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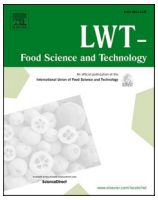

Bottom-up proteomics to investigate the X-ray irradiation effects on soft cheese

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ABSTRACT

Food irradiation is an alternative technological process employed to preserve the hygienic quality of food and to extend its shelf life. In this work, three different types of soft cheese, *mozzarella*, *robiola* and *primosale*, were treated with X-rays at 1.0, 2.0 and 3.0 kGy to evaluate their effect on proteins and peptides using a bottom-up proteomic approach. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-ToF-MS) and liquid chromatography coupled with MS through electrospray interface (LC-ESI-MS) were employed to analyse the peptide mixtures coming from the proteolytic digestion of soft cheese using both trypsin and chymotrypsin enzymes. We discovered 15 peptides most affected by X-rays that were sequenced by tandem MS and identified; ten of these discriminant peptides, generated from the cleavage of β-casein and α-casein (*B. taurus*), were localized on the proteins' secondary structure, highlighting a heightened susceptibility of random coils to X-ray irradiation. No additional post-translational modifications were found to be induced or emphasized by irradiation. For all studied cheese samples, the major spectral differences were encountered at 1.0 kGy, suggesting that such a dose was the most effective in promoting peptide bond cleavage. Some non-enzymatic and/or half-enzymatic peptides belonging to α-S1-, α-S2- and β-casein were also produced when varying X-ray doses.

1. Introduction

Currently, there is a growing consumer demand for natural and healthy food products, driving the exploration of unconventional methods for food preservation. Non-thermal sanitization techniques such as pulsed electric fields, pulsed light, irradiation, high hydrostatic pressure, and ultrasound have gained popularity in meeting these demands (Ricciardi et al., 2019). Among these techniques, food irradiation stands out as a rapid, clean, safe, and effective method for preserving the hygienic quality of food and extending its shelf life, especially for dairy products, without the need for temperature increase (Campaniello et al., 2020; Lacivita et al., 2019; Zianni et al., 2022). Irradiation can be carried out using beam (e-beam), γ-rays, or X-rays (Urbain, 1986). The latter two methods offer superior penetration capabilities and have been successfully applied for degrading pesticides in foodstuffs (Mir et al., 2022), preventing fungal infection and mycotoxin production in vegetables (Hossain et al., 2019; Iqbal, Amjad, Asi, & Ariño, 2012; Liu, Galani Yamdeu, Gong, & Orfila, 2020), and reducing the growth of pathogenic microorganisms by causing direct or indirect DNA damage (Farkas, 2006). In 1921, the U.S. Department of Agriculture (USDA) Bureau of Animals proposed the use of X-rays for food irradiation (O'bryan, Crandall, Ricke, & Olson, 2008), and research and development in this field have continued to evolve over the years. X-ray applications, particularly upon packaging, have been instrumental in preventing post-production contaminations (Lacivita et al., 2019).

Adhering to good irradiation practices, the recommended dose of X-rays on foodstuffs should not exceed 10 kilogray (kGy). For dairy products, only Camembert can be treated, with a maximum dose of 2.5 kGy (EFSA Journal, 2011; Roberts, 2016; Tomaiuolo et al., 2023). Despite the evaluation of irradiated foodstuffs in terms of microbial

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growth, the potential changes induced by X-rays on the food composition are of great significance (Zehi, Afshari, Noori, Jannat, & Hashemi, 2020). Studies have reported the formation of hydrocarbons, 2-alkylcyclobutanones, oxidized cholesterol, and furans (EFSA Journal, 2011; Feng & Ahn, 2016; Bliznyuk et al., 2022; Zianni et al., 2022).

Protein-rich foodstuffs subjected to irradiation undergo structural changes, including cross-linking, aggregation (Dogan, Siyakus, & Severcan, 2007), increased content of ortho-tyrosine (Hein, Simat, & Steinhart, 2000), radical species formation (Hawkins & Davies, 2001; Hellwig, 2019), dimerization, and/or truncation (Audette-Stuart, Houée-Levin, & Potier, 2005; Houée-Levin & Sicard-Roselli, 2001), folding of peptide chains and formation of intramolecular disulfide bonds (Stewart, 2021). Additionally, proteins can undergo modification through reactions with carbohydrates, via the well-known Maillard reaction (Carulli, Calvano, Palmisano, & Pischetsrieder, 2011), by primary and secondary lipid oxidation by-products, heat, metals, polyphenols, reactive oxygen species or free radicals.

To extend the shelf life of nutrient-dense cheese, non-thermal ionizing treatments have been recently employed (Tomaiuolo et al., 2023; Zianni et al., 2023). Cheese, being rich in high-quality proteins, lipids, vitamins, and minerals (Feeney, Lamichhane, & Sheehan, 2021), has a dynamic proteome that responds to various environmental stimuli (Agregán et al., 2021). Proteomics, the study of large-scale proteins and their modifications, becomes crucial in assessing food quality, digestibility, and nutritional values (Dupree et al., 2020; Moradian, Kalli, Sweredoski, & Hess, 2014).

Analytical techniques, particularly mass spectrometry (MS) with soft ionization sources like matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), have revolutionized proteomics (Fenn, Mann, Meng, Wong, & Whitehouse, 1989; Harvey, 1995; Nadler et al., 2017; Schiller et al., 2004). The shotgun proteomic approach, involving enzymatic digestion of protein mixtures followed by tandem MS peptide sequencing (i.e., peptide mass fingerprinting, PMF), allows for comprehensive, rapid, and sensitive characterization of proteins and peptides (McDonald & Yates, 2002; Wu & MacCoss, 2002). High-performance liquid chromatography coupled with tandem MS (RPLC-MS/MS) is also widely employed for peptide separation and identification (Bianco, Ventura, Calvano, Losito, & Cataldi, 2022a, 2022b; Dupree et al., 2020; Fonslow & Yates, 2012; McDonald & Yates, 2002; Zhang, Fonslow, Shan, Baek, & Yates, 2013).

In this study, a bottom-up proteomic approach was applied to investigate the effects of X-ray irradiation on the proteomic profile of three soft cheese types, mozzarella, primosale, and robiola, at dose levels of 1.0, 2.0, and 3.0 kGy. To enhance sequence coverage, trypsin and chymotrypsin were used in solution digestion (Calvano, Rigante, Cataldi, & Sabbatini, 2020). Collected data of peptides from all treated and untreated cheeses were compared by multivariate data analysis based on principal component analysis (PCA). Preliminary findings illustrate as some modified alongside aspecific non-tryptic, semi-tryptic and/or non-chymotryptic peptides can be useful markers of irradiation.

This innovative research contributes to our understanding of how X-ray irradiation impacts the proteomic composition of soft cheeses, providing valuable insights for both the scientific community and consumers.

2. Materials and methods

2.1. Chemicals

Deionized water, LC-MS water, acetonitrile (ACN), formic acid (FA), ammonium bicarbonate, α-cyano-4-chloro-cinnamic acid (CClCA), dithiothreitol (DTT), iodoacetamide, trypsin (proteomics grade from porcine pancreas), and chymotrypsin (sequencing grade from bovine pancreas) were acquired from Sigma-Aldrich (Milan, Italy). RapiGest^{SF} Surfactant was purchased from Waters (Milford, MA, USA). Three types of cheese of different brands (see the following paragraph), namely *mozzarella*, *robiola* and *primosale*, were bought from local markets.

2.2. Samples

Mozzarella cheese used for experiments was from brands Land (*mL*), Gioiella (*mG*) and Saporosa (*mS*). Gioiella was a freshly handmade product without preservatives. For *robiola* cheese the following brands were tested: Nonno Nanni (*rN*, made from bovine milk), Nonno Nanni and Spega from goat milk (*rNg* and *rSg* respectively). The following *primosale* cheese brands were used: Nonno Nanni (*pN*), Osella (*pO*) both from bovine milk and Spega made from goat milk (*pSg*).

2.3. X-ray irradiation procedure

The samples were placed into 500 mL carbon fiber tubes with a diameter of 80 mm and the irradiation treatment was performed using a low-energy X-rays irradiator (RS-2400, Radsource Inc., Suwanee, GA, USA) operating at 150 kV and 45 mA at room temperature. . Electrons (e−) were emitted from a heated tungsten filament and they were accelerated by a potential difference to reach a thin metal layer (gold). Hence, the path of accelerated e−was deflected by positive charges of gold nuclei, emitting X-ray photons (Fig. S1 in Supplementary material). An alanine/electron paramagnetic resonance dosimetry system was employed; the calibration curve-dose amplitude of the alanine signal was obtained with a calibrated ionization chamber (Rad-cal Inc., Monrovia, CA, USA). For this investigation, levels of 1.0, 2.0 and 3.0 kGy at a dose rate of approximately 2 kGy/h were used with a calculate uncertainty of delivery dose around 5%.

2.4. Protein extraction and enzymatic digestion

For proteomic investigations, 2 g of each cheese type (mozzarella, robiola and primosale) at different radiation doses (non-irradiated, 1.0, 2.0 and 3.0 kGy) were mashed in a mortar, added with 10 mL deionized water, and kept under magnetic stirring at 50 °C for 30 min to promote the extraction of serum protein and casein fraction. Previous trials on cheese samples proved that such an approach was time-saving and efficient enough in casein extraction to avoid the casein precipitation step with glacial acetic acid. One hundred μL of the extraction solution and 100 μL RapiGest™ reagent (1 mg/mL) in NH_4HCO_3 50 mM were combined and kept at 60 °C for 1 h to attain protein denaturation. Disulphide bonds scission and thiol moieties alkylation were respectively performed with 20 μL DTT 50 mM (incubated for 20 min at 60 °C) and 20 μL iodoacetamide 150 mM (in dark at room temperature for 15 min). Five μL trypsin solution plus 5 μL chymotrypsin solution (both 20 μg/mL in ammonium bicarbonate 25 mM) were finally added to the mixture. Previous works evidenced that, in a multi-enzyme approach, the proteases work synergically in reaching a higher number of proteolytic cleavage sites providing a significant increase in peptides identification and protein coverage (Calvano et al., 2020; Nardiello, Natale, Palermo, Quinto & Centonze, 2015, 2018; Swaney, Wenger, & Coon, 2010). After overnight incubation at 37 °C, enzymatic digestion was interrupted with 5 μL formic acid 98%. Samples were ultra-centrifuged at 14300 g for 20 min and 6 μL of supernatant were used for RPLC-ESI(+)-FTMS/MS injection or mixed with matrix for MALDI MS analysis. The whole procedure was performed in triplicate.

2.5. MALDI–ToF–MS

A 5800 MALDI-ToF/ToF analyzer (AB SCIEX, Darmstadt, Germany), equipped with a neodymium-doped yttrium lithium fluoride (Nd:YLF) laser (345 nm), was employed in reflectron positive mode at 10 ppm mass accuracy. Sample solutions were mixed 1:1 (v:v) with matrix CClCA (10 mg/mL in 70:30 ACN:H_2O plus 0.1% FA), 1 μL of the mixture was applied on a metallic plate (three spots per sample) and dried at room temperature. To remove salts, each spot was washed with 1 μL H_2O

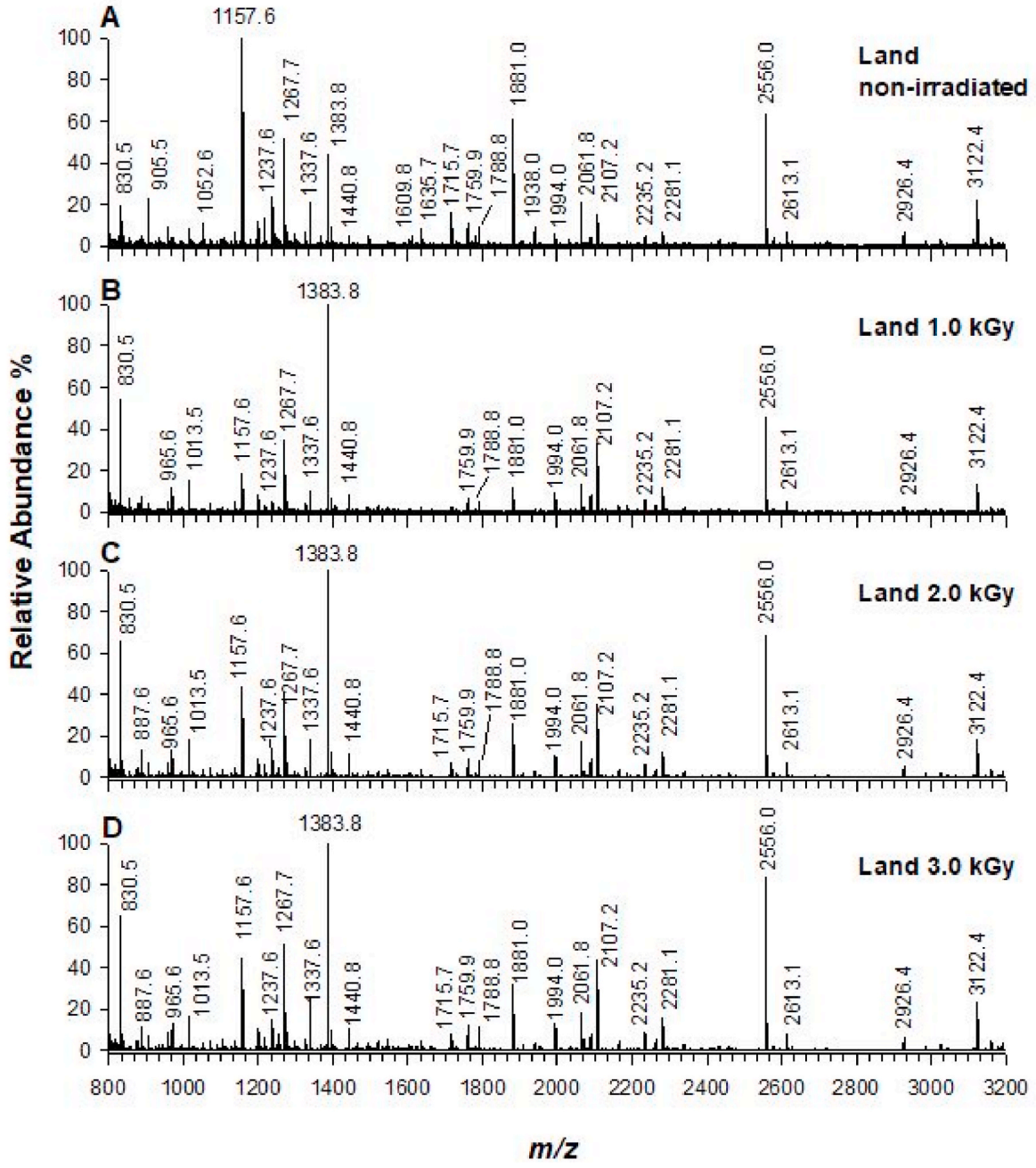

Fig. 1. Peptide mass fingerprinting (PMF) of (A) mozzarella Land control sample (*mL0*), (B) X-rays irradiated samples at 1.0 (*mL1*), (C) 2.0 (*mL2*), and (D) 3.0 kGy (*mL3*).

LC–MS grade. Mass calibration was performed with a mixture of standard peptides: renin, angiotensin I and ACTH. Spectra were acquired in the range *m/z* 800–4000. Final mass spectra were averaged on at least five single spectra each one resulting from 1000 laser shots at a 400 Hz laser pulse rate. The delayed extraction time was set at 240 ns.

2.6. High–resolution RPLC–ESI(+)–FTMS/MS

Reversed-phase liquid chromatographic runs were performed using an Ultimate 3000 HPLC (Dionex, Thermo Scientific, Waltham, MA, USA) coupled with a mass spectrometer apparatus through the HESI (heated electrospray ionization) source. The chromatographic section was composed of a C18 column Aeris™ 3.6 μm WIDEPORE XB-C18 250 × 2.1 mm equipped with a Phenomenex AJO 8783 WIDEPORE C18 (2 × 2.1 mm) guard cartridge (Phenomenex, Torrance, USA) working at 0.200 mL/min flow and 40 °C. The mobile phases were H_2O (A phase) and ACN (B phase) both with 0.1% FA in volume. The mobile phase elution gradient was: min 0-2 at 5% B, min 2-20 from 5% to 60% B, min 20-22 from 60% to 100% B, min 22-26 at 100% B, min 26-30 from 100% to 5% B, min 30-35 re-equilibration at 5% B.

High resolution and <5 ppm accuracy mass spectra were obtained with a Q-Exactive mass spectrometer equipped with a quadrupole/FT-Orbitrap™ analyzer (Thermo Scientific, Waltham, MA, USA). Mass spectrometer parameters were at *m/z* range 200–2000; capillary temperature 320 °C; source heater temperature 45 °C; sheath gas flow rate 35 arbitrary units (a.u.); spray voltage 3.5 kV; S-Lens RF Level 60%. Tandem MS experiments were achieved in HCD mode (High Energy Collisional Dissociation) with 35% a.u. collisional energy. Resolving power was set at 140,000 and 70,000 for full scan and MS/MS respectively. Tandem MS experiments were performed also in CID (collision-

Table 1
Survey of the proteins identified by MALDI-ToF-MS of X-ray irradiated soft cheese samples of mozzarella Land (*mL*).

Protein	Accession number	Protein coverage (%)	Number of peptides
α-S2-casein	P02663	60.8	20
β-casein	P02666	51.8	14
β-lactoglobulin	P02754	48.9	9
α-S1-casein	P02662	38.8	18
α-lactalbumin	P00711	28.9	5
κ-casein	P02668	16.8	4

induced dissociation) fragmentation mode with 35% a.u. collisional energy using a Velos Pro analyzer (Thermo Scientific, Waltham, MA, USA). The chromatographic column, mobile phase flow parameters, and elution gradient were the same as described in the previous paragraph while mass spectrometer parameters were at *m/z* range 200–2000; capillary temperature 320 °C; source heater temperature 45 °C; sheath gas flow rate 35 arbitrary units (a.u.); spray voltage 3.5 kV; S-Lens RF Level 60%.

2.7. MS data processing

MALDI-MS and RPLC-ESI-MS(/MS) raw files were processed using the software Data Explorer® 4.11 (©2010 AB SCIEX, Darmstadt, Germany) and Xcalibur™ 4.1 (Thermo Scientific, Waltham, MA, USA) respectively. mMass 5.5.1 was used to extract the peak lists as txt files.

2.8. Statistical data processing

X-ray treatment effects on cheese samples were studied from the proteomic point of view using principal component analysis (PCA) applied to MALDI-ToF-MS spectra. For each sample, three mass spectra replicates were considered. MS peak intensities were normalized to the base peak and the dataset was built using relative percentage intensities of monoisotopic peaks having rel. int. % ≥ 4% and signal-to-noise (S/N) ratio ≥10. The *m/z* peak intensities were used as variables thus, depending on the sample, several variables ranging from 55 to 155 were considered. PCA was performed using the software MATLAB (MathWorks®, Natick, Massachusetts, USA). Data were centered and up to 3 principal components were set.

3. Results and discussion

3.1. MALDI–MS PMF of X-ray irradiated mozzarella soft cheeses

Fig. 1 shows four MALDI-ToF-MS spectra obtained after digestion of a non-irradiated control sample (A) and X-ray irradiated mozzarella Land (*mL*) samples at increasing irradiation doses, *viz.* 1.0 (B), 2.0 (C), and 3.0 kGy (D), respectively. The online software Protein Prospector MS Fit was employed (https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard) to assess peptide's identity based on their *m/z* values by a PMF approach. Depending on the sample, the search was performed using SwissProt 2021.06.18 and UniProtKB 2020.09.02 databases within *Bos taurus* or *Capra hircus* taxonomies. The following search conditions were applied: tolerance of 100 ppm, four acceptable missed cleavages, oxidation of methionine and proline, deamidation of asparagine/glutamine, and phosphorylation of serine/threonine/tyrosine as a variable modification. Carbamidomethylation of cysteine was also set as a constant modification. Mainly peptides coming from the chymotryptic digestion of caseins (α-S1, α-S2, β, and κ-casein) and serum proteins (β-lactoglobulin, α-lactalbumin) were registered as the most probable candidates in every sample, and it was possible to assess the percentage of protein coverage, which is the portion of the mapped sequence. Table 1 lists the identified proteins in "*mL*" samples together with the number of peptides exhibiting theoretical *m/z* matching with the experimental ones within the tolerance set, and the corresponding protein coverages. Regardless of the X-ray irradiation dose, MALDI-ToF-MS spectra of Fig. 1 seemed to be very similar meaning that the same peptides were generated after proteolytic digestion. Nevertheless, on increasing the X-ray dose, some differences were noted mainly due to the relative intensities of peak signals such as at *m/z* 1157.6, 1383.8 and 1881.0. To highlight the likely discriminating peptides, three samples of the same type and cheese brand were investigated by PCA as described in the Statistical Data Processing paragraph. Fig. 2 displays the biplot of "*mL*" irradiated samples at 1.0, 2.0 and 3.0 kGy compared with control ones. The ions at *m/z* 830.5, 1157.6, 1267.7, 1337.6, 1383.8, 1881.0, 2107.2, and 2556.1, represent the variables that mainly affect the principal components. Specifically, the clear separation in the biplot between control and irradiated samples along the principal component 1 (PC1) was mainly due to two peptides at *m/z* 1157.6 and 1383.8. The peak intensity of the last signal increased going from control to irradiated samples, while the signal at *m/z* 1157.6

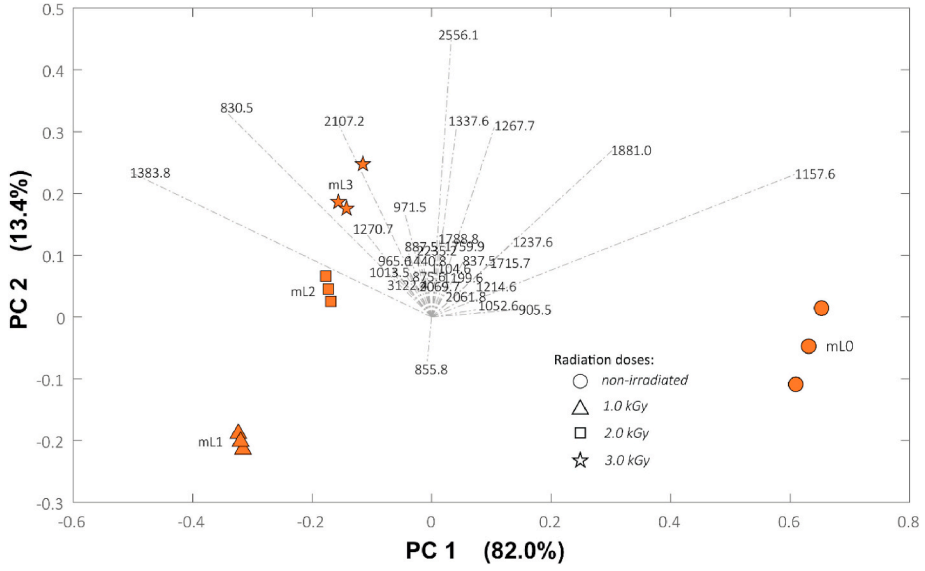

Fig. 2. PCA biplot of X-rays irradiated soft cheese samples of mozzarella Land (*mL1, mL2, mL3*) and control (*mL0*). Only variables with loadings higher than |0.05| in at least one principal component were plotted.

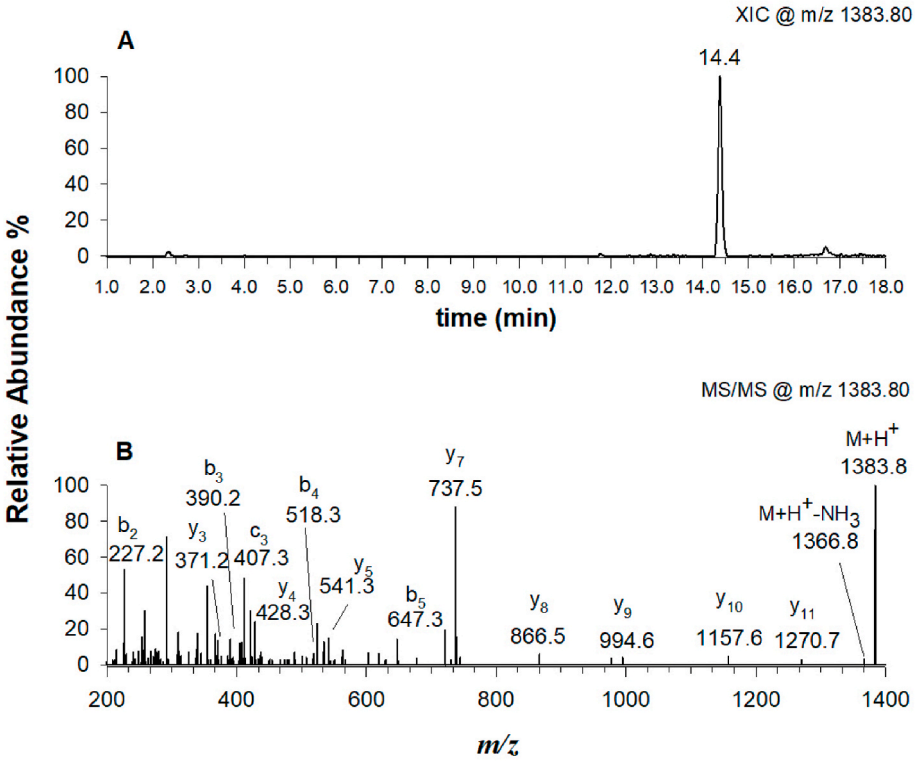

Fig. 3. (A) extracted ion current (EIC) chromatogram of a non-irradiated mozzarella Land sample. (B) HCD tandem MS spectrum of a precursor monocharged ion at experimental *m/z* 1383.7964 assigned to the peptide sequence LLYQEPVLGPVR from β-casein of *B. taurus* (monoisotopic mass 1382.781).

Table 2
Main differences between PMFs of non-irradiated and irradiated mozzarella Land samples.

Experimental *m/z*	Theoretical *m/z*	Peptide sequence	Protein (*B. taurus*)	Peptide sequence position	Rel. abundance % variation
830.5	830.4519	(K)AVPYPQR(D)	β-casein	192–198	+
1157.6	1157.6314	(L)YQEPVLGPVR(G)	β-casein	208–217	–
1267.7	1267.7045	(R)YLGYLEQLLR(L)	α-S1-casein	106–115	+
1337.6	1337.6808	(K)HIQKEDVPSER(Y)	α-S1-casein	95–105	+
1383.8	1383.7995	(F)LLYQEPVLGPVR(G)	β-casein	206–217	+
1881.0	1880.9496	(L)YQEPVLGPVRGPFPIIV(−)	β-casein	208–224	+ (1.0–3.0 kGy)
2107.2	2107.2314	(F)LLYQEPVLGPVRGPFPIIV(−)	β-casein	206–224	+ (0–3.0 kGy)
2556.1	2556.0926	(K)FQSEEQQQTEDELQDKIHPF(A) 1xPhos [S/T]	β-casein	48–67	− (1.0 kGy)

Legend: (+) Relative abundance % increasing. (−) Relative abundance % decreasing.

exhibited an opposite trend. Moreover, the X-ray irradiated samples were rather separated on the PC2 mainly due to peptide at *m/z* 2556.1 whose relative abundance was enhanced from 1.0 to 3.0 kGy of X-ray irradiation dose.

By the same approach, robiola and primosale soft cheeses from various brands were analyzed (see below).

3.2. RPLC-ESI(+)-FTMS/MS of X-ray irradiated mozzarella soft cheeses

Given the complexity of the sample and the presence of isobaric and isomeric peptides, we conducted RPLC-ESI-FTMS and tandem MS analyses to ensure confident identification of peptides. The chromatographic dimension facilitated the separation of complex mixtures, and the FTMS mass accuracy of <5 ppm enabled the differentiation of isomeric peptides. The impact of mass accuracy on distinguishing isobaric peptides is particularly significant when transitioning from low-resolution (LR) to high-resolution (HR). Bioinformatic research revealed that the precursor ion at *m/z* 1383.8, as observed in the MALDI spectrum, could be associated with two possible sequences within the specified tolerance: SRYPSYGLNYY, indicating deamidated asparagine/arginine (N/R) of bovine κ-casein, and/or LLYQEPVLGPVR, originating from β-casein with theoretical monoisotopic *m/z* values of 1383.6216 and 1383.7995, respectively. In HR-RPLC, only one monocharged ion eluted at 14.4 min (as shown in plot A of Fig. 3) from the "*mL*" samples, and it was observed at *m/z* 1383.7964 with a mass accuracy of −2.2 ppm; the corresponding mass spectrum is reported in Fig. 3B. Detailed analysis of the product ion spectrum in Fig. 3A provided proofs for the peptide sequence (F)LLYQEPVLGPVR(G) generated from bovine β-casein. The discriminant ions, which primarily account for the separation observed in the biplot of Fig. 2, were similarly identified and annotated. Table 2 reports the peptide's sequence, both the experimental and theoretical *m/z* values of the monocharged ion, their respective positions in the protein sequence, and the corresponding intensity variations. Fig. 4 presents the HCD-MS/MS spectra of five selected peptides mentioned earlier. It's noteworthy that, except for the doubly charged ion at *m/z* 634.36^{2+} (depicted in panel C of Fig. 4), originating from α-S1-casein, all the distinctive peptides identified in the tryptic/chymotryptic digest were derived from β-casein. The doubly charged ions at *m/z* 941.04^{2+} and 1054.12^{2+} (see panels D and E of Fig. 4), corresponding to the mono-charged peptides at theoretical *m/z* 1880.9496 and 2107.2314 in Table 2, were respectively identified as (L)YQEPVLGPVRGPFPIIV(−) and (F)LLYQEPVLGPVRGPFPIIV(−) of

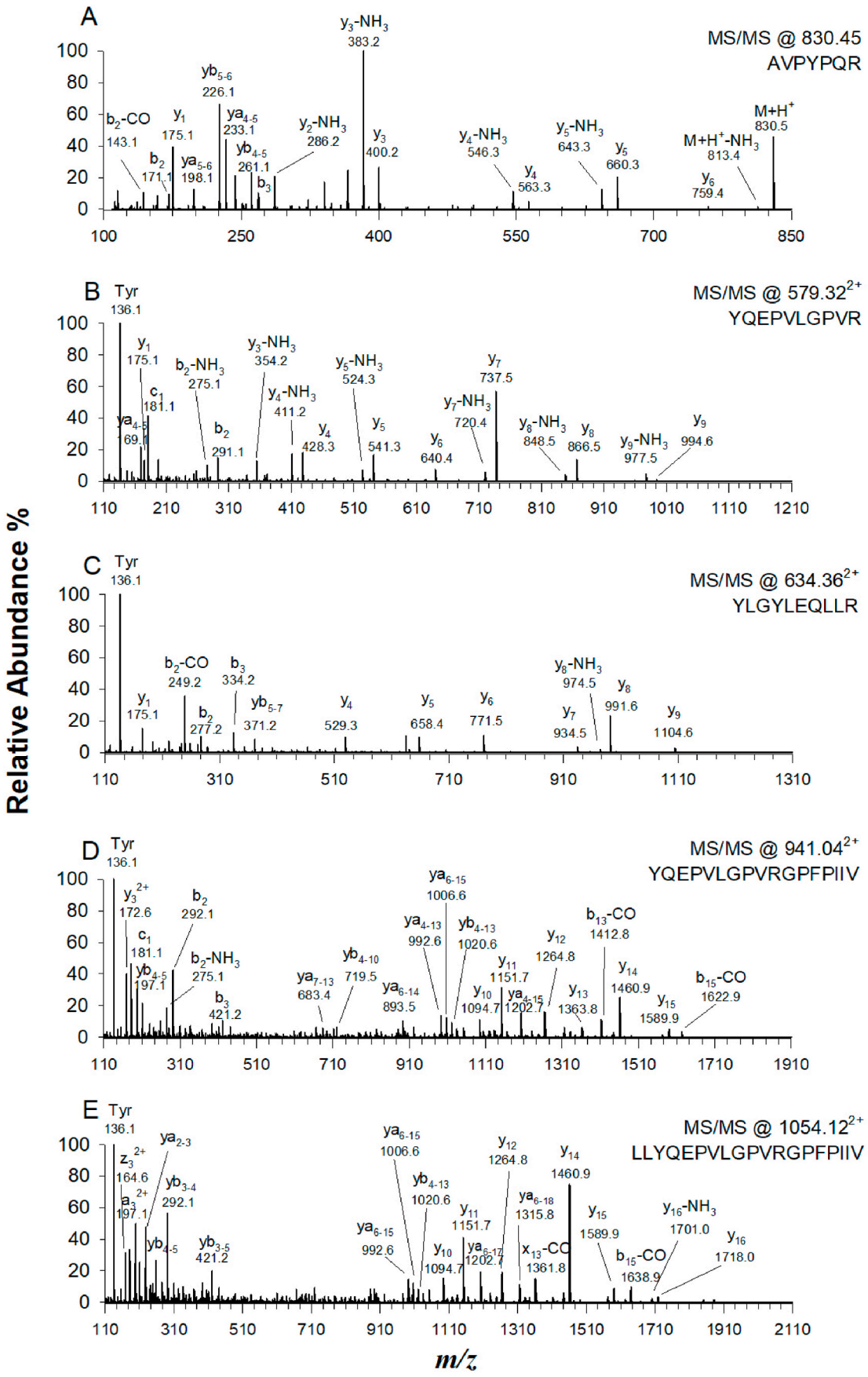

Fig. 4. Tandem MS spectra in positive HCD mode of five discriminant peptides of mozzarella Land samples.

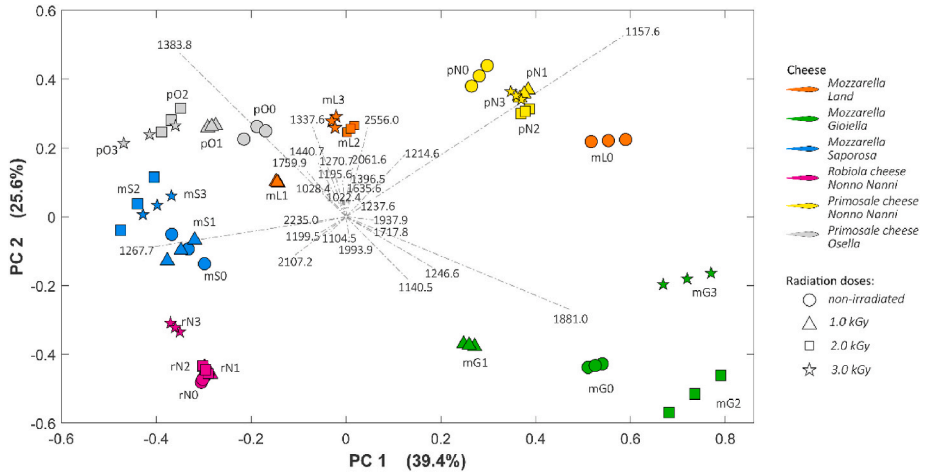

Fig. 5. Biplot of X-rays irradiated soft cheese samples of mozzarella Land (*mL1, mL2, mL3*), Gioiella (*mG1, mG2, mG3*) and Saporosa (*mS1, mS2, mS3*), robiola cheese Nonno Nanni (*rN1, rN2, rN3*) and primosale cheese Nonno Nanni (*pN1, pN2, pN3*), Osella (*pO1, pO2, pO3*) and controls (*mL0, mG0, mS0, rN0, pN0, and pO0*). Only variables with loadings higher than |0.05| in at least one principal component were plotted.

β-casein. We tried to correlate these peptides' susceptibility to X-ray irradiation with the secondary/tertiary structure of their proteins. According to the AlphaFold database, which provides the prediction of 3D protein structures, the amino acid sequence 206–224, LLYQEPVLGPVRGPFPIIV, is located at the end of *B. taurus* β-casein, where the protein is organized into a random coil structure (Jumper et al., 2021; Varadi et al., 2022). Remarkably, six out of eight ions reported in Table 2 (*m/z* 830.5, 1157.6, 1337.6, 1383.8, 1881.0, and 2107.2) belong to random coil regions of β- and α-S1-casein's secondary structure.

Recently, Genové et al. (Genové, Betriu, & Semino, 2022) working

Table 3
Survey of the peptides having variable peak relative abundance % in MALDI-ToF-MS of cheese samples (in bold discriminant peptides resulting also from PCA data). The number in the slot indicates the degree of X-rays absorbed dose where that peptide is variable. Empty slots indicate that no rel. int. % trends were observed.

m/z	Mozzarella			Robiola			Primosale		
	Land (*mL*)	Gioiella (*mG*)	Saporosa (*mS*)	Nonno Nanni (*rN*)	Nonno Nanni goat (*rNg*)	Spega goat (*rSg*)	Nonno Nanni (*pN*)	Osella (*pO*)	Spega goat (*pSg*)
830.5	**+ (0 to 3)**	+ (0–1,2,3)			− (0–3)				
902.5					− (0–3)				
905.5	**− (0 to 3)**	**+ (0 to 1,2,3)**							
965.6	+ (0–3)	**+ (0 to 1); − (1 to 2,3)**		- (1)					
971.5	+ (0–3)	+ (1)		− (0–1,2,3)					
1022.5		+ (1)		− (1)					
1109.6	− (0–3)	+ (3)							
1157.6	**− (0 to 1); + (1 to 2,3);**	**+ (3)**			**− (3)**				
1214.6	- (0–1); + (1–2,3)	+ (3)			− (3)				
1237.6	- (0–1); + (1–2,3)	+ (3)			− (3)				
1246.6	− (0–1,2,3)	+ (0,1 to 2,3)			− (3)				
1252.6	+ (0–3)			3					
1267.7	**− (0 to 1); + (1 to 2,3)**	+ (1)					− (0–1,2,3)	**+ (0 to 3)**	
1270.7	+				− (3)				
1324.7				+ (3)	+ (3)		− (0–1,2,3)		
1337.6	− (0–1); + (1–2,3)	+ (1)		+ (0,1 to 2,3)				+ (0–1,2,3)	
1367.7	− (0–1,2,3)				− (3)				
1383.8	**+ (0 to 1,2,3)**	+ (0–1); − (1–2,3)		**+ (3)**	**− (3)**				
1396.6		+ (3)			− (0–3)				
1759.9		+ (1)		− (0–1); + (1–2,3)			− (0–1,2,3)	+ (0–1,2,3)	
1781.9	− (0–1,2,3)				− (0–3)				
1788.8	− (0–1); + (1–2,3)			+ (3)					
1881.0	**− (0 to 1); + (1 to 2,3)**			− (0–3)					
2061.7		3		3					
2107.2	+	**+ (1)**		− (0–3)				− (1)	
2556.0	**− (0 to 1); + (1 to 2,3)**	**+ (3)**	**+ (0,1 to 2,3)**				− (0–1,2,3)		
2613.1	− (0–1); + (1–2,3)	+ (3)							
2616.1		1		− (0–1,2,3)					
2909.4		1		− (3)					

Legend: 0 = non-irradiated; 1 = 1.0 kGy dose; 2 = 2.0 kGy dose; 3 = 3.0 kGy dose. + = rel. int. % increasing; − = rel. int. % decreasing.

Fig. 6. Position of the peptides susceptible to peak intensity variations in MALDI-MS spectra of soft cheese samples. Red and blue indicate random coil and alpha helix structures, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with amphiphilic self-assembling peptides as a candidate biomaterial for tissue engineering, demonstrated that the transition from β-sheet to random coil configuration is promoted by a temperature increase. Thus, the loss of β-sheet configuration impacts the cleavage site accessibility being more susceptible to protease attack, so thermally denatured samples had a higher extent of degradation than control ones. Similar behaviour may be invoked upon X-ray-treated samples of mozzarella cheeses and trypsin + chymotrypsin digest. In this case, the irradiation can trigger the transition from β-sheet arrangement in control samples to random coil as evidenced by the higher number of peptides generated in this section of secondary structure for treated cheeses.

The RPLC-ESI-MS/MS analyses were performed in the same way for robiola and soft primosale cheeses of various brands (see discussion below).

3.3. PCA of X-ray irradiated mozzarella, robiola and primosale soft cheese

To verify whether these preliminary results on mozzarella Land were cheese-dependent, other soft cheeses were investigated by MALDI-ToF-MS, including all bovine milk ones such as mozzarella Gioiella (*mG*), mozzarella Saporosa (*mS*), robiola Nonno Nanni (*rN*), primosale Nonno Nanni (*pN*), and primosale Osella (*pO*). Mass spectra were examined by PCA and the biplot is illustrated in Fig. 5. The samples *pO* and *mS* did not display significant differences in increasing the X-ray irradiation as their proteome was nearly unaffected (see biplot, Fig. 5). The *primosale* Nonno Nanni (*pN*) exhibited a slight separation between control, *pN0*, and irradiated samples mainly due to peptides at *m/z* 1267.7, 1324.7 and 2556.0 which showed a decreasing trend in relative abundances registered in MALDI spectra (Fig. S2). The main differences were noticed with *mL*, along with *mG* and *rN*. In this last sample, the separation in the biplot was significant at the highest irradiation dose of 3.0 kGy, whereby the peptide at *m/z* 1383.8 from β-casein was much increased. The biplot of *mG* exhibited a more pronounced dispersion for *mG0*, *mG1*, *mG2* and *mG3* samples (Fig. 5). This finding could be explained considering that *mG* is a handcrafted product which contains much fewer artificial preservatives and additives than the other soft cheese investigated here. Indeed, *mG* samples exhibited a minor shelf-life and were more susceptible to X-ray treatments. Moreover, handcrafted production has a lower reproducibility compared to industrial one, thus affecting data variability and dispersion even more than X-ray irradiation itself.

Goat milk samples, *rNg*, *rSg* and *pNg*, were analogously examined and seemed to be more resistant to irradiation treatment. Indeed, in all cases, PCA plots pointed out no significant trend in ions intensities nor discriminating peptides useful to assess any difference between irradiated and non-irradiated samples (see, as an example, MALDI-TOF-MS spectra of samples rNg in Fig. S3).

3.4. X-ray irradiation effect on soft cheese peptides

A manual comparison of the MALDI-ToF-MS spectra of each sample at different irradiation degrees supported statistical treatment to rescue some additional clues. Table 3 lists the peptides exhibiting variable intensity in MALDI-MS spectra for all soft cheese samples. Fifteen of them, i.e., at *m/z* 830.5, 965.6, 971.5, 1157.6, 1214.6, 1237.6, 1246.6, 1267.7, 1324.7, 1337.6, 1383.8, 1759.9, 1881.0, 2107.2 and 2556.0, were susceptible to X-ray irradiation in three or more of the investigated cheeses, with the peak intensity varying differently among the samples (see a few MS/MS spectra in Fig. S4). For example, in mozzarella Land, the peptide sequence at *m/z* 965.6 displays an increasing relative intensity from control (no irradiation) to 3.0 kGy, whereas a different scenario was observed with *mG* where the peak intensity increases from 0 to 1.0 kGy, and again decreases at 3.0 kGy. The same peptide in sample *rN* lowered the peak intensity at dose 1.0 kGy. Typically, the major spectral differences were encountered in cheese samples at 1.0 kGy of X-ray irradiation, thus suggesting that this dose was the most effective in promoting cleavages of peptide bonds. Both samples of goat milk Spega brand showed negligible changes as PMFs of irradiated and control samples. Likewise, primosale cheese was globally less affected by X-ray irradiation, whereby the main differences involved the peptide at *m/z* 1267.7 whose peak intensity changed with irradiation in the soft cheese samples of *pN*, *mL* and *mG*. Most X-rays affected peptides were

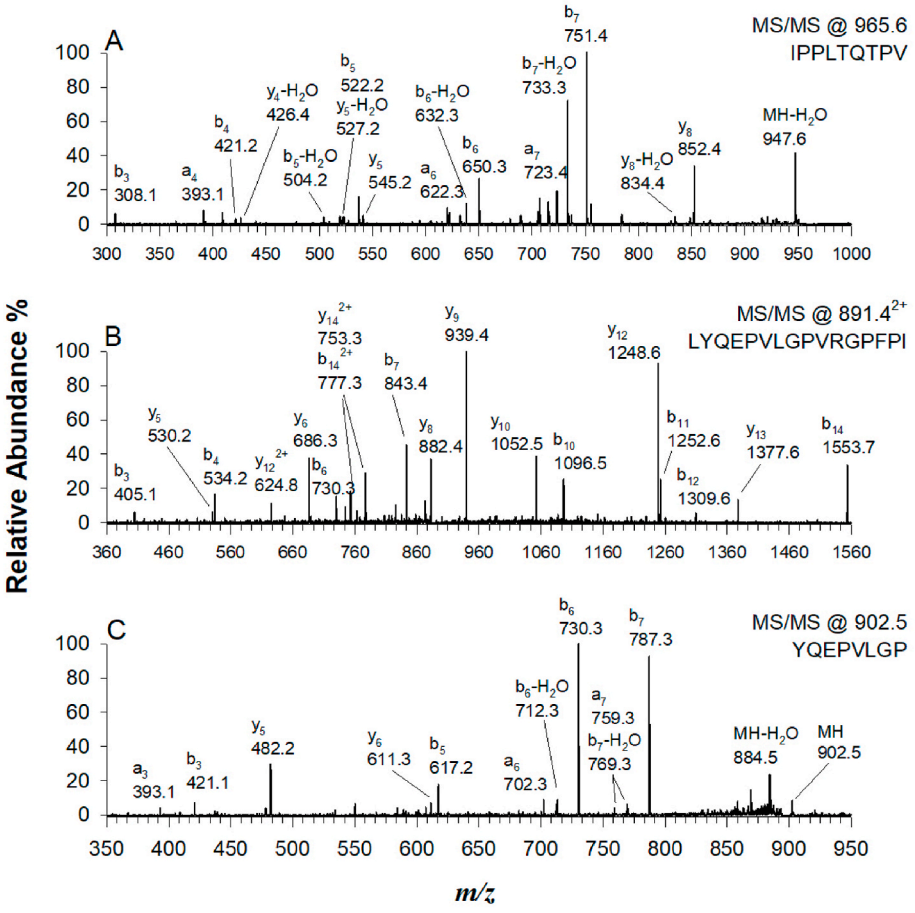

Fig. 7. CID tandem MS spectra of three non-specific, semi-chymotryptic, sequences. Precursor ions at (A) *m/z* 965.6 (N)IPPLTQTPV(V) from β-casein (*B. taurus*), (B) *m/z* 891.4 (L)LYQEPVLGPVRGPFPI(I) from β-casein (*B. taurus*) (*m/z* 1781.9 of the monocharged ion), and (C) *m/z* 902.5 (L)YQEPVLGP(V) β-casein (*C. hircus*).

sequenced through tandem MS experiments. Fig. 6 reports the β- and α-S1-casein amino acid sequences highlighting the 15 peptides found more susceptible to MS peak intensity variation.

3.5. *Non-proteolytic and semi-proteolytic peptides of X-ray irradiated soft cheese*

More exhaustive protein coverages were obtained by bioinformatics searching with Protein Prospector MS-tag to establish peptide identity using the option *"no enzyme"*. With this approach, several peptides were found to be *non-tryptic*, *semi-tryptic* and/or *non-chymotryptic, semi-chymotryptic* meaning that the cleavage did not occur where typically expected when using such proteases. As known, trypsin cleaves at the C-terminal sides of arginine (R) and lysine (K), when they are not followed by a proline (P), while chymotrypsin cleaves at the C-terminal of aromatic amino acids tyrosine (Y), phenylalanine (F), and tryptophan (W). Anyway, the cleavage of leucine (L), methionine (M), alanine (A), asparagine (N), and glutamine (Q) at a lower rate was also reported (Alves et al., 2008; Olsen, Ong, & Mann, 2004; Ziebuhr, 2000). Three examples of tandem MS spectra of monoprotonated peptides at *m/z* 956.6 and 902.5, and a doubly protonated peptide at *m/z* 891.9 are shown in plots (A), (B) and (C) of Fig. 7, respectively. The protonated sequence (N)IPPLTQTPV(V) at *m/z* 965.6 of bovine β-casein is a semi-chymotryptic peptide of mozzarella samples; its peak intensity

Table 4
Summary of the non-specific or semi-specific peptides retrieved. Where not differently indicated, taxonomy must be considered *B. taurus*. When rel. int. % of a certain ion was unvaried with irradiation degrees in MALDI mass spectra, the sample is not listed in the relative column.

	m/z	Sequence	Protein	Sample	
Bovine	965.6	(N)IPPLTQTPV(V)	β-casein	*mL*	+ (0–1,2,3)
				mG	+ (1)
				mS	+ (1)
				rN	− (1)
	1213.2	(P)IVLNPWDQVK(R) [N4 deam]	α-S2-casein		
	1622.9	(H)QGLPQEVLNENLLR(F)	α-S1-casein		
	1781.9	(L)LYQEPVLGPVRGPFPI(I)	β-casein	*mL*	− (0–1,2,3)
	1946.9	(L)AMAASDISLLDAQSAPLRV(Y) [Q13 deam; R18 deam; M2 ox]	β-lactoglobulin	*mG*	+ (2,3)
	2555.4	(P)SFSDIPNPIGSENSGKTTMPLW(−) [N7 deam; S3 phospho; P8 ox; S1 sulfo]	α-S1-casein	*mG*	+ (3)
Goat	902.5	(L)YQEPVLGP(V)	β-casein (*C. hircus*)	*rNg*	− (0–1,2,3)
	1151.7	(P)VLGPVRGPFPI(L)	β-casein (*C. hircus*)	*rNg*	− (0–1,2,3)

Legend: 0 = non-irradiated sample; 1 = 1.0 kGy absorbed dose; 2 = 2.0 kGy absorbed dose; 3 = 3.0 kGy absorbed dose. + = rel. int. % increasing; − = rel. int. % decreasing.

Fig. 8. The position of the non-specific peptides is summarised in Table 4. Red, blue, and green indicate random coil, α-helix and β-sheet structures respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

increased from 0 to 3.0 kGy in mozzarella Land, and up to 1.0 kGy in brands Saporosa and Gioiella. Conversely, the longer peptide (L) LYQEPVLGPVRGPFPI(I) at *m/z* 891.9, generated from β-casein of *B. taurus*, displayed a decreasing intensity from 0 to 3.0 kGy in *mL*. Analogously, the sequence (L)YQEPVLGP(V) at *m/z* 902.5 from β-casein (*C. hircus*) exhibited the highest intensity in non-irradiated samples of goat-milk *rN*. Table 4 gathers all the non-specific sequences identified by tandem MS experiments. As reported in Fig. 8, most of the non-specific peptides correspond to random coil secondary structures (in red), which are possibly more susceptible to X-ray irradiation, being less rigid with more exposed amino acids, and mostly externally localized on the tertiary structure (Jumper et al., 2021; Varadi et al., 2022). However, as inferable from the information reported in Table 4, no simple relationship was found between the peak intensity of proteolytic or non-proteolytic peptides and increasing X-ray irradiation doses. In other cases, no particular trends were observed meaning that some unspecific peptides formation can be independent of irradiation conditions and rather correlated to truncation of tryptic and chymotryptic peptides due to peptide's high length and mass (Alves et al., 2008) such as the ion at *m/z* 1622.9 (H)QGLPQEVLNENLLR(F) from α-S1-casein which is the truncated form of the tryptic peptide (K)HQGLPQEVLNENLLR(F) at *m/z* 1759.9.

4. Conclusions

The present findings give a better knowledge of the X-ray irradiation effects on soft cheese. A bottom-up proteomic strategy was employed with two combined proteases (trypsin and chymotrypsin) and MALDI-ToF to obtain PMFs of the samples at different irradiation degrees including non-irradiated control samples; all data were statistically and manually compared to look for any difference arising after irradiation treatment. The X-ray treatment, at the doses examined, surprisingly did not induce peptide modifications like oxidations or deamidations. However, variations in X-ray absorbed doses did reveal some interesting differences in terms of mass peak relative intensity. Notably, these differences were more pronounced in mozzarella samples, while robiola and primosale showed relatively minor effects. By employing a combination of manual spectra comparison and PCA, we pinpointed 15 ions whose peak intensity was most impacted by changes in X-ray dosage. Conducting RPLC-ESI(+)-FTMS tandem MS experiments allowed us to sequence and identify these ions as peptides originating from α-S1- and β-casein. Moreover, these peptides exhibited a strong correlation with the protein's secondary structure, highlighting a heightened susceptibility of random coils to X-ray irradiation. Some of these peptides, with varying peak relative intensity, were identified as non-proteolytic and half-proteolytic ones. A few were generated through the truncation of longer proteolytic sequences, while others seemed linked to the alteration of random coil structures or exposed tertiary structures after irradiation, affecting proteins such as α-S1-, α-S2-, β-casein, and β-lactoglobulin. Although our findings are intriguing, further studies will be necessary to determine whether the effects of X-ray irradiation on soft cheese proteins could have implications for consumer safety and health.

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CRediT authorship contribution statement

Elena C.L. Rigante: Formal analysis, Investigation, Writing – original draft. **Cosima D. Calvano:** Conceptualization, Methodology, Writing – review & editing. **Giovanni Ventura:** Data curation. **Rosalia Zianni:** Conceptualization, Data curation, Resources. **Maria Campaniello:** Methodology, Resources. **Valeria Nardelli:** Resources. **Annalisa Mentana:** Conceptualization, Resources, Writing – review & editing. **Tommaso R.I. Cataldi:** Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115643.

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