE inhibitory; DPP-IV inhibitory	0.104117			+	
	826.4554	1650.8963	1650.8916	32	V**LP**VPQQMVP**YP**QR	DPP-IV inhibitory; ACE inhibitory	0.350489			+	
	1754.4865	3506.9585	3506.9476	24	A**MPVQ**AVLPF**QEPV**PDP**VR**GLHPVPQPLVPVIA	Immunomodulatory; DPP-IV inhibitory	0.201209			+	
	718.9371	1435.8597	1435.8551	73	GLHPVPQPL**VP**VIA	ACE inhibitory	0.556007			+	
	1649.2221	4944.6444	4944.6332	62	IYT**FP**QPQSL**VY**SHTEP**IPYP**ILPQN**FL**PPLQPAVM	DPP-IV inhibitory; ACE inhibitory	0.0588597				+
	799.9690	1597.9235	1597.9113	33	VMD**VP**KTKETI**IP**K	ACE inhibitory; DPP-IV inhibitory	0.151499				+
	1738.1674	6948.6406	6949.6615	35	EMPLLQ**SPVVPF**TESQSLTLTDLENLH**LP**LP**LL**QSL	DPP-IV inhibitory; ACE inhibitory	0.0550194				+

(continues)

Table 1. Continued.

	Mass observed (Da)	Mr (expt)[a] (Da)	Mr (calc)[b] (Da)	Score	Sequence	Bioactive propertiesc	Potential bioactivity[d]	0[e]	10	20	30
	465.2903	928.5660	928.5593	33	TKET**IIP**K	DPP-IV inhibitory; ACE inhibitory	0.0750801				+
	826.4568	1650.8990	1650.8916	30	V**LP**VPQQMVP**YP**QR	DPP-IV inhibitory; ACE inhibitory	0.350489				+
α-CN-derived peptides	742.9443	1483.8740	1483.8762	19	**ILELA**VVSPIQFR	DPP-IV inhibitory; antioxidant	0.302035	+			
	728.9414	1455.8683	1455.8701	31	K**ILELA**VVSPIQF	DPP-IV inhibitory; antioxidant	0.207185		+		
	533.6612	1597.9616	1597.9556	34	KILDLAV**VSPI**QFR	ACE inhibitory	0.394655			+	
	703.8884	2811.5244	2811.5130	17	ILDLAV**VSPI**QFRQENIDELKDTR	ACE inhibitory	0.0689203			+	
	799.9877	1597.9608	1597.9556	18	RKILDLAV**VSPI**QF	ACE inhibitory	0.259048				+

Note. DPP-IV: dipeptidyl-peptidase IV; ACE: angiotensin-converting enzyme.
[a]Mr (expt): experimental mass.
[b]Mr (calc): calculated mass.
[c]According to the MBPDB database of bioactive milk peptides.
[d]The probability of sequences as bioactive peptides was calculated in the Peptide Ranker (https://distilldeep.ucd.ie/PeptideRanker/ [accessed: 14 March 2024]).
[e]Ripening time in days.

Identification of bioactive peptides

The biofunctionality of peptides was determined using an online database, and ACE inhibition and antioxidant properties were observed, comparable to other cheese studies (Nielsen *et al.*, 2017). In Tables 1 and 2, the peptides identified from camel milk cheeses during 30 days of ripening are presented; these were collected using mesophilic and thermophilic cultures during 30 days of ripening.

The bioactive peptides obtained are fragments of β-CN-derived peptides, which are more sensitive to proteolytic enzymes than α-casein. In all, 21 peptides in cheeses using mesophilic cultures for its production were detected; five peptides of these were detected on the 1st day of ripening. Seven peptides were detected on the 10th day of ripening. Four peptides were detected on the 20th day, and five peptides on the 30th day of ripening. In camel milk cheeses using thermophilic culture for their production, 17 peptides were identified. Of these peptides, four peptides were detected on the 1st day, three peptides each on the 10th day and 20th day of ripening, and four peptides on the 30th day of ripening, which matched the fragments of β-CN-derived peptides.

Less number of peptides were also identified in α-CN origin. In camel cheeses prepared using mesophilic culture in fragments of α-CN origin, one peptide each was found on the 1st, 10th, and 30th day, but two peptides were identified on the 20th day of ripening.

However, slightly more peptides relevant to the α-CN origin were identified in cheeses using thermophilic cultures, compared to mesophilic cultures. There were three peptides on the 1st day, and two peptides each on the 10th, 20th, and 30th day of ripening.

For the first time, 508 novel peptides were identified in camel milk cheeses, of which 52 had potential biological activity, as confirmed by MBPDB.

The precursor MPVQA enclosed in the AMPVQAVLPFQEPVPDPVR sequence, released from β-CN-derived peptides also identified in samples with mesophilic and thermophilic cultures, was described as dipeptidyl-peptidase IV (DPP-IV) inhibitory activity with a maximum inhibitory concentration of 93.3 micromolars (μM) (IC50), had a role in the potential regulation of glycemia in humans (Nongonierma *et al.*, 2018). In addition, the precursor QEPVPDPVR from β-CN-derived peptides matched the peptides derived in the study done by Parastouei *et al.* (2022) and characterised with ACE-inhibitory potential. Most of the identified peptides in our study had common sequences; for example, the GLHPVPQPLVPVIA peptide was

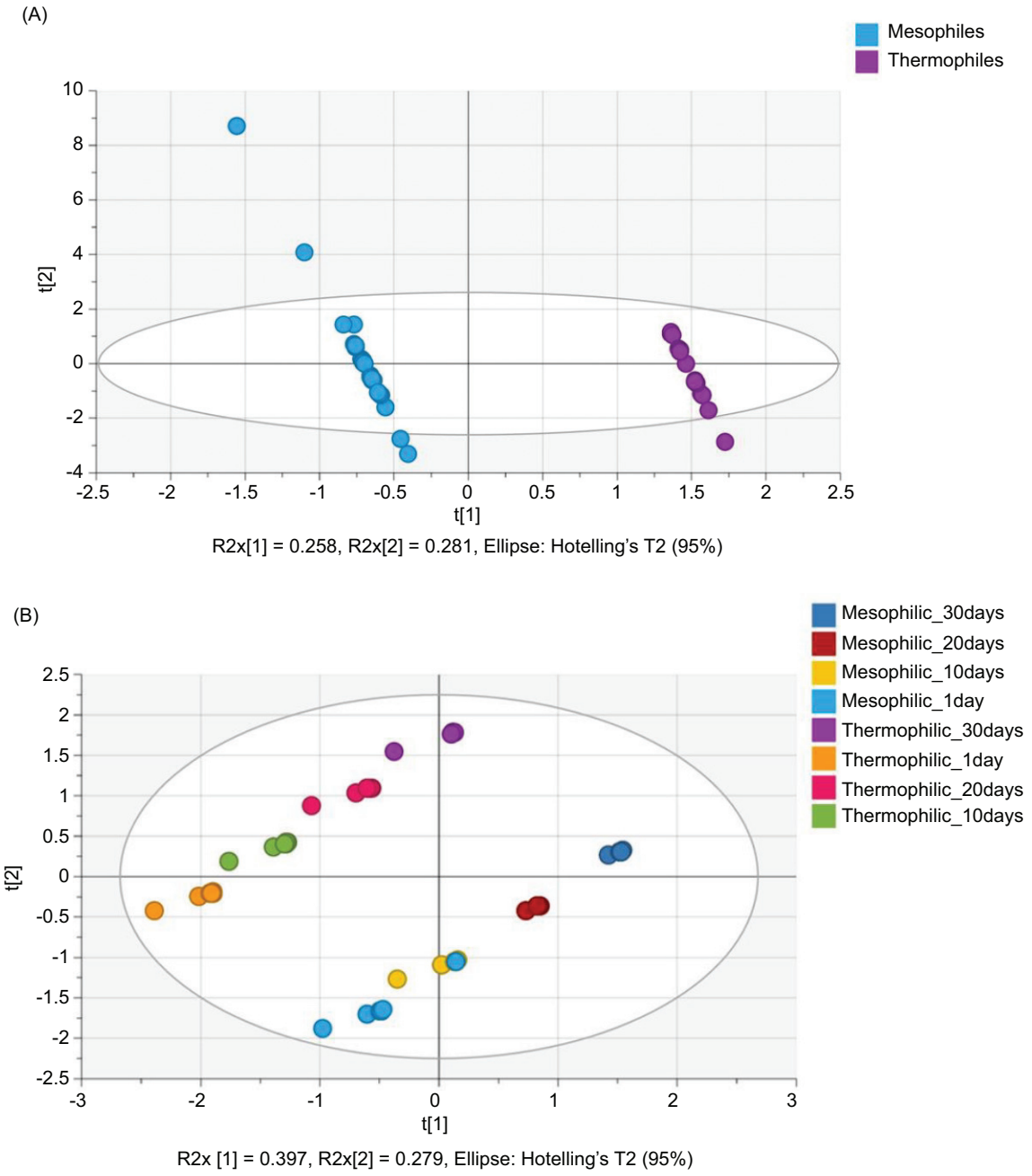
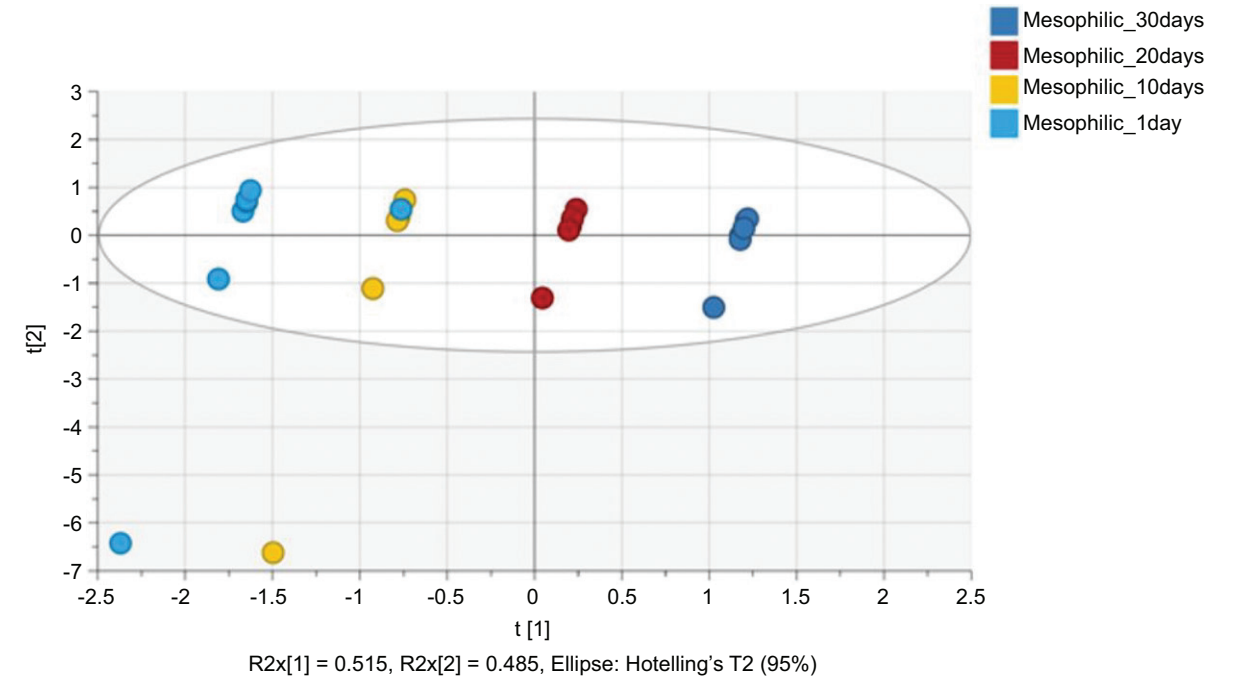


Figure 2. The principal component analysis (PCA) score scatter plot presents (A) the clustering of mesophilic and thermophilic data, and (B) the clustering of mesophilic and thermophilic samples on the 1st, 10th, 20th, and 30th day of ripening. A = 4.

identified in the production of cheese using mesophilic culture on the 1st day and 20th day of ripening, having QP precursor (Gómez-Ruiz *et al.* 2006), VP (Norris *et al.*, 2014), and PVPQP (Gupta *et al.*, 2009; Kohmura *et al.*, 1989), which, as reported, had antioxidant, ACE-inhibiting, antimicrobial, and immunomodulatory properties. ETIIPK peptide was found in cheese ripened by thermophilic starter culture on the 1st and 30th day of ripening, and precursor AVVSPIQF was identified in α-CN derived in cheeses using mesophilic culture. Murali *et al.* (2021) while studying whey protein hydrolysates showed anticancer and anti-inflammatory properties of camel whey. El Hatmi *et al.* (2016) while studying fermented camel milk with *S. thermophilus* LMD-9 found the same peptide AMPVQAVLPFQEPVPDPVR. In our study, the peptide identified in cheese using thermophilic culture on the 1st day of ripening, AMPVQAVLPFQEPVPDPVR (β-CN-derived peptides), matched the data that demonstrated anti-inflammatory properties. The α-CN-derived ILELAVVSPIQFR peptide



(B)

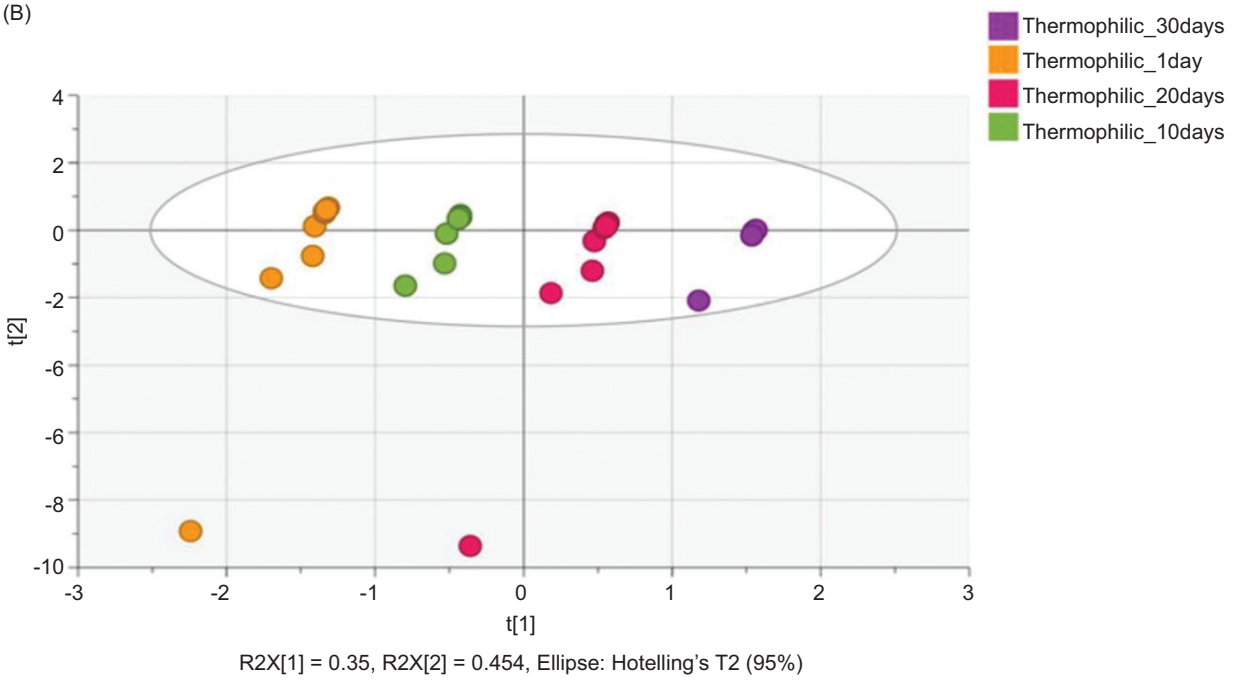


Figure 3. The principal component analysis (PCA) score scatter plot over 30 days of ripening for (A) mesophilic, and (B) thermophilic samples. A = 3.

found in cheeses using thermophilic culture on the 1st day and 30th day of ripening, as well as using mesophilic culture on the 1st day of ripening, was attributed to DPP-IV inhibitory features (Nongonierma *et al.*, 2018). In addition, ILELAVVSPIQFR peptide of α-CN origin showed ACE inhibitory and antioxidant properties (Erhardt *et al.*, 2016). Many potential bioactive peptides, all from β-CN, are ACE inhibitors *in vitro* or have a hypothetical effect *in vivo*. Camel milk fermented with Lacticaseibacillus rhamnosus (Lb. rhamnosus) demonstrated ACE inhibitory properties higher than that of cow's fermented milk (Moslehishad *et al.*, 2013; Murali *et al.*, 2021). ILELAVVSPIQFR, GLHPVPQPLVPVIA, and VLPVPQQMVPYPQR peptides also were identified in camel milk (Cebo and Saadaoui, 2015), but their biofunctionality was not detected. Peptide QENIDELKDTR found in α-CN origin using thermophilic culture on the 20th day of ripening was also detected in camel milk's

Table 2. Identified peptides, and their bioactivity from camel milk cheeses using thermophilic culture during 30 days of ripening.

	Mass observed (Da)	Mr (expt)[a] (Da)	Mr (calc)[b] (Da)	Score	Sequence	Bioactive properties[c]	Potential bioactivity[d]	0[e]	10	20	30
β-CN-derived peptides	350.7175	699.4205	699.4167	37	ETI**IP**K	DPP-IV inhibitory; ACE inhibitory	0.112471	+			
	551.3169	1650.9288	1650.8916	50	VLPVPQQMVPYPQR		0.350489	+			
	697.3905	2089.1497	2089.1030	33	A**MPVQ**AVLPFQEP**VP**DP**VR**	DPP-IV inhibitory; ACE inhibitory	0.359886	+			
	479.6341	1435.8804	1435.8551	72	GLHPVPQPL**VP**VIA	ACE inhibitory	0.556007	+			
	413.7374	1650.9205	1650.8916	17	V**LPVPQ**QM**VPYPQR**	DPP-IV inhibitory; antioxidant	0.350489		+		
	1045.5825	2089.1504	2089.1030	86	A**MPVQ**AVLPFQEP**VP**DP**VR**	DPP-IV inhibitory; ACE inhibitory	0.359886		+		
	1170.0157	3507.0252	3506.9476	20	A**MPVQ**AVLPFQEPVPDP**VR**GLHPVPQPL**VP**VIA	DPP-IV inhibitory; ACE inhibitory	0.201209		+		
	352.6916	703.3686	703.3575	19	VMD**VP**K	ACE inhibitory	0.174862			+	
	465.2986	928.5827	928.5593	39	TKETI**IP**K	DPP-IV inhibitory; ACE inhibitory	0.0750801			+	
	826.4763	1650.9381	1650.8916	62	V**LPVP**QQM**VPYP**QR	DPP-IV inhibitory; ACE inhibitory	0.350489			+	
	1045.5843	2089.1540	2089.1030	48	A**MPVQ**AVLPFQEP**VP**DP**VR**	DPP-IV inhibitory; ACE inhibitory	0.359886			+	
	1170.0172	3507.0297	3506.9476	19	A**MPVQ**AVLPFQEPVPDP**VR**GLHPVPQPL**VP**VIA	DPP-IV inhibitory; ACE inhibitory	0.201209			+	
	479.6371	1435.8894	1435.8551	74	GLHPVPQPL**VP**VIA	ACE inhibitory	0.556007			+	
	350.7211	699.4277	699.4167	37	ETIIPK	DPP-IV inhibitory; ACE inhibitory	0.112471				+
	1045.5883	2089.1621	2089.1030	19	A**MPVQ**AVLPFQEP**VP**DP**VR**	DPP-IV inhibitory; ACE inhibitory	0.359886				+
	1053.5879	2105.1612	2105.0980	49	A**MPVQ**AVLPFQEP**VP**DP**VR**.G	DPP-IV inhibitory; ACE inhibitory	0.359886				+
	1170.0180	3507.0321	3506.9476	17	A**MPVQ**AVLPFQEPVPDP**VR**GLHPVPQPL**VP**VIA	DPP-IV inhibitory; ACE inhibitory	0.201209				+
α–Casein-derived peptides	495.6421	1483.9044	1483.8762	30	**ILELA**VVSPIQFR	DPP-IV inhibitory; antioxidant	0.302035	+			
	494.7610	987.5075	987.4873	28	QENID**EL**K	Antioxidant	0.114525	+			
	478.3096	954.6046	954.5862	19	LLQLEAIR	DPP-IV inhibitory	0.1321	+			
	728.9621	1455.9097	1455.8701	25	K**ILELA**VVSPIQF	DPP-IV inhibitory; antioxidant	0.207185		+		
	664.9101	1327.8057	1327.7751	25	**ILELA**VVSPIQF	DPP-IV inhibitory; antioxidant	0.214756		+		
	490.9727	1469.8964	1469.8606	52	ILDLAV**VSP**IQFR	ACE inhibitory	0.3448			+	
	454.2391	1359.6954	1359.6630	59	QENID**EL**KDTR	Antioxidant	0.104182			+	
	742.9655	1483.9164	1483.8762	18	**ILELA**VVSPIQFR	DPP-IV inhibitory; antioxidant	0.302035				+
	494.7647	987.5149	987.4873	25	QENID**EL**K	Antioxidant	0.114525				+

Notes: DPP-IV: dipeptidyl-peptidase IV; ACE: angiotensin-converting enzyme.
[a]Mr (expt): experimental mass.
[b]Mr (calc): calculated mass.
[c]According to the MBPDB database of bioactive milk peptides.
[d]The probability of sequences as bioactive peptides was calculated in the Peptide Ranker (https://distilldeep.ucd.ie/PeptideRanker/ [accessed: 14 March 2024]).
[e]Ripening time in days.

α-CN (Cebo and Saadaoui, 2015), but bioactivity was not determined.

Discussion

This study aimed to investigate the effect of different starter cultures on the formation of bioactive peptides in camel milk cheese. Camel milk cheese, studied during 30 days of ripening was produced by different starter cultures, and showed peptide profiles throughout the entire ripening period. It should be noted that while the cheeses were briefly treated in hot whey (85–95°C for 5 min), which could reduce the activity of some microbial cultures, spore-forming bacteria and heat-resistant enzymes still could contribute to proteolysis during storage.

Principal component analysis revealed clear clustering patterns depending on both ripening time and type of starter cultures. Samples aged 20–30 days formed well-separated clusters, indicating that prolonged ripening significantly altered peptide profile in both mesophilic and thermophilic cheeses. This confirmed the progressive quality of proteolysis and the accumulation of specific peptides over time. In contrast, samples obtained on the 1st day and 10th day overlapped with each other, suggesting that early proteolytic changes were less pronounced and that peptide differentiation became more distinct after prolonged ripening.

Moreover, PCA plot for thermophilic cheeses (Figure 3B) showed a clear differentiation of samples across four ripening stages, indicating a consistent progression of proteolysis over time in this group. Compared to mesophilic PCA results (Figure 2B), thermophilic samples showed clearer temporal differentiation, which reflected the faster and more intense proteolytic activity typically associated with thermophilic cultures.

However, two outliers were found in the cheese with thermophilic cultures, namely, one sample from day 1 and another from day 20 were located outside the expected clusters. These deviations, absent in mesophilic PCA, could be the result of minor inconsistencies in enzyme–substrate interactions, differences in starter quality, or technical peculiarities. Despite these anomalies, the overall PCA structure remained stable and confirmed the dynamic, culture-dependent evolution of peptide profile during cheese ripening.

Identified peptides had ACE inhibitory (Tagliazucchi *et al.*, 2016), antimicrobial, and DPP-IV inhibitory properties (Nongonierma *et al.*, 2018). Such peptides appeared to be related to each other, which could be the result of a diverse and complex set of proteolytic enzymes acting on the cheese matrix (Gupta *et al.*, 2009; Korhonen and Pihlanto, 2006). The ILELAVVSPIQFR sequence found in α-CN origin in cheeses produced using mesophilic and thermophilic cultures had precursors of shorter sequence peptides such as ILELA, which showed DPP-IV inhibitory activity (Nongonierma *et al.*, 2018). The precursor peptide EL had antioxidant properties (Suetsuna *et al.*, 2000), whereas the precursor peptide VSP had ACE inhibitory properties (Weimann *et al.*, 2009). Based on the above-mentioned results, camel milk cheeses using mesophilic and thermophilic cultures for production contain peptides that have biological activity and beneficial to human health in various ways.

The use of thermophilic cultures in cheese production resulted in the earlier and more intense formation of key peptides, such as VLPVPPQQQMVPYPQR and GLHPVPQPLVPVIA, which exhibit ACE inhibitory and antioxidant activities. These peptides were detected on day 0 and persisted throughout the maturation period, indicating the high proteolytic activity of thermophilic bacteria at elevated temperatures (Fernández-Esplá and Rul, 1999). In contrast, cheeses made with mesophilic cultures had a broader peptide profile and contained longer sequences, such as IYTFPFPQPQSLVYSHT EPIPYPPYPILPQQNFLPPLQPAVM. This indicates slower but more sustained proteolysis. Some peptides with potential immunomodulatory functions, such as EMPLLQSPVVVPFTESQSLTLTDLTD LENLHLPLPLPLLQSL, were detected only in cheeses prepared using mesophilic cultures. This is probably due to specific enzyme–substrate interactions inherent to the mesophilic microbiota.

Although there was some overlap in the identities of peptides between two culture types, there were significant differences in the timing, number, and diversity of bioactive sequences. These results suggested that thermophilic cultures could be more suitable for the accelerated production of specific short-chain bioactive peptides, whereas mesophilic cultures had greater peptide complexity and a longer generation time. It is obvious from the results that the vast majority of peptides originate from β-CN, while the number of peptides that originate from α-CN is significantly less, as shown in Table 2. Considering the overall peptide profile, the greater presence of β-CN peptides is mainly explained by the fact that this casein fraction is more susceptible to hydrolysis. This is due to its open conformation, mainly the high content of proline in its primary chain (Bhat *et al.*, 2016).

One of the methodological limitations of this study was reliance on conventional LC-MS/MS techniques, which typically employed data-dependent acquisition (DDA) and were limited to targeted peptide identification. This approach could overlook low-abundance or unexpected bioactive peptides, especially in complex cheese matrices.

In contrast, recent advances in untargeted proteomics using high-resolution mass spectrometry (HRMS), such as Orbitrap or Q-TOF instruments, allow for deeper and more comprehensive peptide profiling with improved sensitivity and resolution (Toldrá *et al.*, 2021). In spite of this, research in the field of biopeptides offers promising opportunities, as various strains are used in the food industry, including cheese production, which show significant utility. Biopeptides have the potential to replace some pharmaceuticals, thus embodying the idea of "health through nutrition" (Kurbanova *et al.*, 2022). From a broader food science perspective, identifying peptides with potential biological activity (e.g. ACE inhibitors, antioxidants, and DPP-IV inhibitors) highlights the potential of camel milk cheese as a functional food product. The data obtained in this study would contribute to improve cheese production technology, as it provided insights into the peptide profiles generated during the fermentation of camel milk using specific cultures.

In addition, camel milk cheeses were studied previously as well for their biopeptide profile. While these properties require further *in vitro* and *in vivo* validation, our findings enrich databases on peptides from lesser-studied milk sources and pave the way for developing bioactive ingredients for dairy-based health products. The future studies must focus on determining the bioactivity of peptides in camel milk cheeses *in vivo* and *in vitro* to fill this gap in knowledge about their functionality and potential health effects, and to provide producers and consumers officially substantiated information on functionality and potential health outcomes of locally produced cheeses.

Conclusions

In this study, camel milk was fermented by mesophilic and thermophilic cultures. The peptides were analysed using LC-MS/MS through the Mascot 2.6.1 software. The biological activity of peptides and their precursors was identified in novel, 52 identified peptides using the MBPDB and Peptide Ranker database. Of the identified peptides, 21 were derived from β-CN in cheese samples using mesophilic culture, while five peptides were derived from α-CN. On the other hand, 17 peptides were identified in cheese using thermophilic culture, with nine peptides derived from α-CN. β-CN- and α-CN-derived peptides differ using mesophilic and thermophilic cultures in producing camel cheese during 30 days of ripening. According to the MBPDB database, they have ACE inhibitory, antioxidant, antimicrobial, and DPP-IV inhibitory properties. Furthermore, this study demonstrated that thermophilic cultures promoted earlier and more intense release of specific bioactive peptides, which persisted throughout the ripening and were associated with ACE inhibitory and antioxidant activity. In contrast, mesophilic cultures resulted in a broader and more diverse peptide profile, such as longer sequences with potential immunomodulatory effects. In spite of some overlap, marked differences were observed in peptide diversity, timing, and functioning. PCA confirmed distinct culture- and time-dependent evolution of peptide profiles, with thermophilic samples exhibiting more defined temporal separation. These findings suggested that culture type must be selected strategically to modulate the biofunctional potential of camel milk cheese during ripening.

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Author Contributions

All authors contributed equally to this article.

Competing Interests/Conflict of Interest

The authors had no relevant financial interests to disclose.

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