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Peptidome profiles and bioactivity elucidation of buffalo-milk dairy products after gastrointestinal digestion

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ABSTRACT

Buffalo milk is highly appreciated for its nutritive properties and highly employed in dairy products, despite this the release of bioactive peptides has not been investigated thoroughly. The aim of this work was to characterize in detail the bioaccesible peptides from buffalo-milk dairy products. Six products were subjected to *in vitro* simulated gastrointestinal digestion and then analyzed by LC-HRMS. The identified peptides were 165 in Yoghurt, 152 in Scamorza, 146 in Mozzarella, 136 in Grana and Ricotta, 120 in Ice Cream samples, belonging to both buffalo caseins (α_{s1}-, β-, k-CN) and whey proteins (α-LA, β-LG). The identified peptide sequences were subjected to a database driven bioactivity search. Results highlighted a wide range of potential bioactive peptides, including antihypertensive, immunomodulatory, antimicrobial, antidiabetic, anticancer and antioxidant activity. These data evidence the content of healthy peptides released from buffalo-milk dairy products and suggest that the specific technological process influence their bioaccessibility.

1. Introduction

In the constant search for healthy compounds capable to improve and maintain the wellness state and to prevent the onset of chronic degenerative pathologies, bioactive peptides represent a relevant class of molecules. Peptides are small molecules with weight < 10 kDa and can be present in foods as natural components or can be generated by chemical or enzymatic hydrolysis of the parent proteins (Coda, Rizzello, Pinto, & Gobbetti, 2012). Usually peptides are latent, when encrypted into proteins, and become active when released after proteolysis (Hayes, Stanton, Fitzgerald, & Ross, 2007). They are involved in numerous physiological functions in the human body, such as in gastrointestinal, cardiovascular, immune, endocrine, and nervous systems. Many studies proved that various animal and vegetable matrices contain bioactive peptides and their daily consumption seems to be associated with a reduced risk of developing chronic diseases (Capriotti et al., 2015). In particular, milk and dairy products are considered as the most important source of peptides, positively modulating physiological and metabolic functions and thus exerting beneficial effects on human health (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2016). World milk production has doubled in the last decades and it is noteworthy that buffalo have supplied about 13% of the total world milk production in the last few years (International Dairy Federation, 2014), with an annual growth rate of ~3%, higher than cow milk. Dairy buffalo production has been a local tradition of the Caucasian countries, Asia and Egypt, where fresh buffalo milk, dahi (cultured sour milk), ghee (butter oil) and yoghurt are popular. In Italy, especially in the southern regions, the dairy buffalo industry is flourishing thanks to the popularity of buffalo mozzarella cheese.

Buffalo milk is higher in total solids, fat (7 to 8%) and proteins (4.2 to 4.5%) compared to cow's milk (average fat and protein content of 3.9% and 3.2%, respectively). Buffalo milk also contains less cholesterol, more tocopherols and vitamin A than cow's milk (Ahmad, Anjum, Huma, Sameen, & Zahoor, 2013).

Buffalo milk proteins possess a high homology to their cow counterparts (D'Ambrosio et al., 2008). In order to perform their biological

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activity, the encrypted sequences must be able to reach the target tissues in sufficient amount. In particular, their bioavailability after ingestion is influenced by some factors such as the physical properties of the molecule, metabolism and interactions with other molecules; as well as the stability of the proteins to various digestive steps, its release from the matrix (bioaccessibility) and the efficiency of its passage through the gastro-intestinal mucosa. Many studies have been focused on the peptides released from a single buffalo proteins, but a comprehensive study on buffalo-milk commercial dairy products has never been performed (Abdel-Hamid, Otte, De Gobba, Osman, & Hamad, 2017). Thus, this work is aimed to characterize in detail the peptidomic profile of typical buffalo-milk dairy products. For this purpose, an *in vitro* simulated gastrointestinal digestion was carried out on six buffalo dairy products namely: Grana, Ice Cream, Yoghurt, Mozzarella, Ricotta and Scamorza. The resulting isolated peptide digests were characterized by high resolution mass spectrometry. Subsequently a database-driven specific bioactivity assessment was performed for each identified sequence. A large number of potential bioactive peptides have been identified, endowed with established health-promoting properties, including antihypertensive, immunomodulatory, antimicrobial, antidiabetic, anticancer and antioxidant. Differences in the released peptides were justified on the basis of the manufacturing process involved.

2. Material and methods

2.1. Chemicals

Liquid Chromatography-Mass spectrometry (LC-MS) grade water (H_2O) was obtained by a Direct-8 Milli-Q system (Millipore, Milan, Italy). LC-MS grade acetonitrile (ACN), additives formic acid (HCOOH) and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (St. Louis, Mo, USA). The Strata-X™ polymeric reversed phase SPE cartridge was purchased from Phenomenex® (Castel Maggiore,Bologna, Italy). Unless stated otherwise, all other reagents and compounds were purchased from Sigma Chemicals Company (Sigma, Milan, Italy).

2.2. In vitro gastrointestinal digestion

Buffalo-milk dairy product samples (Grana, Ice Cream, Mozzarella, Ricotta, Scamorza and Yoghurt) were kindly donated by San Salvatore dairy factory (Giungano, SA, Campania, Italy). The procedure was performed according to Tenore et al. (2015). The oral phase of digestion was omitted, due to the absence of starchy matrices in the buffalo-milk dairy samples. The lyophilized samples (1 g) were solubilized in 20 mL of deionized water and the pH was adjusted to 2 with HCl 0.1 M. The mixture was incubated with pepsin (from porcine gastric mucosa) (1:100 enzyme/protein ratio, w/w) at 37 °C for 2 h in a Thermomixer comfort (Eppendorf, Hamburg, Germany) and the reaction was stopped by heating the solution at 95 °C for 15 min. The digests were incubated in a solution of $HCOONH_4$ 10 mM adjusted to pH 7.5 with formic acid and then incubated with pancreatin (from porcine pancreas), chymotrypsin (from bovine pancreas) and bile salts (all in 1:100 enzyme/protein ratio, w/w) at 37 °C for 2 h; then, the reaction was stopped bringing the solution to pH 2. The mixture was centrifuged at 4000*g* at 4 °C for 10 min (Mikro 220R centrifuge, Hettich, Germany), filtered on 0.45 μm filters (Phenex RC membrane, Phenomenex, Bologna, Italy), lyophilized and stored at − 80 °C. The samples were defatted prior SPE by employing different organic solvents. The resulting pellet was thus subjected to SPE extraction to purify and concentrate the digests. The peptide fraction was solubilized in distilled water and loaded on a Strata-X 33 μm Polymeric Reversed Phase SPE cartridge, 500 mg sorbent, surface area of 760–820 m^2/g (Phenomenex®) previously equilibrated in distilled water, then eluted with MeOH 2% v/v formic acid and finally re-lyophilized and stored at − 20 °C. Lyophilized samples were solubilized in a mixture of water:ACN, 65:35 v/v.

2.3. UHPLC PDA conditions

UHPLC analysis were performed on a Nexera UHPLC system (Shimadzu, Kyoto, Japan) consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A_{R5} degasser, an SPD-M20A photo diode array detector (equipped with a 2.5 μL detector flow cell volume), a CTO-20A column oven, a SIL-30AC autosampler.

The optimal mobile phase consisted of 0.1% TFA/H_2O v/v (A) and 0.1% TFA/ACN v/v (B). Analysis was performed in gradient elution as follows: 0–2.00 min, isocratic at 1% B; 2–45.00 min, 1–40% B, then five minutes for column re-equilibration. Flow rate was 1.8 mL/min. Column oven temperature was set to 40 °C. Injection volume was 5 μL of final peptide digest (6.6 mg/mL). UHPLC analysis was performed with Ascentis® Express Peptide ES-C18 (150 × 4.6 mm × 2.7 μm, 160 Å). The following PDA parameters were applied: sampling rate 12,5 Hz; detector time constant, 0.240 s; cell temperature, 40 °C. Data acquisition was set in the range 190–800 nm and chromatograms were monitored at 214 and 220 nm at the maximum absorbance of the compounds of interest.

2.4. UHPLC-HRMS analysis of peptide fraction

HRMS experiments were performed on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) through an electrospray source. Peptide separation was carried out employing an Accela 600 LC system, an Ascentis® Express Peptide ES C18 150 × 2.1 mm × 2.7 μm column (Supelco, Bellefonte, PA, USA) was used. Mobile phases were (A): 0.1% HCOOH in H_2O v/v and (B) ACN plus 0.1% HCOOH v/v. The separation was performed in gradient mode at a flow of 0.3 mL/min as follows: 0–22 min, 0–30% B; 22–27 min, 30–70% B; 27–28 min, 70–95% B; hold for 1 min, 29–34 min, returning to initial condition. Column oven was set to 45 °C. Two microliters of sample was injected. The MS parameters were set as follows: spray voltage was set at + 3.5 kV; sheath gas, 30 (arbitrary units); auxiliary gas, 10 (arbitrary units); capillary temperature, 250 °C. Data-dependent mode MS/MS was performed over the *m/z* range of 300–2000, at 30,000 resolution. MS/MS spectra collection parameters: collision energy, 35%; isolation window, 2 m/z; minimum signal threshold, 150; monoisotopic precursor, enabled. Ion trap and Orbitrap ion injection times were set to 50 and 100 ms, respectively. Automatic gain control (ACG) was set to 2×10^5 for full Fourier Transform Mass spectrometry (FTMS) scan and 3×10^4 ions in MS/MS mode for the linear ion trap. Dynamic exclusion repeat, 1; repeat duration, 30 s; list size, 50; exclusion duration of 30 s.

2.5. Peptide sequence identification

Raw MS/MS data files were converted in mzXML format, and a free trial of PEAKS 7.5 software (Bioinformatics Solutions Inc., Waterloo, Canada) was employed for peptide sequence determination. Search was performed using a database (DB) search tool, by searching against Swiss-Prot/UniProt database (Release 2015_11) taxonomy *Bubalus bubalis*, with an improved algorithm that validates and assists the database search with *de novo* sequencing results with the following settings enzymes: pepsin, trypsin, chimotrypsin; peptide charges from + 1 to + 4, monoisotopic precursor mass; fragmentation mode, CID (y and b ions); precursor mass tolerance, 10 ppm; fragment mass tolerance of 0.5 Da; oxidation (M) and phosphorylation (S, T, Y) were used as dynamic modifications. To assess the peptide bioactivity, the following free databases: BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) and EROP-Moscow (http://erop.inbi.ras.ru/) were consulted (Piovesana et al., 2015).

3. Results and discussions

3.1. Overview of the peptides released after simulated gastrointestinal digestion of buffalo-milk dairy products

The simulated gastrointestinal digestion protocol allowed us to mimic the physiological and biochemical conditions and the sequence of events that occur during *in vivo* gastrointestinal digestion. The highly acidic environment in the stomach lumen determined the milk parent proteins denaturation and consequently exposition of the protein's peptide bonds. The consequent action of the enzymes present in the stomach such as pepsin, and in the small intestine such as trypsin, chymotrypsin and pancreatin, allowed hydrolysis of casein and/or whey proteins, generating several small peptides. Digestion process was monitored by RP-UHPLC-DAD, while, the peptide identification was carried out by UHPLC-Orbitrap-based tandem mass spectrometry (MS/MS). The chromatographic profiles and total ion current chromatograms (TIC) relative to the gastrointestinal digestion of each dairy products are reported in Fig. 1 and the supplementary material file (Fig. S1), respectively. The choice to employ a narrow bore (2.1 mm I.D) column of the same packing is dictated to obtain higher sensitivity in the MS coupling, without losing the resolution and elution order obtained on the 4.6 mm I.D column employed for DAD analyses. MS/MS spectra were employed for sequence determination and the complete list of peptides including retention times, peptide sequences, precursor proteins, positions, and masses are reported in supplementary material file (Tables S1A–S6A). The identified peptides were 165 in Yoghurt, 152 in Scamorza, 146 in Mozzarella, 136 in Grana and Ricotta, 120 in Ice Cream samples, belonging to buffalo caseins α_{s1}-, β-, and k-CN and to buffalo whey proteins α-lactalbumin and β-lactoglobulin. Among the different proteins sources it can be observed from Fig. 2A that, except for Ricotta, almost 50% of the identified peptides belong to β-CN. This depends from a major degradation of β-CN, in particular at the C-terminal portion with L^{208}-Y^{209} and Y^{209}-Q^{210} residues together with the N-terminal portion with A^{1}-R^{2}, moreover also α_{s1}-CN was prone to degradation at the N-terminal portion, within the region comprising residues A^{1}-R^{2} and, in particular, F^{39}-F^{40} and F^{40}-V^{41} as highlighted in previous work (Sommella et al., 2016). It should be pointed out that, since Ricotta is made from a ratio of whey-milk 5:1, in this sample peptides derived from whey proteins, and in particular from α and β-

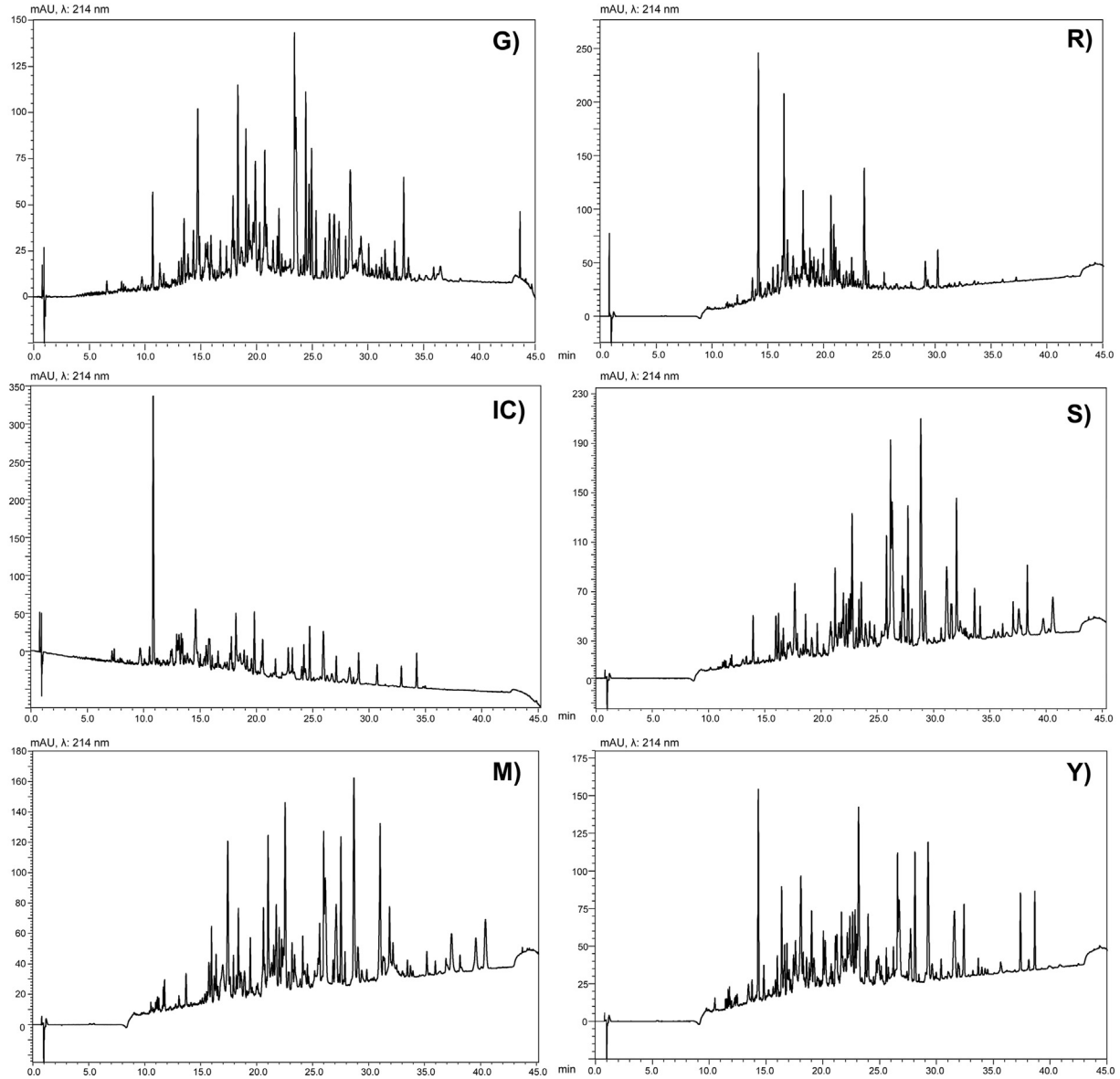

Fig. 1. Chromatographic profiles acquired by UHPLC-DAD of peptides released after simulated gastrointestinal digestion of buffalo Grana (G), Ice Cream (IC), Mozzarella (M), Ricotta (R), Scamorza (S) and Yoghurt (Y).

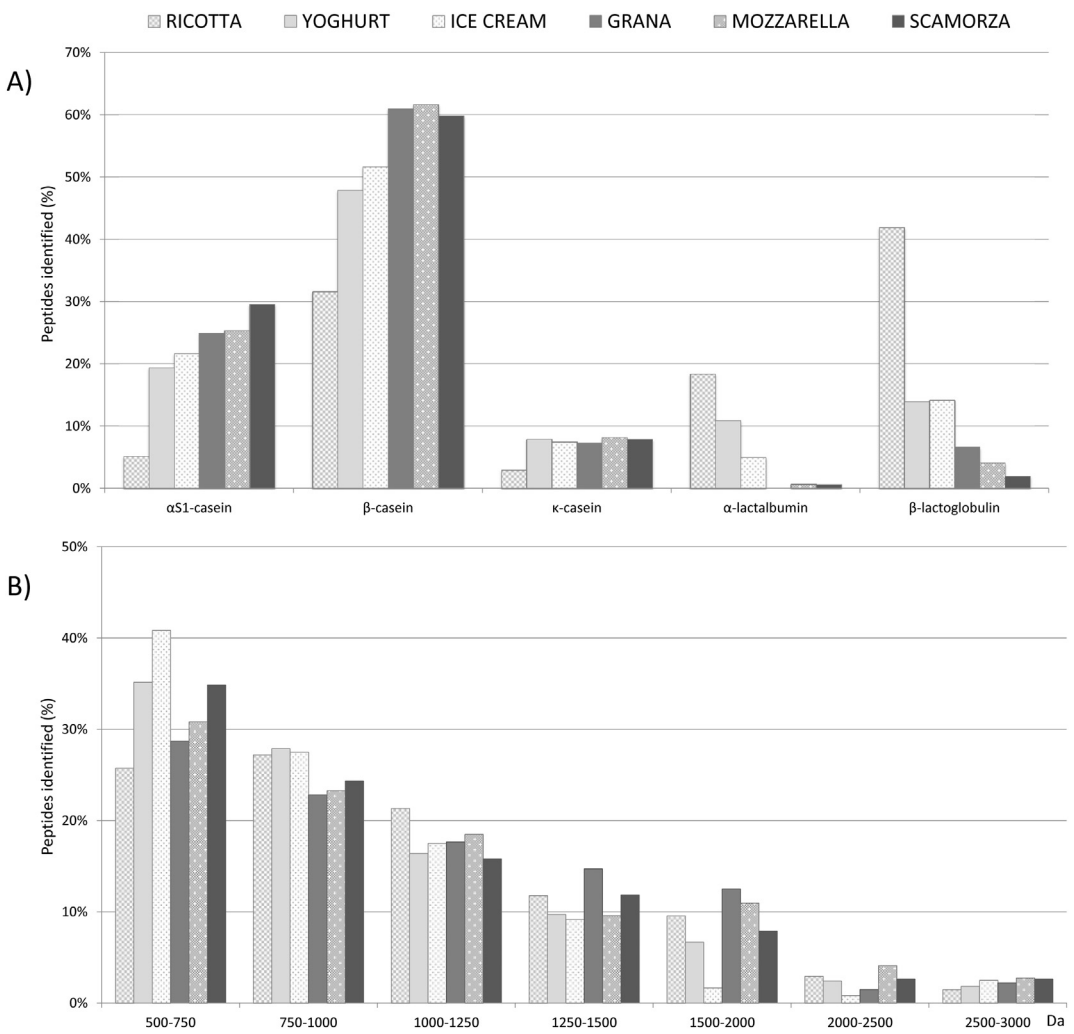

Fig. 2. (A) Relative contribution of each parent protein (%) in the release of bioaccessibility peptides in intestinal lumen; (B) Mass range order (500–3000 Da) of the identified peptides.

lactoglobulin, were the most represented. Since the parent proteins were basically identical for all samples partial overlap between the identified peptides was observed. It should be pointed out that short peptides (< 300 Da) were not investigated in this study, besides the MS scan conditions, usually these peptides, which are usually highly polar, are better analyzed by hydrophilic interaction chromatography (HILIC), that offers a different selectivity and better ionization efficiency with respect to RPLC (Sommella et al., 2015; Zenezini Chiozzi et al., 2016), and this actually under evaluation.

3.2. *Influence of manufacturing processes on the bioaccessibility of buffalo-milk peptides*

Fig. 2B shows the peptides released after *in vitro* gastrointestinal digestion of buffalo-milk dairy products, clustered on the basis of their molecular weight (Da). In particular, the peptides identified in the present study ranged from 5 to 26 amino acid residues, thus between 500 Da and 3000 Da. The graph shown in Fig. 2B highlights that, after gastrointestinal digestion of each sample, there is a different distribution of the released peptides. This aspect could be related to the different technological processes used in buffalo-milk manufacture. As showed in Fig. 3, each of six commercial samples analyzed have been produced following a specific technological process which could influence the bioaccessibility of peptides (Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998). Previous investigations demonstrated that several technological factors play an important role on milk protein organization in both the colloidal casein and the serum phases of milk (Donato & Guyomarc'h, 2009).

When milk proteins are subjected to thermal processing (Grana, Mozzarella, Ricotta and Scamorza production), whey proteins may undergo a denaturation process, which determines a protein unfolding and an exposure of hydrophobic groups. The increase in temperature and exposure time determine the denaturation of α-lactalbumin which forms complexes with large denatured β-lactoglobulin aggregates, and both proteins bind to the surface of casein micelles through intermolecular disulfide bonds (Jang & Swaisgood, 1990). Contrariwise, at low temperature (Yoghurt and Ice Cream production) the complex between β-lactoglobulin and κ-casein is less resistant, being mainly driven by hydrophobic interactions (Corredig & Douglas, 1996; Jang & Swaisgood, 1990). During refrigerated storage of milk and dairy products, β-casein as well as calcium, magnesium and phosphorus dissociate from the casein micelles due to weakening of hydrophobic interactions. Thus, the relative amount of β-casein in the micellar phase is decreased significantly and casein concentration, in water phase, increases.

The formation of complexes between whey proteins and κ-casein is also correlated to the coagulation process, fundamental step of cheese manufacturing. The addition of proteolytic enzymes in buffalo milk causes hydrolysis of κ-casein into para-κ-casein, which is known to bridge the casein micelles (López, Jordán, Hellin, Castillo, & Laencina, 1997). A further important factor, influencing the hydrolysis process, is the high fat content of buffalo-milk dairy products. In fact, Pierri et al.

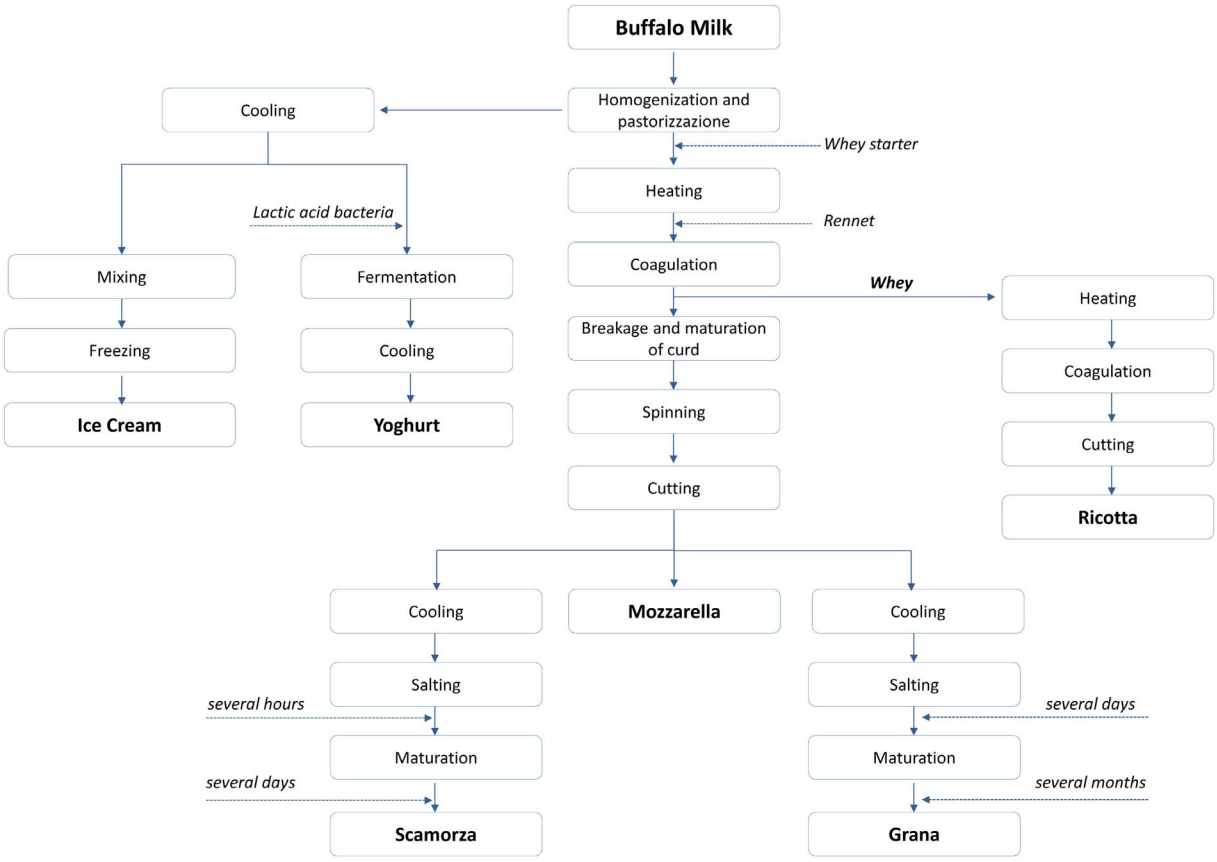

Fig. 3. Schematic representation of the different technological processes used in buffalo-milk commercial dairy products manufacture.

(2013) have hypothesized a stabilizing role of the lipid fraction in inhibiting whole milk casein degradation.

It can be concluded that high temperature and rennet adding used for the Ricotta, Mozzarella, Scamorza and Grana manufacturing in addition to their high fat content (22%, 24%, 32% and 33% w/w, respectively), strengthen the interaction between whey proteins and casein micelles (Anema & Li, 2003; Corredig & Douglas, 1996). These stable interactions make the milk proteins less available to the proteolysis of digestive enzymes, consequently the peptides released from these samples have high molecular weight (> 1000 Da). In contrast, the low temperatures used for the production and storage of Ice Cream and Yoghurt and their low fat content (6–9% w/w) determined weaker interactions between milk proteins leading to an increased proteolytic activity and therefore the release of low molecular weight peptides (< 1000 Da).

3.3. Identification of potential bioactive peptides

All of the identified peptides, obtained after *in vitro* gastrointestinal digestion of six buffalo-milk dairy products, were submitted to bioactivity search. A large number of potential bioactive peptides with various biological activities such as antihypertensive, immunomodulatory, antimicrobial, antidiabetic, anticancer and antioxidant were identified (Tables S1B–S6B). Interestingly, several milk-derived peptides reveal multifunctional properties since some regions in the primary structure of milk protein, considered “strategic zones”, contain overlapping peptide sequences (Fiat, Migliore-Samour, Jollès, Drouet, & Bal dit Sollier, & Caen, 1993). The identified peptide fragments in buffalo-milk dairy products with identical sequences to previously reported bioactive peptides are listed in bold (Table 1). As showed in Fig. 4, ACE inhibitor peptides represent the main class of bioactive compounds identified. For instance, α_{s1}-CN peptide with sequence **FVAPFPEVFG** (f39–48) has been reported to exhibit strong ACE inhibitory activity (Robert, Razaname, Mutter, & Juillerat, 2004) together with a large number of β-CN derived peptides. For example, **LHLPLP**L (f148–154) identified from milk fermented with *Enterococcus faecalis*, and **YQEPVLGPVRGPFPIIV** (f208–224), obtained from bovine casein by *Lactobacillus casei Shirota* (Rojas-Ronquillo et al., 2012). Finally, several ACE-inhibitory peptides derived from α-lactoalbumin as KGY**GGVSLPEW** (α-LA, f38–44) and from β-lactoglobulin such as **VLDTDY**K (β-LG, f112–117) L**DAQSAPL**R (β-LG, f51–57), were identified (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000).

In addition, for each sample is possible identify a second main bioactivity profile. After gastrointestinal digestion of Grana product many peptides with immunomodulatory properties have been identified. In detail, casein-derived immunopeptides including fragments of α_{s1}- and β-CN as **TTMPLW** (f209–213) and **YQEPVLGPVRGPFPIIV** (f208–224), respectively, stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages, induce a significant proliferative response in rat lymphocytes and exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous administration of peptides (Coste et al., 1992). Although the immunomodulatory mechanism is not clear, it seems to exist a relationship between the immune system and opioid peptides.

Ice Cream product digestion led to the formation of several neuropeptides. The main class is represented by β-casomorphins (BCMs), a group of opioid peptide agonists, formed from proteolytic digestion of β-casein playing a crucial role in the response to pain and stress. Neocasomorphin-6 (**YPVEP**F, f129–134) showed opioid activity in Guinea Pig Ileum (GPI) assay and receptor affinity with IC_{50} of 59 mM and 92 mM, respectively (Jinsmaa & Yoshikawa, 1999). Neocasomorphin-6 contains aliphatic amino acid X in YPX sequence instead of aromatic ones as in the case of β-casomorphin (YPF-) and hemorphin

Table 1
Bioactive peptides identified in intestinal digesta of buffalo-milk commercial dairy products.

Product	Potential bioactivity	t_r (min)	Mass	Error ppm	Protein	Amino acid	Peptide sequence	Peptide containing the sequence
Grana	Immunomodulator	11.72	561.2833	− 3.6	α_{S1}-Casein	209–213	K.TTMPL.W	**TTMPL**W
		23.63	1716.9926	− 1.2	β-Casein	209–224	Y.QEPVLGPVRGPFPIIV	Y**QEPVLGPVRGPFPIIV**
		24.08	1880.0559	− 2.2	β-Casein	208–224	L.YQEPVLGPVRGPFPIIV	**YQEPVLGPVRGPFPIIV**
		24.55	1993.1400	− 1.6	β-Casein	207–224	L.LYQEPVLGPVRGPFPIIV	**LYQEPVLGPVRGPFPIIV**
Ice cream	Neuropeptide	2.68	608.2918	− 0.6	κ-Casein	54–58	L.SRYPS.Y	**SRYPS**Y
		10.62	651.3955	1.9	β-Casein	185–190	K.VLPVPQ.K	K**VLPVPQ**
		10.87	561.2833	1.5	α_{S1}-Casein	209–213	K.TTMPL.W	**TTMPL**W
		14.56	750.3588	− 0.4	β-Casein	129–134	K.YPVEPF.T	**YPVEPF**
		20.24	801.5112	0.9	β-Casein	148–154	N.LHLPLPL.L	**LHLPLPL**
		22.74	1108.5593	− 3.1	α_{S1}-Casein	39–48	F.FVAPFPEVFG.K	F**FVAPFPEVFG**K
Mozzarella	Antimicrobial	4.96	761.4072	− 1.5	κ-Casein	64–69	Y.YQQKPV.A	Y**YQQKPV**A
		5.29	832.4443	− 2.4	κ-casein	64–70	Y.YQQKPVA.L	
		10.42	1222.5829	− 1.9	β-Casein	57–66	M.EDELQDKIHP·F	T**EDELQDKIHP**
		20.44	904.4694	− 1.5	α_{S1}-Casein	39–46	F.FVAPFPEV.F	**FVAPFPEV**F
		21.63	1667.9034	− 3.3	β-Casein	208–222	L.YQEPVLGPVRGPFPI.I	**YQEPVLGPVRGPFPI**
		24.33	1880.0559	− 3.5	β-Casein	208–224	L.YQEPVLGPVRGPFPIIV	**YQEPVLGPVRGPFPIIV**
Ricotta	Antidiabetic	3.85	978.4043	− 3.6	α-Lactalbumin	51–59	F.HTSGYDTQA.I	F**HTSGYDTQA**
		9.42	1244.5771	− 1.2	β-Lactoglobulin	143–153	R.TPEVDDEALEK.F	**TPEVDDEALEK**
		9.89	968.5178	− 1.4	β-Lactoglobulin	64–72	E.LKPTPEGDL.E	**LKPTPEGDL**
		15.84	1210.6445	− 1.3	β-Lactoglobulin	65–75	L.KPTPEGDLEIL.L	L**KPTPEGDLEIL**
		16.50	529.2900	− 2.8	β-Casein	218–222	R.GPFPI.I	**GPFPI**LV
		18.16	843.4127	− 2.0	α-Lactalbumin	38–45	Y.GGVSLPEW.V	GY**GGVSLPEW**
Scamorza	Anticancer	19.91	801.5112	− 0.4	β-Casein	149–155	L.HLPLPLL.Q	NL**HLPLPLL**
		20.28	801.5112	− 1.6	β-Casein	148–154	N.LHLPLPL.L	N**LHLPLPL**L
		20.39	1044.5968	− 0.6	β-Casein	146–154	V.ENLHLPLPL.L	**ENLHLPLPL**L
		20.57	915.5541	− 1.6	β-Casein	147–154	E.NLHLPLPL.L	**NLHLPLPL**L
		22.71	1108.5593	− 0.7	α_{S1}-Casein	39–48	F.FVAPFPEVFG.K	F**FVAPFPEVFG**K
Yoghurt	Antioxidant	3.72	645.3156	− 0.7	β-Casein	115–120	K.EAMAPK.H	**EAMAPK**
		3.75	608.2918	− 0.6	κ-Casein	54–58	L.SRYPS.Y	**SRYPS**
		6.21	1180.5724	− 1.6	α_{S1}-Casein	95–104	K.HIQKEDVPSE.R	**HIQKEDVPSE**R
		9.06	673.3435	− 1.9	β-Casein	192–197	K.AVPYPQ.R	**AVPYPQ**R
		9.31	779.4905	0.7	β-Casein	185–191	K.VLPVPQK.A	**VLPVPQK**
		11.48	632.3533	− 0.6	κ-Casein	46–50	K.YIPIQ.Y	**YIPIQ**Y

(YPW-). However, accumulating evidence indicates that the aliphatic moiety also contribute to onset of opioid activity. Contrariwise, gastrointestinal hydrolysis of κ-CN releases the **SRYPS**Y (f54–58) neuropeptide with opioid antagonistic properties, known as casoxin 6. This peptide binds selectively to μ- and κ-receptors and not to δ-receptors. It has been reported that the casoxin 6 antagonized the morphiceptin effect in the GPI assay and the dynorphin A effect in the rabbit vas deferens (Yoshikawa, Tani, & Chiba, 1988). Other neuropeptides in Ice Cream gastrointestinal digest (L**HLPLP**L, β-CN f148–154) are prolyl oligopeptidase (POP) inhibitors. The POP activity is involved in cognitive and neurological functions (Myöhänen, García-Horsman, Tenorio-Laranga, & Männistö, 2009). Moreover, POP is suspected to be involved in pathological conditions such as Parkinson's and Alzheimer's diseases (Myöhänen et al., 2009). Finally, also a group of ACE-

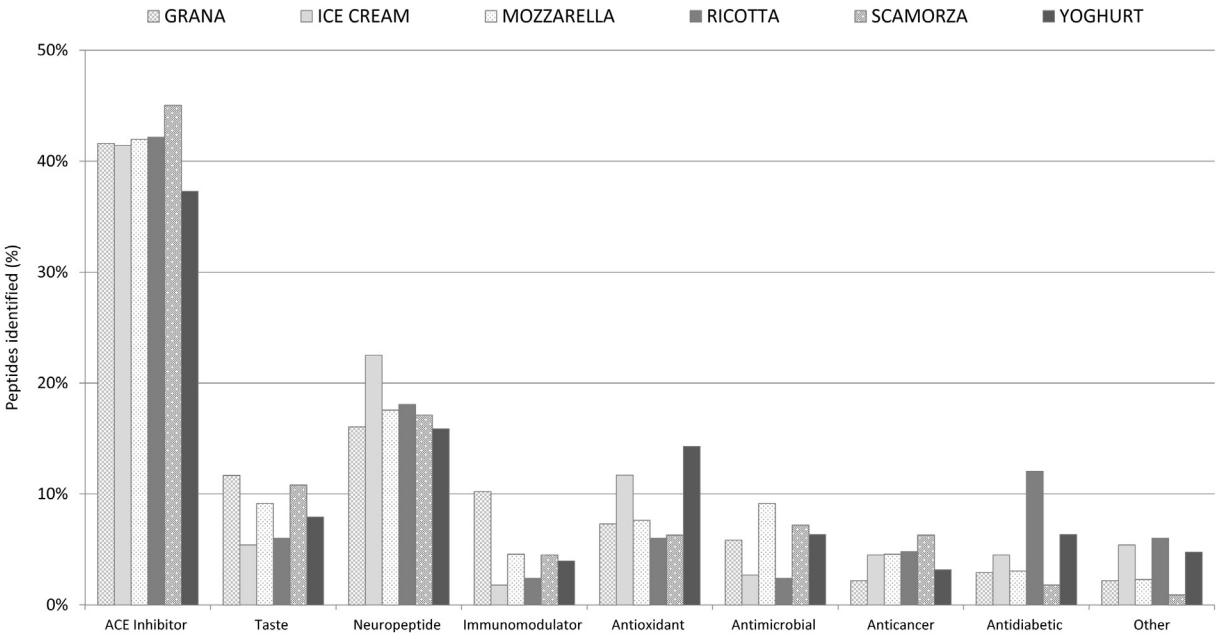

Fig. 4. Bioactive properties of identified peptides after gastrointestinal digestion of six buffalo-milk dairy products.

inhibitory peptides, identified in Ice Cream digesta, can be considered as neuropeptides (F**FVAPFPEVFGK**, α_{s1}-CN f39–48; K**VLPVPQ**, β-CN f185–190; **TTMPLW**, β-CN f209–213). In fact, ACE enzyme, expressed by neuronal cells, is capable in cleaving, amyloid-β as shown *in vitro*, *ex vivo* and recently *in vivo* (Hemming, Selkoe, & Farris, 2007).

Gastrointestinal digestion of buffalo Mozzarella product, releases antimicrobial peptides. A clear example is represented by the two κ-CN derived peptides (Y**YQQKPV**A, f64–69; Y**YQQKPVA**, f64–70) showing antimicrobial activity against *E. coli* ATCC 25922 (López-Expósito, Minervini, Amigo, & Recio, 2006). In addition, two peptides, with antimicrobial activity, casecidin 17 and casecidin 15, were identical to sequences in the C-terminal of bovine β-casein (**YQEPVLGPVRGPFPIIV**, β-CN f208–224; **YQEPVLGPVRGPFPI**, β-CN f208–222). Hence, buffalo Mozzarella represents an important source of antibacterial peptides potentially useful for the development of novel supporting therapies to the classic antimicrobial protocol.

In gastrointestinal digesta of Ricotta product, several Dipeptidyl Peptidase IV (DPP-IV) inhibitor peptides were identified. The DPP-IV enzyme is known to inactivate the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), two gut derived-hormones that play crucial roles in glucose regulation by stimulating pancreatic glucose-dependent insulin, suppressing glucagon release, promoting β-cell proliferation and survival, retarding gastric emptying and modulating appetite (Lim & Brubaker, 2006). For these reasons, the inhibition of the enzyme dipeptidyl-peptidase IV (DPP-IV) is an effective pharmacotherapeutic approach for the management of type 2 diabetes. Recent findings have suggested that dietary proteins could be precursors of peptides able to inhibit DPP-IV. In details, peptides derived from α-lactalbumin such as GY**GGVSLPEW** (α-LA, f38–45) and F**HTSGYDTQA** (α-LA, f51–59) identified in Ricotta digesta, show strong binding but low DPP-IV inhibitory activity, probably due to unspecific protein binding (Lacroix & Li-Chan, 2014). An example is the peptide **TPEVDDEALEK** (β-LG, f143–153), that showed moderate inhibitory activity (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013) or the peptide derived from bovine β-casein (**GPFPI**LV, β-CN f218–222) that showed higher potency (Zhang et al., 2016). However, peptides with a third Pro resistant to the hydrolysis of DPP-IV are thought to be more potent DPP-IV inhibitors (Lacroix & Li-Chan, 2014). The **LKPTPEGDL** (β-LG, f64–72) and L**KPTPEGDLEIL** (β-LG, f65–75) peptides show both high affinity and non-competitive inhibitory activity of DPP-IV. The data show that the buffalo Ricotta product is an important source of DPP-IV inhibitory peptides, and can be used as a food in the prevention or co-adjuvant therapy for the management of type 2 diabetes.

On the other hand, the peptides **ENLHLPLPL** (f146–154) and **NLHLPLPL** (f147–154) of β-casein and the peptide **FVAPFPEVFG** (f39–48) of α_{s1}-casein, respectively, were identified in gastrointestinal digesta of Scamorza product. These peptides are able to inhibit the enzymatic activity of Matrix Metalloproteases (MMPs) (Pender & MacDonald, 2004). The MMPs represent a family of several enzymes that collectively are able to degrade virtually all extracellular matrix proteins. Several studies have documented the importance of the increased expression of MMP-2 and MMP-9 activities in the development and progression of colon inflammatory diseases and cancer (Roeb et al., 2001). Peptides derived from α_{s1}-casein inhibit MMP-2 and MMP-9 rather than MMP-7, whereas peptides derived from β-casein inhibit the MMPs with equivalent potencies. These peptides also selectively inhibited the enzymatic activities of prolyl-amino-peptidases, prolyl-aminodipeptidases, and prolyl-endopeptidases in human colon carcinoma cells (Juillerat-Jeanneret, Robert, & Juillerat, 2011).

Milk proteins can be considered as a carrier for the delivery of antioxidant peptides in the gastrointestinal tract, since the antioxidant peptides are encrypted in the protein sequences, thus preserved from oxidation and degradation. Gastrointestinal digestion of parent protein determines a slow and continuous release of antioxidant peptides and amino acids, accumulating in the gastrointestinal tract. This aspect suggests that the major physiological effects are locally explicated, protecting the gastrointestinal tract itself from the oxidative damage and the onset of oxidative diseases, such as cancer, coronary heart diseases and neurodegenerative disorders (Tagliazucchi, Helal, Verzelloni, & Conte, 2016). In particular, after κ- and α_{s1}-casein hydrolysis, antioxidant compounds such as the peptides **YIPIQY** (κ-CN, f46–50), **SRYPS** (κ-CN, f54–58) and **HIQKEDVPSER** (α_{s1}-CN, f95–104) in Yoghurt digesta were detected, suggesting that the amino acids tyrosine and serine were active against the hydroxyl radicals. Other antioxidant peptides derived from buffalo β-casein after simulated gastrointestinal digestion of Yoghurt were identified. An example is **EAMAPK** peptide (β-CN, f115–120), which has showed antioxidant activities in a wide concentration range (5–150 μg/mL), inhibiting ROS release and increasing an antioxidant response, as Nrf2 pathway activation and SOD expression (Pepe et al., 2016). The activity of hexapeptide could be ascribed to the presence of a methionine residue, which can be oxidized to sulfone, and to the proline residue, which can form a stable free radical adducts generating hydroxyproline derivatives. The **VLPVPQK** (β-CN, f185–191) and **AVPYPQR** (β-CN, f192–197) peptides also possess a strong antioxidant potential (Rival, Boeriu, & Wichers, 2001).

4. Conclusions

Our approach, based on the comprehensive peptidomic profiling after simulated gastrointestinal digestion of six buffalo-milk dairy products, highlighted a wide presence of peptides endowed with peculiar bioactivities and recognized health benefits.

342 peptides belonging to both buffalo caseins [α_{s1}-CN (74), β-CN (146), k-CN (27)] and whey proteins [α-LA (32), β-LG (63)] were characterized but only one-third of them (90 peptides) are reported to possess biological properties in literature. The potential bioactivities of the remaining peptides are undisclosed and deserves future investigations. These results could drive the pharmaceutical sector to the discovery of new active compounds and the dairy industry to realize health-enhancing products using the buffalo-milk as functional matrix which could be enriched with other bioactive extracts, in order to obtain a synergic or additive associations.

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