Journal of Functional Foods xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

In vitro antioxidant properties of digests of hydrolyzed casein and caseinophosphopeptide preparations in cell models of human intestine and osteoblasts

Michela Bottani[a], Stefano Cattaneo[b], Valentina Pica[b], Milda Stuknytė[b], Ivano De Noni[b,*], Anita Ferraretto[a,c]

[a] *IRCCS Istituto Ortopedico Galeazzi, Laboratory of Experimental Biochemistry & Molecular Biology, via R. Galeazzi 4, 20161 Milan, Italy*
[b] *Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, via G. Celoria 2, 20133 Milan, Italy*
[c] *Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, LITA, via Fratelli Cervi 93, 20090 Segrate, Italy*

ARTICLE INFO

Keywords:
Caseinophosphopeptides
Gastrointestinal digestion
Antioxidant activity
Caco-2/HT-29 co-culture
Osteoblast-like cells Saos-2
ROS

ABSTRACT

Three commercial samples consisting of enriched calcium-free caseinophosphopeptides (CPP), enriched calcium-bound caseinophosphopeptides (Ca-CPP) and an enzymatically hydrolyzed casein (hCN) were *in vitro* digested according to COST-Infogest protocol. As assessed by UPLC-HR-MS/MS, the digests contained 207–235 unique caseinophosphopeptides, and the species presenting the cluster sssEE were more abundant in CPP digest. The antioxidant activity at three different doses of each digest was firstly evaluated on human intestinal Caco-2/HT-29 70/30 co-culture. In presence of AAPH, hCN and CPP digests displayed a dose-dependent antioxidant activity equal or even greater than Vitamin C. In presence of Fe^{2+}, the digests exerted an antioxidant activity mainly at the highest dose. Antioxidant activities of the intestinal metabolized digests was then evaluated on human osteoblast (Saos-2) cells. The digests exerted an antioxidant activity in presence of AAPH, but not in presence of Fe^{2+}. These results highlight milk-derived peptides as potential dietary supplements for gut and bone health.

1. Introduction

The oxidative damage is at the base of aging-associated modifications that together with a low grade inflammation can concur to chronic diseases onset, high health expenditure and poor quality of life (Liguori et al., 2018). These concerns boosted the research to exploit the potential of food components with antioxidant properties as an alternative therapy to drugs (Korhonen & Pihlanto, 2003). This approach emerges as a new promising field in human nutrition and, for this reason, attracted interest for developing new dietary supplements. So far, three different antioxidant actions have been recognized: i) scavenging of reactive oxygen species (ROS), or primary antioxidant mechanism, which is based on a direct physical interaction between antioxidants and free radicals; ii) metal chelation, or secondary antioxidant mechanism; iii) modulation of enzymes involved in ROS production and destruction. The ROS are unstable radicals and non-radicals physiologically produced during cellular metabolism, and they play a role in cell functions such as signaling, ion transportation and regulation of gene expression (Lü, Lin, Yao, & Chen, 2010). Nonetheless, an excessive ROS production promotes damage to cell macromolecules (DNA, proteins and lipids) inducing an oxidative stress caused by direct interactions between ROS and the same macromolecules.

Bovine caseinophosphopeptides are phosphorylated peptides enzymatically released from casein (CN) during cheese ripening (López-Expósito, Amigo, & Recio, 2012) and/or from *in vitro* and *in vivo* enzymatic digestion (Boutrou et al., 2013; Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, & O'Brien, 2009). Caseinophosphopeptides are capable to bind and carry divalent cations (Meisel, 1997), and this feature has been related to chemical features such as length, level of phosphorylation, negative net charge, distribution of phosphorylated residues, and presence of the cluster Ser(P)-Ser(P)-Ser(P)-Glu-Glu (sssEE) in their primary sequence (Ferraretto, Gravaghi, Fiorilli, & Tettamanti, 2003; Gravaghi et al., 2007). The cation-binding activity of caseinophosphopeptides has been demonstrated to have biological interest, since it positively affects calcium and iron availability (Bouhallab & Bouglé, 2004) and scavenges free radicals (Kitts, 2005). The antioxidant properties of caseinophosphopeptides have been only studied *in vitro* using aqueous solution (Chiu & Kitts, 2003; Kitts, 2005), oil-in-water emulsions (Díaz, Dunn, McClements, & Decker, 2003), phosphatidylcholine liposomes (Kansci, Genot, Meynier, Gaucheron, &

* Corresponding author.
E-mail address: ivano.denoni@unimi.it (I. De Noni).

https://doi.org/10.1016/j.jff.2019.103673
Received 26 July 2019; Received in revised form 24 October 2019; Accepted 8 November 2019

Chobert, 2004), and ground beef (Díaz & Decker, 2004). All these studies have highlighted the caseinophosphopeptide ability to induce free radical scavenging activity in addition to a Fenton reaction inhibition as a consequence of metal quenching.

García-Nebot, Cilla, Alegría, and Barberá (2011) demonstrated that caseinophosphopeptides are capable to decrease the damage induced by lipid peroxidation and to exert protective action against H_2O_2 treatment in Caco-2 cells. (Laparra, Alegría, Barberá, & Farré, 2008) did not evidence this effect, but an increase in the GSH-reductase and a modulation of cell cycle phase activity have been detected. Milk-derived peptides are also known for their anti-osteopenic activity through the inhibition of bone oxidative damage, bone-resorbing cytokines in ovariectomized rats (Mada et al., 2017), and the promotion of calcium uptake and osteoblast differentiation (Donida et al., 2009).

The study of the biological effectiveness of caseinophosphopeptides implies to ascertain whether the antioxidant activity is retained after gastrointestinal digestion. In this regard, using a Caco-2 cell line, (Laparra et al., 2008) demonstrated that *in vitro* gastrointestinal digestion of caseinophosphopeptides influenced their antioxidant potential due to the degradation or release of caseinophosphopeptides from CN.

Based on these evidences and data reported in literature, in the present study we aimed to evaluate the antioxidant properties of three commercial samples consisting of: i) enzymatically hydrolyzed bovine CN (hCN); ii) enriched preparation of calcium-free bovine caseinophosphopeptides (CPP); iii) enriched preparation of calcium-bound bovine caseinophosphopeptides (CaCPP). Ultra-Performance Liquid Chromatography-High Resolution tandem Mass Spectrometry (UPLC-HR-MS/MS) was adopted for identifying the caseinophosphopeptides present in the samples after *in vitro* static simulated gastrointestinal digestion (SGID) according to the COST-Infogest protocol (Brodkorb et al., 2019). The antioxidant activity of digests was evaluated *in vitro* using a co-culture of Caco-2/HT-29 human intestinal cells under oxidative conditions represented by a ROS generator and Fe^{2+} ions. Then, the antioxidant activity of the basolateral content of the intestinal cell co-culture, mimicking the intestinal metabolizate, was assessed *in vitro* on human osteoblast-like Saos-2 cells.

2. Materials and methods

Cell culture media and reagents for *in vitro* SGID and biological assays were from Sigma-Aldrich (St. Louis, MO, USA), FBS was from EuroClone (Pero, Italy). The eluents for UPLC were from Panreac Química (Castellar del Vallès, Spain) and those for HR-MS/MS from Thermo Fisher Scientific (San Jose, CA, USA) if not diversely specified.

2.1. Commercial samples

Three commercial powdered samples were considered. The hCN sample represented an enzymatic hydrolyzate of bovine CN. CPP and CaCPP samples were enriched preparations of calcium-free and calcium-bound bovine caseinophosphopeptides, respectively. As reported by the corresponding technical data sheets, the caseinophosphopeptide contents (%, w/w) of hCN, CPP and CaCPP were 26, 90.5 and 95, respectively. The calcium content (%, w/w) was 0.015 and 6.6 in CPP and CaCPP, respectively. The calcium content was not reported for hCN.

2.2. In vitro static simulated gastrointestinal digestion (SGID)

To simulate the *in vivo* physiological digestive process, the static protocol proposed by (Brodkorb et al., 2019) was used omitting the oral phase. For each sample, 2.5 g of powder was resuspended in 2.5 mL of MilliQ water. To inhibit enzyme activity, at the end of digestion 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride was added, the samples were frozen in liquid nitrogen and stored at −80 °C. Subsequently, centrifugation at 4 °C (Eppendorf centrifuge 5810 R, Eppendorf, Hamburg, Germany) was performed at 3100*g* for 20 min to separate the insoluble components. The osmolality of the digests was measured using an osmometer (Micro Osmometer Type 6/6M, Löser Messtechink, Berlin, Germany). If necessary, it was corrected to obtain physiological values of 300 mOsm/kg H_2O. This step avoided osmotic shocks to *in vitro* intestinal co-culture after administration of the digest. To adjust osmolality, MilliQ water was added to samples with osmolality greater than 300 mOsm/kg H_2O and mannitol (0.1143 M) was used for samples with lower values.

Three different protein concentrations (2.4, 3.2, and 6.4 μg/μL) of each *in vitro* digests were used.

2.3. Identification of caseinophosphopeptides in digested samples

The identification of caseinophosphopeptides was performed in hCN, CPP and CaCPP digests after enrichment of phosphorylated peptides using the High-Select™ Fe-NTA Phosphopeptide Enrichment Kit (Thermo Fisher Scientific) according the manufacturer's indications. The enriched fractions were vacuum–dried and kept at −24 °C. Before chromatographic fractionation, samples were re-suspended in 50 μL of 0.1% (v/v) formic acid.

The caseinophosphopeptide-enriched fractions were separated on an Acquity UPLC BEH300 C18 column (150 × 2.1 mm, 1.7 μm) (Waters, Milford, MA, USA) using an Acquity UPLC module (Waters), and the peptides were identified by HR-MS/MS on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) as described by (De Luca et al., 2016). The MS data were processed and the caseinophosphopeptides were identified using the Proteome Discoverer 1.4 software (Thermo Fisher Scientific). The MS and MS/MS spectra were matched against the database of *Bos taurus* (UniProt taxon ID 9913) CN considering their genetic variants (Farrell et al., 2004). Settings were as follows: enzyme, No-Enzyme; mass accuracy window for precursor ions, 5 ppm; mass accuracy window for fragment ions, 0.02 Da; no fixed modifications; variable modifications: phosphorylation of serine and threonine, deamidation of asparagine and glutamine, oxidation of methionine. The false discovery rate of peptide identification was set to FDR = 0.01 (Strict).

2.4. Human in vitro cell models

As an *in vitro* model of human intestine, a 70% Caco-2/30% HT-29 co-culture was seeded on the porous membrane of transwell wells (Millicell, Merck, Darmstadt, Germany). The co-culture was used at the 6th day of post confluence, when it was composed of both mucus secreting cells and absorptive enterocytes in a proportion similar to the human small intestine (Ferraretto et al., 2018). Moreover, it presented the functional digestive and absorptive features of human small intestine.

As an *in vitro* model of human osteoblasts, Saos-2 cells (Saos-2 HTB-85, ATCC, LGC standards, Sesto San Giovanni, Italy) were grown and subcultured by passaging weekly in 75 cm^2 flasks containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS, 4 mM L-Glutamine, 0.1 mg/L streptomycin, 100,000 U/L penicillin, 0.25 mg/L amphotericin-B in a humidified atmosphere of 5% CO_2 at 37 °C (Donida et al., 2009).

2.5. In vitro digest metabolization by intestinal cells (IM digests)

To mimic *in vitro* the changes occurring at the intestinal cell level, each digest (in 400 μL HBSS buffer) was administered in the apical chamber of transwell wells (Millicell, Merck) where the 70% Caco-2/30% HT-29 co-culture was seeded. After 2 h, the contents of the basolateral chambers, named IM-hCN, IM-CPP and IM-CaCPP, were recovered and administered to Saos-2 cells.

2.6. Cell viability assay (MTT)

Cell viability assay (MTT) allowed to evaluate cell proliferation and viability on the basis of the mitochondrial metabolic activity (Riss, Moravec, Niles, Duellman, Benink, Worzella, & Minor, 2004). Both the co-culture and the Saos-2 cells were plated at 40,000 cells/cm^2 in 24 multiwell plates and used at 80% of confluence. The cell viability was monitored in presence of the three digests alone or in combination with either with 2,2′-azobis(2-methylpropionamide) dihydro-chloride (AAPH), able to generate peroxyl radicals, or with $FeCl_2$ (Fe^{2+}) that can induce the Fenton reaction. The same assay was repeated with the IM digests. As a reference, the possible effects of AAPH, Fe^{2+}, the digestion buffer (DB) and mannitol, at the different doses added to the samples, were studied. Cells were incubated with the samples for 2 h at 37 °C and subsequently with MTT for 4 h to evaluate the formazan salt formation. Results were expressed and reported as percentage *vs* control of non-treated cells (CTR).

2.7. Cell-based antioxidant assay (CAA)

The cell-based antioxidant assay (CAA) measured the ROS production in living cells by means of the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Wan, Liu, Yu, Sun, & Li, 2015). Both co-culture and Saos-2 cells were plated at 40,000 cells/cm^2 in a black 96-well plate (CELLSTAR, Greiner Bio-One, Kremsmünster, Austria) in their complete medium. All the samples here considered, hCN, CPP, CaCPP, IM-hCN, IM-CPP, IM-CaCPP, were administered alone or in combination with 250 μM either AAPH or Fe^{2+} in HBSS buffer. In addition, the prooxidant activity of AAPH and iron, as well as the antioxidant activity of 1 mM Vitamin C (Vit C) were evaluated. The fluorescence ($\lambda_{excitation}$ = 485; $\lambda_{emission}$ = 538) was measured immediately after sample administration and every 15 min till 120 min by a Wallac Victor^2 1420 Multilabel Counter plate reader (Perkin Elmer, Waltham, MA, USA). All results were expressed as relative CAA units according to (Hidalgo et al., 2018) after subtracting the DB value from the digest readings. Positive CAA values indicated antioxidant activity *vs* CTR (CAA value of 0, representing the basal oxidative status of the control of non-treated cells), while negative CAA values indicated oxidant activity *vs* CTR. Moreover, positive values significantly different from CTR were also compared to Vit C effect, whereas negative values significantly different from CTR were also compared to AAPH or Fe^{2+} activities.

2.8. LDH release

The colorimetric assay was performed using the Cytotoxicity detection kit (LDH, Roche Diagnostics, Mannheim, Germany). The assay was based on the measurement of lactate dehydrogenase (LDH) activity released into the medium from the cytosol of cells with a damaged membrane. Both co-culture and Saos-2 cells were plated at 40,000 cells/cm^2 in a 24-well plate (Greiner Bio-One) in their complete medium. All the samples here considered (hCN, CPP, CaCPP, IM-hCN, IM-CPP, IM-CaCPP) were administered alone or in combination with 250 μM either AAPH or Fe^{2+} in a complete HBSS solution. Results were expressed as relative LDH release (% *vs* control of non-treated cells, CTR) after subtracting the DB value from the digest readings.

2.9. Statistical analysis

Statistically significant differences among digest or IM digest mean values at the three different doses were established by one-way ANOVA followed by Bonferroni post hoc *t*-test with the SPSS 20 statistical software (SPSS, Chicago, IL, USA). In the case of comparisons among mean values and AAPH, Fe^{2+} or Vit C, an unpaired Student's *t*-test with two-tailed distribution has been performed. A $P < 0.05$ was considered significant, and it was represented by different letters for digests or IM digests at different doses, or symbols for comparisons with positive and negative oxidation controls.

Table 1
Qualitative profile of caseinophosphopeptides revealed in hCN, CPP and CaCPP digests. (n, number of unique caseinophosphopeptides; sssEE, number of caseinophosphopeptides containing the cluster sssEE; 1P/2P/3P, mono-, di- and tri-phosphorylated, precursor CNs, and molecular weight (MW) as a percentage (%) of total peptides).

	hCN	CPP	CaCPP
n	207	235	220
sssEE (n)	12	41	19
1P/2P/3P (%)	63/23/12	39/30/22	62/23/9
α_{S1}-CN/α_{S2}-CN/β-CN (%)	37/31/19	37/38/21	34/26/26
MW < 2 kDa (%)	96	91	88

3. Results

3.1. Qualitative profile of caseinophosphopeptides in hCN, CPP and CaCPP in vitro digests

After *in vitro* SGID, the UPLC-HR-MS/MS analysis of digests revealed for hCN, CPP and CaCPP the qualitative profiles of caseinophosphopeptides summarized in Table 1.

In detail, about the same number of unique caseinophosphopeptides were present in hCN, CPP and CaCPP digests. In all samples 26–35% and 60–62% of total peptides were smaller than 1 kDa and in the MW range of 1–2 kDa, respectively. Monophosphorylated caseinophosphopeptides accounted for most of the identified species, followed by di- and tri-phosphorylated forms. In the hCN and CaCPP digests, the ratio among mono-, di- and tri-phosphorylated peptides was almost the same. Contrarily, a quite similar proportion of the differently phosphorylated forms was recorded for the CPP digest. The peptides containing the cluster sssEE represented 6%, 17% and 9% of total peptides found in hCN, CPP and CaCPP, respectively. In general, they were shorter forms of the cluster-containing peptides α_{S2}-CN f(53–70), β-CN f(1–25) and β-CN f(2–25). The peptide β-CN f(1–25)3P was identified only in the digest of CaCPP, whereas 4, 11 and 6 shorter forms (8–19 amino acids long) of the β-CN f(1–25) peptide presenting 3 to 5 phosphorylations were detected in the hCN, CPP and CaCPP digests (data not shown). Overall, the digests shared forty caseinophosphopeptides (Table 2).

3.2. Cell viability of the 70% Caco-2/30% HT-29 co-culture and Saos-2 cells after digest and IM digest administration

After *in vitro* SGID, the hCN, CPP and CaCPP digests were administered to the 70% Caco-2/30% HT-29 co-culture to mimic *in vitro* interactions at the intestinal epithelium. Digest administration *per se* did not significantly affect intestinal cell viability. In presence of AAPH or Fe^{2+}, only in the case of CPP and CaCPP digests administration some slight but significant ($P < 0.05$) decrements in cellular viability were observed (Fig. 1A, B, C). These very small entity decrements, about 10–20% *vs*. control of non-treated cells, CTR, are presumably due to little variations in the number of living cells in each well, without consequences on the cell morphology and functions.

Successively, the IM-hCN, IM-CPP and IM-CaCPP samples were administered to Saos-2 cells. Similarly, the administration of IM digests, alone or in combination with AAPH or Fe^{2+}, did not modify the Saos-2 cell viability (Fig. 1D, E, F). In addition, DB, mannitol, AAPH and Fe^{2+} were individually assayed for cell viability without revealing any significant ($P < 0.05$) effect (Supplementary Fig).

Table 2
Common caseinophosphopeptides among hCN, CPP and CaCPP digests. Some of them were previously reported in literature as released *in vivo* after CN or milk ingestion (references are indicated in the parentheses). (S) indicates phosphoserine; (S, T) indicates phosphorylation at both, Ser and Thr, residues.

Casein	Fragment	Sequence	Phosphorylation	Reference
α_{S1}-CN	35–42	EKVNELsK	1 (S)	Barbé et al. (2014)
α_{S1}-CN	37–42	VNELsK	1 (S)	
α_{S1}-CN	43–47	DIGsE	1 (S)	Sanchón et al. (2018)
α_{S1}-CN	43–53	DIGsEStEDQA	2 (S,T)	
α_{S1}-CN	43–53	DIGsESTEDQA	1 (S)	Sanchón et al. (2018)
α_{S1}-CN	43–54	DIGsEStEDQAM	2 (S,T)	
α_{S1}-CN	43–58	DIGsEsTEDQAMEDIK	2 (S)	Barbé et al. (2014)
α_{S1}-CN	44–52	IGSEstEDQ	2 (S,T)	Sanchón et al. (2018)
α_{S1}-CN	59–65	QMEAEsI	1 (S)	
α_{S1}-CN	109–118	LEIVPNsAEE	1 (S)	Boutrou et al. (2013); Sanchón et al. (2018)
α_{S1}-CN	109–119	LEIVPNsAEER	1 (S)	Barbé et al. (2014); Boutrou et al. (2013)
α_{S1}-CN	110–118	EIVPNsAEE	1 (S)	Sanchón et al. (2018)
α_{S1}-CN	110–119	EIVPNsAEER	1 (S)	Barbé et al. (2014); Boutrou et al. (2013); Sanchón et al. (2018)
α_{S1}-CN	111–119	IVPNsAEER	1 (S)	Barbé et al. (2014)
α_{S2}-CN	7–17	VsssEESIIsQ	4 (S)	
α_{S2}-CN	13–17	SIIsQ	1 (S)	
α_{S2}-CN	13–18	SIIsQE	1 (S)	
α_{S2}-CN	13–19	SIIsQET	1 (S)	
α_{S2}-CN	14–17	IIsQ	1 (S)	
α_{S2}-CN	126–136	EQLstsEENSK	3 (2S, T)	
α_{S2}-CN	126–136	EQLStsEENSK	2 (S,T)	
α_{S2}-CN	129–136	sTsEENSK	2 (S)	
α_{S2}-CN	138–146	TVDMEStEV	1 (T)	Boutrou et al. (2013)
α_{S2}-CN	138–146	TVDMEsTEV	1 (S)	Boutrou et al. (2013)
α_{S2}-CN	139–146	VDMEStEV	1 (S)	
β-CN	6–16	LNVPGEIVEsL	1 (S)	Barbé et al. (2014); Sanchón et al. (2018)
β-CN	7–16	NVPGEIVEsL	1 (S)	Sanchón et al. (2018)
β-CN	7–17	NVPGEIVEsLs	2 (S)	Chabance et al. (1998)
β-CN	8–16	VPGEIVEsL	1 (S)	Sanchón et al. (2018)
β-CN	11–16	EIVEsL	1 (S)	
β-CN	12–16	IVEsL	1 (S)	
β-CN	32–38	KFQsEEQ	1 (S)	
β-CN	33–37	FQsEE	1 (S)	
β-CN	33–38	FQsEEQ	1 (S)	
β-CN	33–39	FQsEEQQ	1 (S)	
β-CN	33–40	FQsEEQQQ	1 (S)	
β-CN	34–39	QsEEQQ	1 (S)	
β-CN	163–167	LSLsQ	1 (S)	
κ-CN	122–131	INTIAsGEPT	1 (S)	
κ-CN	124–131	TIAsGEPT	1 (S)	

3.3. Antioxidant activity of in vitro digests on 70% Caco-2/30% HT-29 co-culture

The digested samples alone did not exert any significant ($P < 0.05$) prooxidant action on the 70% Caco-2/30% HT-29 co-culture (data not shown). This finding confirmed previous results of García-Nebot et al. (2011), who tested *in vitro* tryptic digests of bovine CN on Caco-2 cells.

When combined with AAPH, digested hCN administered to cells at 6.4 μg/μL induced an antioxidant effect 2.6 times higher than Vit C (Fig. 2A). CPP digest acted as an antioxidant till 3.3 times higher than Vit C, with a dose-dependent behavior (Fig. 2B). Finally, the CaCPP sample showed no significant ($P < 0.05$) effects (Fig. 2C).

Digests administered to the co-culture in presence of Fe^{2+} induced prooxidant or antioxidant effects. In detail, hCN was more oxidant than Fe^{2+} at 2.4 and 3.2 μg/μL doses, whereas at 6.4 μg/μL it exerted an antioxidant activity 4.4 times higher than Vit C (Fig. 2D). The administration of CPP digest along with Fe^{2+} increased the cell oxidative status at the lowest dose, but it behaved as antioxidant at higher doses, thus resulting 3.5 and 3.7 times more effective than Vit C (Fig. 2E). The administration of CaCPP digest and Fe^{2+} displayed a prooxidant effects at the minor and intermediate doses, while the antioxidant activity of the highest dose was 1.5 times higher than Vit C (Fig. 2F).

3.4. Antioxidant activity of IM digests on Saos-2 cells

The administration of IM-hCN, IM-CPP and IM-CaCPP did not cause any significant ($P < 0.05$) modification of the basal cell oxidative status of Saos-2 cells (data not shown). The combination of the three IM digests with AAPH resulted in a dose-dependent antioxidant activity, although some differences among the samples were observed. Indeed, IM-hCN, IM-CPP and IM-CaCPP showed antioxidant effects 3.2–4.9, 2.4–5.3 and 1.2–4.6 times higher than that of Vit C, respectively (Fig. 3). In presence of Fe^{2+}, IM-hCN displayed an antioxidant activity only at the minor dose, and 2 times higher than Vit C (Fig. 3D). However, IM-CPP and IM-CaCPP did not induce any significant ($P < 0.05$) effects (Fig. 3E and F).

3.5. LDH release by 70% Caco-2/30% HT-29 co-culture and Saos-2 cells after administration of digests or IM digests

Digest administration in presence of Fe^{2+} did not induce membrane damage of the intestinal cells (Fig. 4A, B, C, white bars). In presence of AAPH, all digests showed a dose-dependent protective effect compared to AAPH administration alone ($P < 0.05$). Similarly, the administration of IM digest, in combination with Fe^{2+} or AAPH, did not affect the membrane integrity of Saos-2 cells (Fig. 4D, E, F). All IM digests showed a significant ($P < 0.05$) protective effect *vs* AAPH at the highest dose, and, only in the case of IM-hCN, even at the intermediate dose.

4. Discussion

In the present study, we investigated three commercial samples,

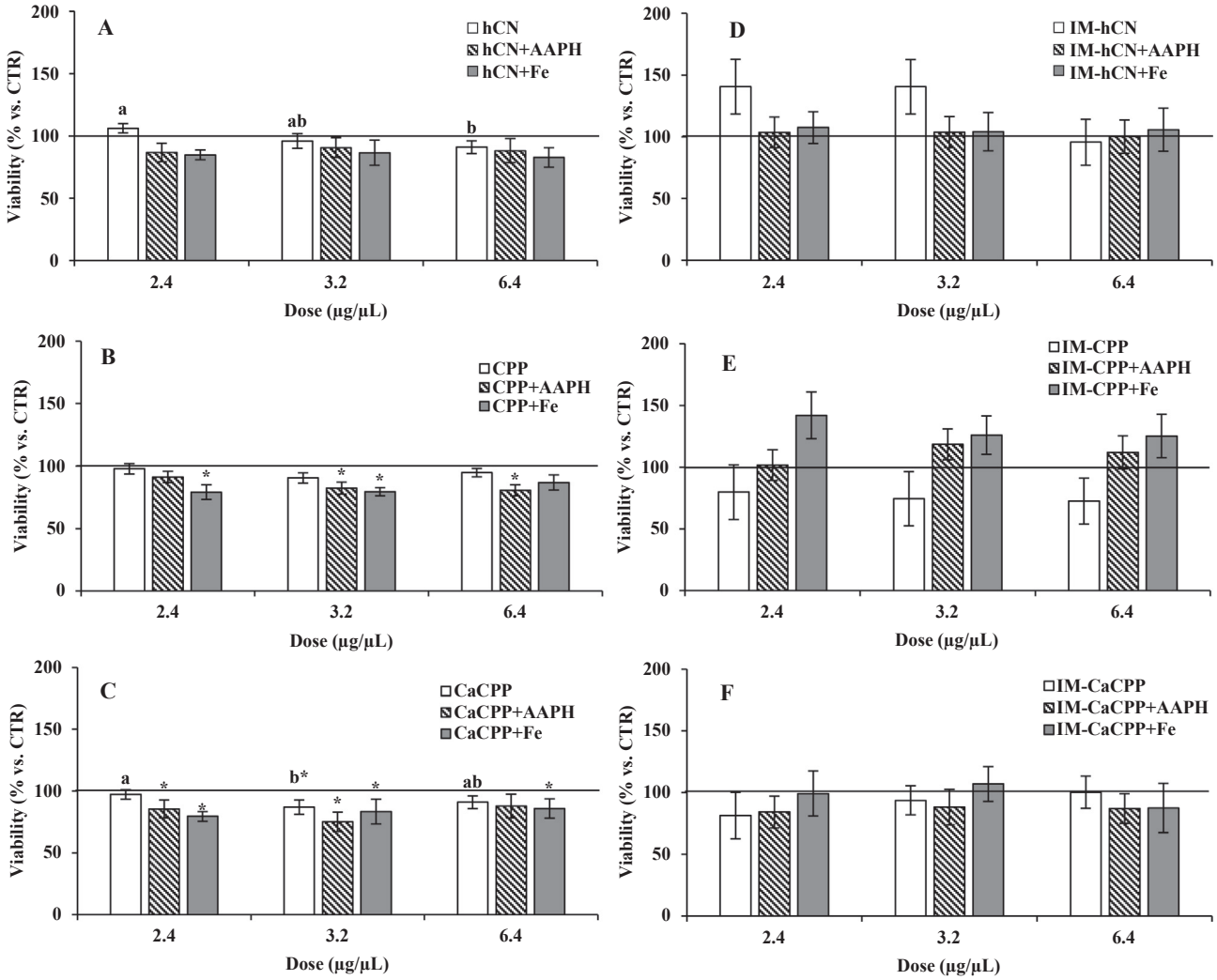

Fig. 1. Caco-2/HT-29 70/30 co-culture cell and Saos-2 cell viability after incubation with *in vitro* digests and IM digests. (A, B, C) Effect of hCN, CPP and CaCPP administration on co-culture cells. (D, E, F) Effect of IM-hCN, IM-CPP and IM-CaCPP administration on Saos-2 cells. Asterisks indicate statistical differences from control of non-treated cells (CTR, 100), while letters indicate differences ($P < 0.05$) among the three tested doses of the same sample.

bovine hCN, CPP and CaCPP, as potential antioxidant dietary supplements. To simulate the *in vivo* physiological digestive process, the *in vitro* SGID according to the internationally-approved COST-Infogest digestion protocol (Brodkorb et al., 2019) was performed. All digests were characterized for their qualitative profile of caseinophosphopetides. Similar number of caseinophosphopetides was revealed in all of them. Despite this, tri-phosphorylated forms and cluster sssEE containing peptides were most abundant in CPP digest. The cluster sssEE represents the sequences α_{S1}-CN f(66–70), α_{S2}-CN f(8–12), α_{S2}-CN f(56–60) and β-CN f(17–21) of the native bovine CNs (Farrell et al., 2004), and it is well characterized as conferring a strong cation-binding activity to caseinophosphopeptides. The latter, in turn, have been demonstrated to have biological interest for positive effects on calcium and iron availability (Bouhallab & Bouglé, 2004) and free radical scavenging (Kitts, 2005). Indeed, some of the caseinophosphopeptides in common among the three digests were previously revealed in different *in vitro* and *in vivo* (Table 2) studies. Recently, (Sanchón et al., 2018) reported the caseinophosphopeptides released from the α_{S1}-CN f (43–53) region after *in vitro* or *in vivo* CN digestion. Some of these peptides were found in the hCN, CPP and CaCPP digests (Table 2). The same authors also showed that the peptides originated *in vivo* from the N-terminal region of β-CN were shorter in length compared to those obtained *in vitro*. This feature also applied to N-terminal β-CN peptides revealed in hCN, CPP and CaCPP digests, which however contained some peptides identical to those released *in vivo* (Sanchón et al., 2018). Research studies of García-Nebot and collaborators (García-Nebot et al., 2011; García-Nebot, Alegría, Barberá, Contreras, & Recio, 2010; García-Nebot, Alegría, Barberá, Gaboriau, & Bouhallab, 2015) also reported identical peptides after *in vitro* digestion [α_{S2}-CN f(138–146)1P, β-CN f (6–16)1P, β-CN f(7–16)1P, β-CN f(11–16)1P, β-CN f(33–39)1P and β-CN f(33–40)1P].

The capability of caseinophosphopeptides to chelate metal ions mainly accounts for their secondary antioxidant activity (Kitts, 2005), and in this regard the key role in chelation is exerted by the sssEE cluster (Miquel, Alegría, Barberá, & Farré, 2006). The formation of iron-phosphopeptide complexes by peptides carrying the sssEE cluster occurs at a certain Fe^{2+} to Ser(P) molar ratio (Kitts, 2005). Overall, the binding of phosphopeptides with Fe^{2+} could produce stable iron-phosphopeptide complexes, and this phenomenon is the basis for reduction of ROS formation through the Fenton reaction. In literature, little information is available regarding the antioxidant activity of specific caseinophosphopeptides. As a matter of fact, only the cluster-containing peptide β-CN f(1–25)4P has been extensively studied (Bouhallab et al., 2002; Kibangou et al., 2005). The peptide β-CN f (1–25)3P was identified only in the digest of CaCPP, while shorter and/or different phosphorylated forms of β-CN f(1–25), still containing the cluster and detected in our digests. Based on this, it could be hypothesized all these peptides could also elicit antioxidant properties

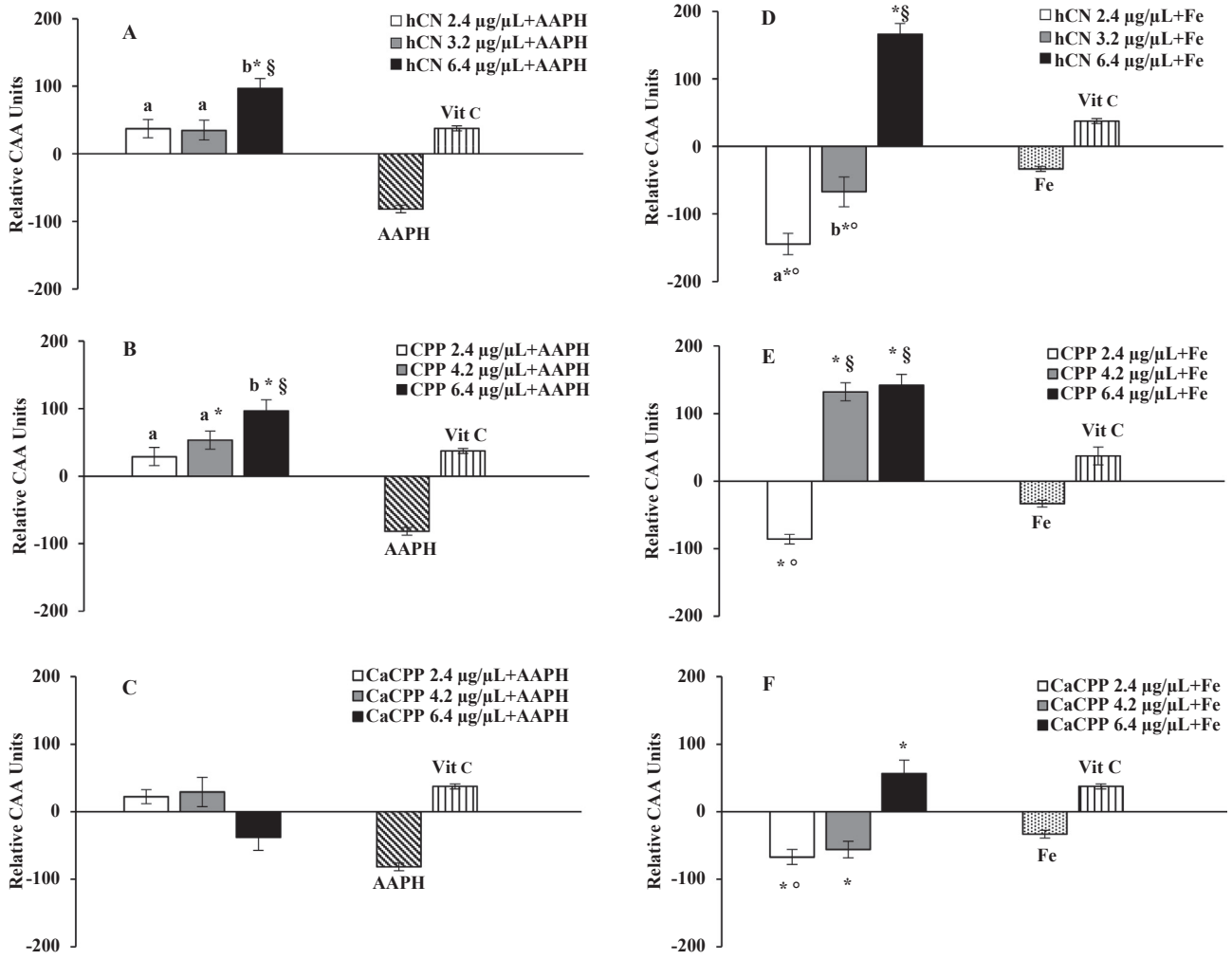

Fig. 2. Effect of hCN, CPP and CaCPP on the oxidative state of Caco-2/HT-29 70/30 co-culture cells. For each sample, hCN (panels A, D), CPP (panels B, E) and CaCPP (panels C, F), the effect of the three different doses was evaluated with and without the presence of AAPH and Fe^{2+}. Asterisks indicate statistical differences from control of non-treated cells (CTR, relative CAA Units = 0), while letters indicate differences ($P < 0.05$) among the three tested doses within the same sample. §, °, # symbols indicate statistically significant ($P < 0.05$) differences between digest administration in presence of the oxidants, in comparison to Vit C, Fe^{2+} and AAPH, respectively.

similar to β-CN f(1–25)4P.

The antioxidant activity was tested using three different doses of the digests to evaluate a possible dose-effect relationship. Firstly, we investigated the prooxidant or antioxidant effects of digests on intestinal cells. Indeed, some dietary components, including iron, can directly interact with enterocytes by increasing their oxidative stress (Seril et al., 2002). In presence of AAPH, hCN and CPP digests displayed a primary dose-dependent antioxidant activity, sometimes more pronounced than Vit C and likely due to scavenging of free radicals (Fig. 2A, B and C). Instead, the CaCPP digest displayed a minor primary antioxidant activity, which did not significantly modify the basal oxidative status of intestinal cells. This different behavior was likely related to calcium preloading. The complexes between calcium ions and caseinophosphopeptides in CaCPP were demonstrated to be particularly stable (Perego et al., 2015). This feature likely impaired phosphopeptide interaction with ROS, thus reducing the primary antioxidant activity of CaCPP (Kitts, 2005).

In presence of Fe^{2+}, which promotes ROS production through the Fenton reaction, the activity of digests appeared more heterogeneous and dose-dependent (Fig. 2C, D and E). At the minor doses they behaved as prooxidants in a way comparable or higher than Fe^{2+}, while at the maximum tested dose they acted as antioxidants as much or more than Vit C. The prooxidant action revealed at the lowest digest doses is in accordance with the same activity displayed by both, a preparation of enriched caseinophosphopeptides (Díaz & Decker, 2004) and known Fe^{2+} chelators such as EDTA (Frankel, Satué-Gracia, Meyer, & German, 2002). As stated above, the formation of phosphopeptides/Fe^{2+} complexes occurs at a precise Fe^{2+} to Ser(P) molar ratio (Kitts, 2005). This might explain the dose-dependent secondary antioxidant activity of the studied digests. Indeed, in literature prooxidant action has been detected when the iron level stoichiometrically exceeded that of molecules with chelating capability (Díaz & Decker, 2004). This condition likely occurred when the lowest doses of the studied digests were tested. It has to be mentioned that the Fe^{2+} amount (250 µM) employed in the present work to mimic an oxidative stress, and capable to trigger the Fenton reaction, hardly occurs *in vivo*. Anyway, at the highest doses the opposite effect was recorded (Fig. 2), and a LDH release, indicating the damaged plasma membrane upon oxidative stress was not evidenced for all the tested doses.

The secondary antioxidant activity of CPP digest, unlike the two other samples, was already evident at the intermediate dose. This finding could be explained considering that CPP digest has the highest number of caseinophosphopeptides carrying the sssEE cluster, as well as the highest number of peptides with three or more phosphorylation sites (Table 1). Moreover, CPP digest comprised the highest number of caseinophosphopeptide sequences from α_{S1}-CN and α_{S2}-CN (Table 1),

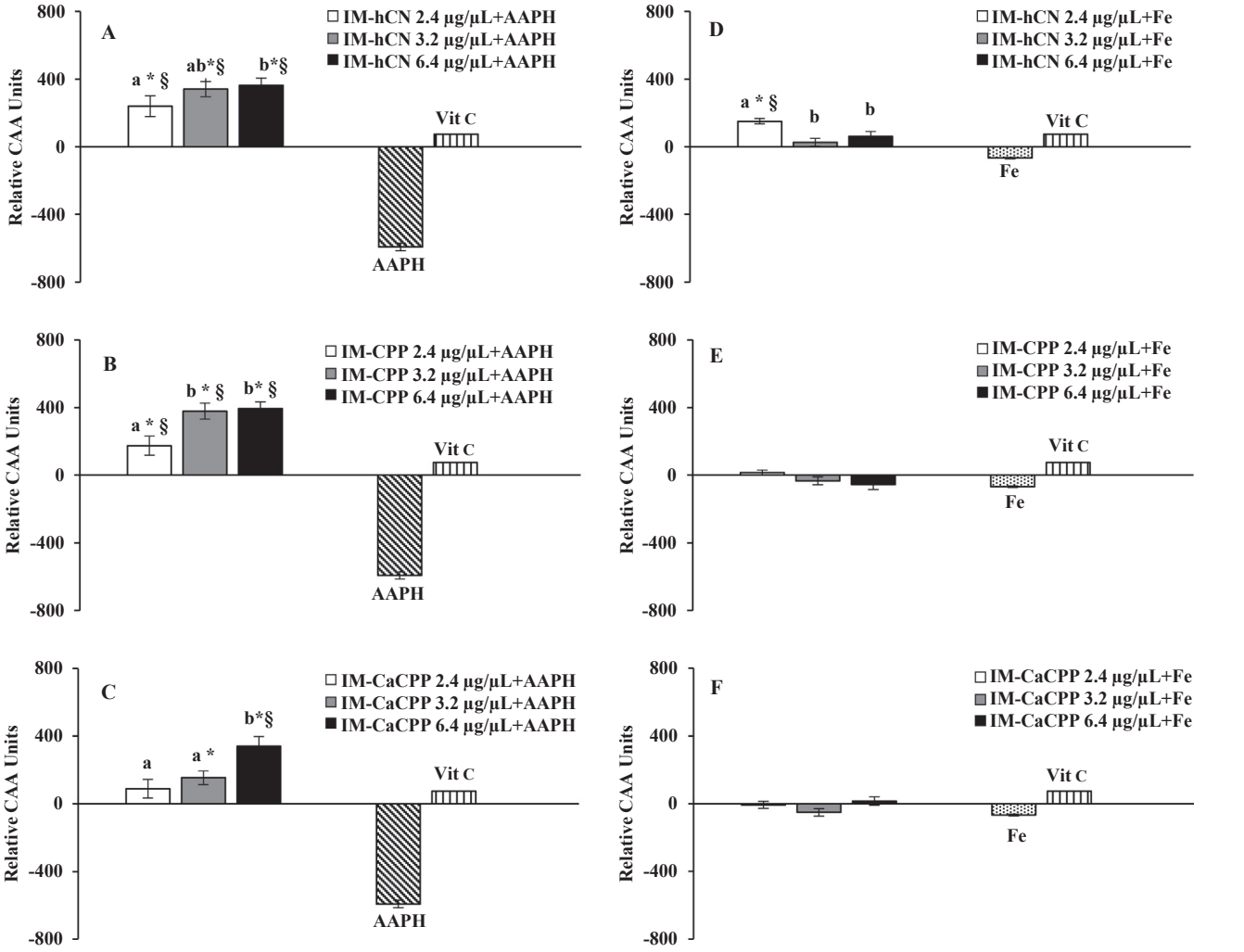

Fig. 3. Effect of IM-hCN, IM-CPP and IM-CaCPP on the oxidative state of Saos-2 cells. For each sample, IM-hCN (panels A, D), IM-CPP (panels B, E) and IM-CaCPP (panels C, F), the effect of the three different doses was evaluated with and without the presence of AAPH and Fe^{2+}. Asterisks indicate statistical differences from control of non-treated cells (CTR, relative CAA Units = 0), while letters indicate differences ($P < 0.05$) among the three tested doses within the same sample. §, °, # symbols indicate statistically significant differences ($P < 0.05$) between digest administration in presence of the oxidants, in comparison to Vit C, Fe^{2+} and AAPH, respectively.

compared to hCN and CaCPP. In this regard, cluster-containing peptides from α_{S1}-CN have been reported to strongly chelate Fe^{2+} and, consequently, to reduce the bioavailability of this metal (Bouhallab et al., 2002; Kibangou et al., 2005). All together these features make CPP digest more capable to bind and retain Fe^{2+}, a property already described for caseinophosphopeptides originated from α_S-CN (Bouhallab & Bouglé, 2004). In the case of CaCPP digest, it can be speculated that almost all Ser(P) residues of caseinophosphopeptides were bound to calcium ions. Therefore, an antioxidant activity based on Fe^{2+} binding was detectable only at the major dose, when free Ser(P) likely overcame the number of Fe^{2+} ions. This explanation is in agreement with the suggested saturation mechanism proposed by (Rival, Boeriu, & Wichers, 2001), who negatively correlated caseinophosphopeptide chelating properties with increasing Fe^{2+} concentrations. Analogously, the most important prooxidant effect was exerted by hCN, which contained the lowest amount of caseinophosphopeptides and the highest proportion of mono- and di-phosphorylated peptides. This likely accounted for its minor capacity to bind Fe^{2+}, especially at the minor doses.

To take into account the *in vitro* changes occurring at the intestinal cell level, the IM digests were administered *in vitro* to Saos-2 cells. In presence of AAPH, these digests exerted an antioxidant activity higher than that of Vit C. On the contrary, the secondary antioxidant activity (i.e. Fe^{2+} chelation) was retained only by the IM-hCN digest when administered at the lowest dose. As far as the LDH release is concerned, the administration of IM digest, in combination with Fe^{2+} or AAPH, did not affect the membrane integrity of Saos-2 cells (Fig. 4D, E, F). At the highest dose, all IM digests showed a significant protective effect *vs* AAPH, and even at the intermediate dose in the case of IM-hCN and IM-CPP. Interestingly, digest and IM digest from hCN showed the highest secondary antioxidant activity, probably due to the presence of peptides other than caseinophosphopeptides. The possibility for CN-derived peptides to reach bone and equally exert an antioxidant activity is far to be unlikely (Chabance et al., 1998). Unfortunately, it was not possible to determine by HR-MS/MS the (caseinophospho)peptidic profile of the IM digests due to the very low amount and negligible concentration of peptides of available (basolateral) samples. Nonetheless, the obtained results suggest metabolization and transport at *in vitro* intestinal epithelium to modify the bioactivity of the initial digests administered to the intestinal co-culture. Anyway, the antioxidant activity exerted by the IM digests at the osteoblast level is worth of noting. Indeed, the usefulness to ingest antioxidants is based on experimental evidences that ROS are signaling molecules in the regulation of bone metabolism, since the oxidative stress caused by the production of superoxide by osteoclast leads to bone resorption (Rajput, Wairkar, & Gaud, 2018).

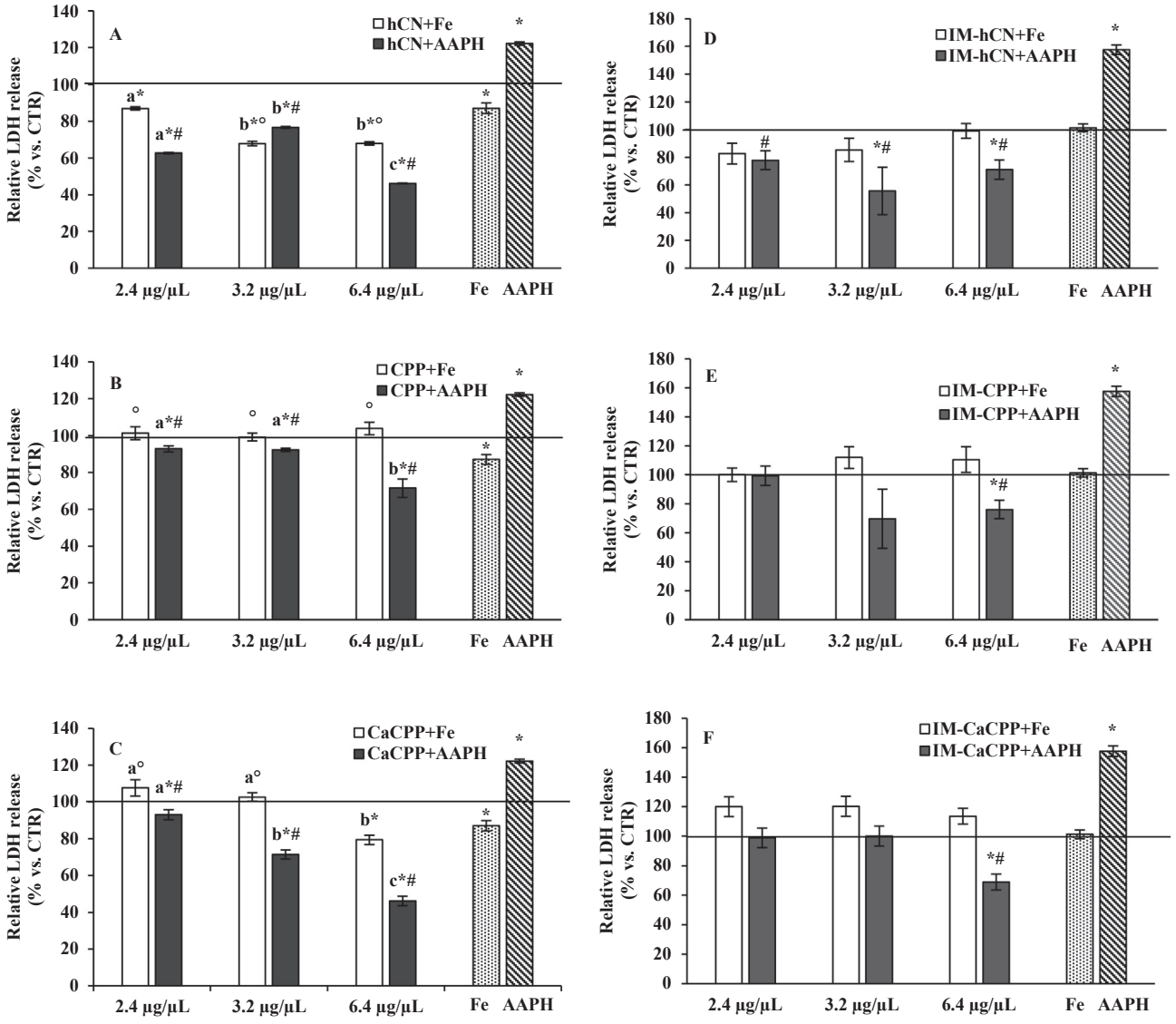

Fig. 4. LDH release by Caco-2/HT-29 70/30 co-culture and Saos-2 cells after incubation with *in vitro* digests and IM digests, respectively. (A, B, C) Effect of hCN, CPP and CaCPP administration on co-culture cells. (D, E, F) IM-hCN, IM-CPP and IM-CaCPP administration on Saos-2 cells. *, °, and # indicate statistical differences from control of non-treated cells (CTR, 100), Fe^{2+} and AAPH, respectively; letters indicate differences ($P < 0.05$) among the three tested doses of the same sample.

5. Conclusions

The results here presented showed that CN hydrolysate and caseinophosphopeptide preparations submitted to SGID exerted primary and secondary antioxidant properties at *in vitro* intestinal level in presence of AAPH and Fe^{2+}. Moreover, an antioxidant activity in presence of AAPH was revealed for IM digests in cell model of human osteoblasts, Saos-2 cells. Overall, these results claim the possibility to consider both caseinophosphopeptide-enriched preparations and CN hydrolyzates as potential tools for delivering milk-derived antioxidants addressed to support *in vivo* gut and bone health.

Funding sources

Part of this work has been funded by the Italian Ministry of Health (Ricerca Corrente).

Ethical statement

The authors declare that the research did not include any human subjects and animal experiments.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103673.

References

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., ... Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion: Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63*, 147–156. https://doi.org/10.1016/j.foodres.2014.02.015.

Bouhallab, S., & Bouglé, D. (2004). Biopeptides of milk: Caseinophosphopeptides and mineral bioavailability. *Reproduction Nutrition Development, 44*(5), 493–498. https://doi.org/10.1051/rnd:2004053.

Bouhallab, S., Cinga, V., Aít-Oukhatar, N., Bureau, F., Neuville, D., Arhan, P., ... Bouglé, D. (2002). Influence of various phosphopeptides of caseins on iron absorption. *Journal of Agricultural and Food Chemistry, 50*(24), 7127–7130. https://doi.org/10.1021/jf025554v.

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., ...

Leonil, J. (2013). Sequential release of milk protein–derived bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition, 97*(6), 1314–1323. https://doi.org/10.3945/ajcn.112.055202.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., ... Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Chabance, B., Marteau, P., Rambaud, J., Migliore-Samour, D., Boynard, M., Perrotin, P., ... Fiat, A. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie, 80*(2), 155–165. https://doi.org/10.1016/S0300-9084(98)80022-9.

Chiu, S. C. K., & Kitts, D. D. (2003). Antioxidant characterization of caseinophosphopeptides from bovine milk. In Nutraceutical Beverages Chemistry, Nutrition, and Health Effects (pp. 279–289). https://doi.org/10.1021/bk-2004-0871.ch021.

De Luca, P., Bruschi, S., Maggioni, M., Stuknytė, M., Cattaneo, S., Bottani, M., ... Ferraretto, A. (2016). Gastrointestinal digestates of Grana Padano and Trentingrana cheeses promote intestinal calcium uptake and extracellular bone matrix formation in vitro. *Food Research International, 89*, 820–827. https://doi.org/10.1016/j.foodres.2016.10.008.

Díaz, M., & Decker, E. A. (2004). Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. *Journal of Agricultural and Food Chemistry, 52*(26), 8208–8213. https://doi.org/10.1021/jf048869e.

Díaz, M., Dunn, C. M., McClements, D. J., & Decker, E. A. (2003). Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 51*(8), 2365–2370. https://doi.org/10.1021/jf025984l.

Donida, B. M., Mrak, E., Gravaghi, C., Villa, I., Cosentino, S., Zacchi, E., ... Ferraretto, A. (2009). Casein phosphopeptides promote calcium uptake and modulate the differentiation pathway in human primary osteoblast-like cells. *Peptides, 30*(12), 2233–2241. https://doi.org/10.1016/j.peptides.2009.08.003.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., ... Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk—Sixth revision. *Journal of Dairy Science, 87*(6), 1641–1674. https://doi.org/10.3168/jds.S0022-0302(04)73319-6.

Ferraretto, A., Bottani, M., De Luca, P., Cornaghi, L., Arnaboldi, F., Maggioni, M., ... Donetti, E. (2018). Morphofunctional properties of a differentiated Caco2/HT-29 cocultureas an in vitro model of human intestinal epithelium. *Bioscience Reports, 38*(2), https://doi.org/10.1042/bsr20171497 BSR20171497.

Ferraretto, A., Gravaghi, C., Fiorilli, A., & Tettamanti, G. (2003). Casein-derived bioactive phosphopeptides: Role of phosphorylation and primary structure in promoting calcium uptake by HT-29 tumor cells. *FEBS Letters, 551*(1–3), 92–98. https://doi.org/10.1016/S0014-5793(03)00741-5.

Frankel, E. N., Satué-Gracia, T., Meyer, A. S., & German, J. B. (2002). Oxidative stability of fish and algae oils containing long-chain polyunsaturated fatty acids in bulk and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 50*(7), 2094–2099. https://doi.org/10.1021/jf0111458.

García-Nebot, M. J., Alegría, A., Barberá, R., Contreras, M. del M., & Recio, I. (2010). Milk versus caseinophosphopeptides added to fruit beverage: Resistance and release from simulated gastrointestinal digestion. *Peptides, 31*(4), 555–561. https://doi.org/10.1016/j.peptides.2009.12.021.

García-Nebot, M. J., Alegría, A., Barberá, R., Gaboriau, F., & Bouhallab, S. (2015). Effect of Caseinophosphopeptides from α s - and β-Casein on Iron Bioavailability in HuH7 Cells. *Journal of Agricultural and Food Chemistry, 63*(30), 6757–6763. https://doi.org/10.1021/acs.jafc.5b02424.

García-Nebot, M. J., Cilla, A., Alegría, A., & Barberá, R. (2011). Caseinophosphopeptides exert partial and site-specific cytoprotection against H2O2-induced oxidative stress in Caco-2 cells. *Food Chemistry, 129*(4), 1495–1503. https://doi.org/10.1016/j.foodchem.2011.05.129.

Gravaghi, C., Del Favero, E., Cantu, L., Donetti, E., Bedoni, M., Fiorilli, A., ... Ferraretto, A. (2007). Casein phosphopeptide promotion of calcium uptake in HT-29 cells - Relationship between biological activity and supramolecular structure. *FEBS Journal, 274*(19), 4999–5011. https://doi.org/10.1111/j.1742-4658.2007.06015.x.

Hidalgo, A., Ferraretto, A., De Noni, I., Bottani, M., Cattaneo, S., Galli, S., & Brandolini, A. (2018). Bioactive compounds and antioxidant properties of pseudocereals-enriched water biscuits and their in vitro digestates. *Food Chemistry, 240*, 799–807. https://doi.org/10.1016/j.foodchem.2017.08.014.

Kansci, G., Genot, C., Meynier, A., Gaucheron, F., & Chobert, J.-M. (2004). β-Caseinophosphopeptide (f1–25) confers on β-casein tryptic hydrolysate an antioxidant activity during iron/ascorbate-induced oxidation of liposomes. *Le Lait, 84*(5), 449–462. https://doi.org/10.1051/lait:2004019.

Kibangou, I. B., Bouhallab, S., Henry, G., Bureau, F., Allouche, S., Blais, A., ... Bouglé, D. L. (2005). Milk proteins and iron absorption: Contrasting effects of different caseinophosphopeptides. *Pediatric Research, 58*(4), 731–734. https://doi.org/10.1203/01.PDR.0000180555.27710.46.

Kitts, D. D. (2005). Antioxidant properties of casein-phosphopeptides. *Trends in Food Science & Technology, 16*(12), 549–554. https://doi.org/10.1016/j.tifs.2005.08.009.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides - opportunities for designing future foods. *Current Pharmaceutical Design, 9*(16), 1297–1308. https://doi.org/10.2174/1381612033454892.

Laparra, J. M., Alegría, A., Barberá, R., & Farré, R. (2008). Antioxidant effect of casein phosphopeptides compared with fruit beverages supplemented with skimmed milk against H2O2-induced oxidative stress in Caco-2 cells. *Food Research International, 41*(7), 773–779. https://doi.org/10.1016/j.foodres.2008.06.002.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., ... Abete, P. (2018). Oxidative stress, aging, and diseases. *Clinical Interventions in Aging, 13*, 757–772. https://doi.org/10.2147/CIA.S158513.

López-Expósito, I., Amigo, L., & Recio, I. (2012). A mini-review on health and nutritional aspects of cheese with a focus on bioactive peptides. *Dairy Science and Technology, 92*, 419–438. https://doi.org/10.1007/s13594-012-0066-5.

Lü, J.-M., Lin, P. H., Yao, Q., & Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: Experimental approaches and model systems. *Journal of Cellular and Molecular Medicine, 14*(4), 840–860. https://doi.org/10.1111/j.1582-4934.2009.00897.x.

Mada, S. B., Reddi, S., Kumar, N., Kumar, R., Kapila, S., Kapila, R., ... Ahmad, N. (2017). Antioxidative peptide from milk exhibits antiosteopenic effects through inhibition of oxidative damage and bone-resorbing cytokines in ovariectomized rats. *Nutrition, 43–44*, 21–31. https://doi.org/10.1016/j.nut.2017.06.010.

Meisel, H. (1997). Biochemical properties of regulatory peptides derived from mil proteins. *Biopolymers, 43*(2), 119–128. https://doi.org/10.1002/(SICI)1097-0282(1997)43:2<119::AID-BIP4>3.0.CO;2-Y.

Miquel, E., Alegría, A., Barberá, R., & Farré, R. (2006). Casein phosphopeptides released by simulated gastrointestinal digestion of infant formulas and their potential role in mineral binding. *International Dairy Journal, 16*(9), 992–1000. https://doi.org/10.1016/j.idairyj.2005.10.010.

Perego, S., Del Favero, E., De Luca, P., Dal Piaz, F., Fiorilli, A., Cantu, L., & Ferraretto, A. (2015). Calcium bioaccessibility and uptake by human intestinal like cells following in vitro digestion of casein phosphopeptide-calcium aggregates. *Food and Function, 6*(6), 1796–1807. https://doi.org/10.1039/c4fo00672k.

Phelan, M., Aherne-Bruce, S. A., O'Sullivan, D., FitzGerald, R. J., & O'Brien, N. M. (2009). Potential bioactive effects of casein hydrolysates on human cultured cells. *International Dairy Journal, 19*(5), 279–285. https://doi.org/10.1016/j.idairyj.2008.12.004.

Rajput, R., Wairkar, S., & Gaud, R. (2018). Nutraceuticals for better management of osteoporosis: An overview. *Journal of Functional Foods, 47*, 480–490. https://doi.org/10.1016/j.jff.2018.06.013.

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2004). Cell Viability Assays. In Assay Guidance Manual. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23805433.

Rival, S. G., Boeriu, C. G., & Wichers, H. J. (2001). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of Agricultural and Food Chemistry, 49*(1), 295–302. https://doi.org/10.1021/jf0003911.

Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chemistry, 239*, 486–494. https://doi.org/10.1016/j.foodchem.2017.06.134.

Seril, D. N., Liao, J., Ho, K.-L. K., Warsi, A., Yang, C. S., & Yang, G.-Y. (2002). Dietary Iron Supplementation Enhances DSS-Induced Colitis and Associated Colorectal Carcinoma Development in Mice. *Digestive Diseases and Sciences, 47*(6), 1266–1278. https://doi.org/10.1023/A:1015362228659.

Wan, H., Liu, D., Yu, X., Sun, H., & Li, Y. (2015). A Caco-2 cell-based quantitative antioxidant activity assay for antioxidants. *Food Chemistry, 175*, 601–608. https://doi.org/10.1016/j.foodchem.2014.11.128.