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Review

Dairy protein hydrolysates: Peptides for health benefits

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ABSTRACT

During food digestion, proteins are hydrolysed into a large variety of peptides. Some of these peptides are structurally similar to sequences acting in the organism as endogenous signals, or hormones. Therefore, homologous food peptides can interact with the same receptors or enzymes in the organism, and in this form, exert an agonistic or antagonistic activity. The evidence of the potential of these dietary peptides to modulate numerous physiological conditions has been mainly achieved using *in vitro* assays; however, it is accepted that health evidence has to be based on *in vivo* trials (animals or humans) since the activity of these molecules depends on the ability of these peptides to reach the target tissue in an active form, which in turn depends on their structure. This article reviews the literature on the physiological effects of milk-derived bioactive peptides with special emphasis in the evidence achieved in animal and human trials.

Contents

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1. Introduction

It is recognised that diet is one of the environmental factors that influences our health and the development of certain diseases. Among the different food components, protein constitutes a major nutrient with daily ingests between 95 and 120 g of protein, which is hydrolysed into a large variety of peptides during gastrointestinal digestion. Some of these peptides share structural characteristics with endogenous peptides that act in the organism as hormones, neurotransmitters, or regulatory peptides. These exogenous food-derived peptides can interact with the same receptors in the organism and exert an agonistic or antagonistic activity. There are many examples of food-derived peptides structurally similar to endogenous peptides; one of the most representative examples is opioid peptides, which have been demonstrated to behave as opioid receptors ligands (Teschemacher, 2003). This group of food-derived bioactive peptides was the first described in 1979 (Henschen, Lottspeich, Brantl, & Teschemacher, 1979) and since then, and especially during the last two decades, much effort has been dedicated to the identification of peptides with different physiological effects. However, the level of evidence built for these food peptides is diverse. For instance, for antihypertensive peptides, activity has been demonstrated in animal models and clinical trials, but for other peptides, bioactivity has been just proven in cell cultures or *in vitro* assays. In addition, there are many examples of lack of correlation between *in vitro* and *in vivo* results, mainly due to further degradation of peptides during gastrointestinal digestion, or the impossibility to reach the target organ in a sufficient amount to exert the physiological effect.

On the other hand, absorption of food-derived peptides is not a requirement to exert a biological function in the organism. The gut is considered to be the largest endocrine organ in the body with a large range of endogenous peptides secreted and receptors expressed. The gastrointestinal tract is in contact with food digests and therefore, with an important quantity (and variety) of food-derived peptides. For this reason, the effects of peptides on different intestinal functions and health are attracting an increasing interest (Moughan, Fuller, Han, Kies, & Miner-Williams, 2007; Shimizu & Hachimura, 2011). It cannot be disregarded that given the low bioavailability described for some bioactive peptides, certain observed physiological effects might be mediated through interaction with receptors located at the gut.

The aim of this paper is to review the literature on the physiological effects of milk-derived bioactive peptides with special emphasis in the evidence achieved in animal and human trials. A short section deals with the advances on hydrolysis technologies. Finally, a section with future trends, and challenges on dairy peptides research is included.

2. Technology: dairy protein hydrolysates

During the last two decades, there has been a growing interest in the use of dairy hydrolysates containing bioactive peptides as agents for maintaining general health and preventing chronic human diseases. As a result, several technologies, mainly based on the enzymatic hydrolysis, have been developed for the production of these bioactive hydrolysates (Hernández-Ledesma, Recio, Ramos, & Amigo, 2002; Korhonen & Pihlanto, 2006; McDonagh & Fitzgerald, 1998; Phelan, Aherne, Fitzgerald, & O'Brien, 2009). This strategy is the main choice, but some disadvantages in the method have been reported, such as the need for using chemical or thermal processes, to stop the proteolysis reaction that could affect the final attributes of the hydrolysed proteins (Kosseva, Panesar, Kaur, & Kennedy, 2009). Immobilisation of enzyme molecules over different supports could overcome this problem, allowing the separation of the enzyme from the protein mixture reaction and, furthermore making possible its reuse (Madadlou, Sheehan, Emam-Djomeh, & Mousavi, 2011; Marqués et al., 2011; Rocha, Gonçalves, & Texeira, 2011). In the food industry, the use of enzymatic membrane reactors represents an interesting technology that allows protein hydrolysis and the subsequent separation of peptides generated by microfiltration or chromatography techniques, such as size exclusion or ion-exchange chromatography (Welderufael, Gibson, & Jauregui, 2012). Some studies have applied these methodologies for the recovery of caseinophosphopeptides (CPPs), antimicrobial, and angiotensin-converting enzyme (ACE)-inhibitory peptides from bovine casein (CN) hydrolysates (Recio & Visser, 1999a; Wu et al., 2013; Zhao, Xu, Yang, & Katiyo, 2013).

Moreover, alternatively to traditional methods, subcritical water hydrolysis strategies have been proposed for dairy hydrolysates production without the need for acids, bases or enzymes. The study of Espinoza, Morawicki, and Hager (2012) demonstrated successfully subcritical water hydrolysis of a whey protein isolate (WPI) and evaluated how treatment conditions (temperature and reaction time) affected the degree of hydrolysis, and the production, composition and concentration of peptides and free amino acids released. In addition, high hydrostatic pressure causes substantial modifications to milk proteins, and ultimately influences their functional properties. The potential utilities of high pressure treatments in dairy technologies have been reviewed (Chawla, Patil, & Singh, 2011; Da Cruz et al., 2010; López-Fandiño, 2006). As an example, some studies employing high pressure conditions have yielded protein hydrolysates with enhanced antioxidant and anti-inflammatory effects in intestinal epithelial cells exposed to hydrogen peroxide (Piccolomini, Iskandar, Lands, & Kubow, 2012).

3. Health benefits

3.1. Effects on the cardiovascular system

Cardiovascular diseases have become a worldwide health problem that goes beyond socio-economic barriers and equally affects men and women. Diet plays a key role in the development of the most significant risk factors of these diseases, such as hypertension, obesity, diabetes, low-grade systemic inflammation, and atherosclerosis. In recent years, bioactive milk peptides have gained interest because of their notable antihypertensive, antioxidant, anti-inflammatory and hypocholesterolaemic effects. In this section, the most current scientific information from cell culture, animal experiments and clinical studies on the role of milk proteins-derived peptides on cardiovascular diseases is summarised and discussed.

3.1.1. Antihypertensive peptides

Elevated blood pressure is one of the major independent risk factors for cardiovascular disease (Erdmann, Cheung, & Schröder, 2008). ACE (EC 3.4.15.1) is one of the main regulators of blood pressure; thus, inhibition of this enzyme is considered as one of the strategies for the treatment of hypertension. In recent years, antihypertensive effects of some peptides derived from milk proteins have been evaluated by *in vitro* and *in vivo* studies, becoming the best known class of bioactive peptides. One of the greatest challenges in developing milk peptides as antihypertensive food ingredients has been proving their *in vivo* efficacy. It mainly depends on the capacity of peptides, after being orally ingested, to reach the target organs in an intact and active form. In the last years, studies demonstrating the bioavailability of potential antihypertensive peptides have been carried out. Most of these studies aimed to evaluating the resistance of bioactive sequences to gastrointestinal

digestion, and their capacity to be absorbed using cell line models (Contreras, Sancho, Recio, & Mills, 2012; Picariello et al., 2013; Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008).

The dairy peptides have been evaluated using spontaneously hypertensive rats (SHR), and the peptide sequences, doses and maximum decrease of systolic blood pressure (SBP) have been summarised in some reviews (Fitzgerald, Murray, & Walsh, 2004; Hernández-Ledesma, Contreras, & Recio, 2011; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012b).

As shown in Table 1, two strategies have been used to release antihypertensive milk peptides: milk protein hydrolysis and fermentation. Hydrolysates of whole milk protein, caseinates and

Table 1
Antihypertensive activity in spontaneously hypertensive rats of peptide derived milk proteins by enzymatic hydrolysis and fermentation.[a]

Procedure	Enzyme/microorganism	Protein fragment	Peptide sequence	Dose (mg kg^{-1})	Decrease in SBP (mm Hg)	Reference
Hydrolysis	Trypsin	α_{S1}-CN f(23–34)	FFVAPFPGVFGK	100.0	−34.0	Karaki et al. (1990)
		α_{S1}-CN f(194–199)	TTMPLW	100.0	−13.6	Miguel, Manso, López-Fandiño,
		β-CN f(177–183)	AVPYPQR	100.0	−10.0	Alonso, and Salaices (2007)
		CMP f(106–112)	MAIPPKK	10.0	−28.0	
	Pepsin	α_{S1}-CN f(90–94)	RYLGY	5.0	−25.0	Contreras et al. (2009)
		α_{S1}-CN f(143–149)	AYFYPEL	5.0	−20.0	Recio et al. (2006)
		α_{S2}-CN f(89–95)	YQKFPQY	5.0	−15.0	Ruiz-Giménez et al. (2010)
		α_{S2}-CN f(203–208)	PYVRYL	3.0	−23.4	
		Lfcin f(20–25)	RRWQWR	10.0	−16.7	
		Lfcin f(22–23)	WQ	10.0	−11.4	
	Gastric and pancreatic enzymes	α-La f(50–53)	YGLF	0.1	−23.4	Nurminen et al. (2000)
	Pepsin, chymotrypsin and trypsin	κ-CN f(22–24)	IAK	4.0	−20.7	Miguel, Gómez-Ruiz, Recio,
		κ-CN f(61–66)	YAKPVA	6.0	−23.1	and Aleixandre (2010)
		κ-CN f(76–86)	WQVLPNAVPAK	7.0	−18.4	
		κ-CN f(98–105)	HPHPHLSF	10.0	−15.7	
	Proteinase K	β-CN f(59–61)	VYP	8.0	−21.0	Abubakar et al. (1998)
		β-CN f(59–64)	VYPFPG	8.0	−22.0	
		β-CN f(80–90)	TPVVVPPFLQP	8.0	−8.0	
		β-Lg f(78–80)	IPA	8.0	−31.0	
		BSA f(221–222)	FP	8.0	−27.0	
	Proteinase of *Lb. helveticus* CP790	α_{S1}-CN f(104–109)	YKVPQL	2.0	−13.0	Maeno et al. (1996)
		α_{S2}-CN f(189–192)	AMPKPW	2.0	−5.0	
		α_{S2}-CN f(190–197)	MKPWIQPK	2.0	−3.0	
		α_{S2}-CN f(198–202)	TKVIP	2.0	−9.0	
		β-CN f(140–143)	LQSW	2.0	−2.0	
		β-CN f(169–174)	KVLPVP	2.0	−32.2	
		β-CN f(169–175)	KVLPVPQ	2.0	−31.5	
	Thermolysin	β-Lg f(58–61)	LQKW	10.0	−18.1	Hernández-Ledesma et al. (2007)
		β-Lg f(103–105)	LLF	10.0	−29.0	
	AS1.398 neutral protease	κ-CN f(15–18)	DERF	300.0[b]	n.d.[b]	Jiang et al. (2010)
		κ-CN f(25–30)	RYPSYG	–	–	
	Flavourzyme + *S. thermophilus* and *Lb. bulgaricus*	κ-CN f(58–61)	YPYY	3.4	−15.9	Tsai et al. (2008)
	Prozyme 6 and mixture of lactic acid bacteria	α_{S1}-CN f(162–164)	GVW	3.3	−22.0	Chen et al. (2007)
		β-Lg f(17–19)	GTW			
Fermentation	*Lb. helveticus* CPN4	α_{S1}-CN f(146–147)	YP	2.0	−32.1	Maeno et al. (1996)
	Lb. helveticus and *S. cerevisiae*	β-CN f(74–76)	IPP	0.3	−28.3	Nakamura, Yamamoto, Sakai,
		β-CN f(84–86)	VPP	0.6	−32.1	Okubo et al. (1995); Nakamura, Yamamoto, Sakai, and Takano (1995)
	E. faecalis	β-CN f(58–76)	LVYPFPGPIPNSL-	6.0	−14.9	Miguel et al. (2006);
		β-CN f(133–138)	PQNIPP	3.0	−25.3	Quirós et al. (2007)
		β-CN f(133–139)	LHLPLP	10.0	−7.7	
		β-CN f(134–138)	LHLPLPL	7.0	−23.5	
		β-CN f(197–206)	HLPLP	10.0	−16.2	
		β-CN f(201–209)	VLGPVRGPFP VRGPFPIIV	10.0	−16.1	
	Caprine kefir	β-CN f(58–68)	LVYPFTGPIPN	10.0	−28.0	Miguel et al. (2010)
	Manchego cheese	α_{S1}-CN f(102–109)	KKYNVPQL	10.0	−11.5	Miguel et al. (2010); Gómez-Ruiz, Ramos, and Recio (2002)
	Gouda cheese	α_{S1}-CN f(1–9)	RPKHPIKHQ	6.1	−9.3	Saito, Nakamura, Kitazawa,
		β-CN f(60–68)	YPFPGPIPN	7.5	−7.0	Kawai, and Itoh (2000)
	Enzyme-modified cheese	β-CN f(102–104)	MAP	3.0	−17.0	Tonouchi, Suzuki, Uchida,
	Lc. lactis NRRL B-50571	κ-CN f(98–110)	HPHPHLSFMAIPP	35.0	−17.7	and Oda (2008)
	Lc. lactis NRRL B-50572	β-CN f(69–77)	SLPQNIPPL	50.0	−16.7	Rodríguez-Figueroa et al. (2012, 2013)
		κ-CN f(35–40)	YPSYGL	–	–	Rodríguez-Figueroa et al. (2012, 2013)
		β-CN f(194–209)	VLGPVRGPFP			
		κ-CN f(35–40)	YPSYGL			

[a] Adapted from Martínez-Maqueda et al. (2012b); SBP, systolic blood pressure.
[b] Effect observed after administration of casein hydrolysate.

whey proteins using gastric and pancreatic enzymes, alone or in combination, have shown antihypertensive activity in SHR. As an example, the tryptic CN hydrolysate containing the peptide corresponding to α_{S1}-CN f(23–34) has been patented and commercialised as antihypertensive agent under the name of peptide C12®. More recently, in a pepsin casein hydrolysate, two peptides derived from α_{S1}-CN, with sequences RYLGY and AYFYPEL, have demonstrated potent SBP reducing effects in SHR (Contreras, Carrón, Montero, Ramos, & Recio, 2009). The action of pepsin is also required to release the antihypertensive fragments RRWQWR and WQ from peptide lactoferricin (LFcin) (Ruiz-Giménez et al., 2010). Combination of gastric and pancreatic enzymes has been reported to be a successful strategy to produce potent antihypertensive peptides from milk proteins (Gómez-Ruiz, Ramos, & Recio, 2007; Nurminen et al., 2000). Similarly, the use of food-grade enzymes derived from microorganisms have become usual for the release of peptides with demonstrated SBP reducing effects in SHR (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Chen, Tsai, & Pan, 2007; Hernández-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007; Jiang, Tian, Brodkorb, & Huo, 2010; Maeno, Yamamoto, & Takano, 1996; Tsai, Chen, Pan, Gong, & Chung, 2008) (Table 1).

Another strategy to produce antihypertensive peptides is the use of the proteolytic system of lactic acid bacteria proteins during the elaboration of fermented milks and cheeses. The most representative example is the production of the β-CN-derived peptides, IPP and VPP, in sour milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Calpis®) with potent decreasing effects on the SBP of SHR (Nakamura et al., 1995; Nakamura, Yamamoto, Sakai, & Takano, 1995). Other peptides derived from β-CN released after milk fermentation with *Enterococcus faecalis*, with sequences LHLPLP and HLPLP, have also shown antihypertensive effect in this rat model (Miguel, Recio, Ramos, Delgado, & Aleixandre, 2006; Quirós et al., 2007). In recent studies, fermented milks with *Lactococcus lactis* NRRLB-50571 and NRRLB-50572 have presented important SBP, diastolic blood pressure (DBP), and heart rate-lowering effects in SHR (Rodríguez-Figueroa, González-Córdova, Torres-Llanez, Garcia, & Vallejo-Cordoba, 2012; Rodríguez-Figueroa, González-Córdova, Astiazaran-Garcia, Hernández-Mendoza, & Vallejo-Cordoba, 2013).

Most of the human intervention studies have focused on the tripeptides IPP and VPP. Two meta-analyses performed with the published data of 17 and 12 clinical trials have been reported by Pripp (2008) and Xu, Qin, Wang, Li, and Chang (2008), respectively. These meta-analyses have reported an average decrease in SBP of 5.1 and 4.8 mm of Hg, respectively. A recent meta-analysis carried out with 28 clinical trials has reported a mean reduction of SBP of 1.66 mm of Hg (Qin et al., 2013). However, other clinical trials have reached controversial results. In fact, Engberink et al. (2008) and Usinger, Jensen, Flambard, Linnneberg, and Ibsen (2010) did not find an effect on human blood pressure in Dutch and Danish subjects when they daily consumed fermented milks containing peptides IPP and VPP. Recently, the meta-analysis of Cicero, Gerocarni, Laghi, and Borghi (2011), which included 18 trials, has reported that IPP and VPP reduce SBP in Asian subjects while this effect was lower in Caucasian individuals. Moreover, Cicero, Aubin, Azais-Braesco, and Borghi (2013) have found that these peptides can significantly decrease SBP in European subjects, as previously shown in Japanese subjects, although the effects were more noteworthy in middle-age adults. These findings suggest that genetics and/or dietary patterns might exert an important influence on the antihypertensive effects of peptides IPP and VPP, with further studies being needed to confirm this hypothesis. Moreover, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) (ESFA, 2009) has concluded that the evidence presented to date on the antihypertensive effects of peptides VPP and IPP is insufficient to establish a cause/effect relationship between the consumption of these peptides and the maintenance of normal blood pressure and, therefore, further studies are required.

Other milk protein-derived hydrolysates and peptides have been tested in clinical trials. The consumption of a whey protein hydrolysate for 6 weeks resulted in a reduction of SBP and DBP of hypertensive subjects, although peptides responsible for the observed effects have not been identified yet (Pins & Keenan, 2006). More recently, it was reported that peptides RYLGY and AYFYPEL could also reduce blood pressure in hypertensive humans (Recio et al., 2011). A yoghurt enriched with these peptides was administrated to 71 hypertensive subjects (divided in placebo and active substances groups), and 50 normotensive volunteers that only received active substance. After 6 weeks of consumption, the hypertensive patients showed a notable reduction in their SBP, while no significant changes in blood pressure were detected in both the placebo and the normotensive groups.

3.1.2. *Anti-inflammatory and antioxidant properties of peptides*

Chronic inflammation is involved in many age-related diseases, such as atherosclerosis, vascular diseases, arthritis, cancer, diabetes, osteoporosis, dementia, obesity, and metabolic syndrome (Yu & Chung, 2006). Different cytokines play a pivotal role as mediators in the production of biomarkers implied in the progression of inflammation and the endothelial dysfunction. The down-regulation of these cytokines by food components, including peptides, may retard or alleviate inflammation, thus exerting beneficial effects against cardiovascular diseases (Tompa et al., 2010). To date, only the commercial peptide NOP-47, derived from whey, has demonstrated in a clinical study to enhance the vascular function by modulating glucose levels and biomarkers of inflammation and oxidative stress in healthy individuals (Ballard et al., 2009).

In vitro, a preliminary study using lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages suggests the potential role of a yak CN hydrolysate with alcalase to prevent inflammation-related disorders because of its ability to reduce the secretion of pro-inflammatory cytokines, and the production of nitric oxide (NO). Also, this hydrolysate has demonstrated to exert radical scavenging activity that might contribute to its beneficial properties (Mao, Cheng, Wang, & Wu, 2011).

Obesity is accompanied by low-grade chronic inflammation in different tissues such as adipose tissue, liver, pancreas, muscle and brain (Troncon Rosa, Zulet, Marchini, & Martínez, 2012). The inflammatory response associated to obesity provokes the activation of cytokines and transcriptional factors, and the penetration of macrophages in the adipose tissue, resulting in an unresolved response associated with insulin resistance, endothelial dysfunction and subsequent cardiovascular disorders (Chung et al., 2009). Dietary interventions targeted at reducing adipose tissue inflammation can be a valuable alternative for improving the general metabolic profile (Siriwardhana et al., 2013). However, Pal and Ellis (2010, 2011) did not observe significant reductions in inflammatory biomarkers of overweight and obese subjects after neither acute nor chronic supplementation with sodium caseinate or WPI compared with the controls. These results are in agreement with those reported by Lee, Skurk, Hennig, and Hauner (2007) that did not show any significant difference after administration of skimmed milk to mild hypertensive subjects. These authors suggested that potential bioactive peptides could be hydrolysed by intestinal or plasma peptidases, losing their anti-inflammatory activity. The lack of concluding findings makes difficult guaranteeing the anti-inflammatory activity of peptides derived from CN or whey proteins, and more studies are needed to confirm it.

Oxidative stress is another responsible factor for the initiation or evolution of cardiovascular diseases. Natural antioxidants provide additional benefits to the endogenous defence strategies in the battle against oxidative stress (Erdmann et al., 2008). Food-derived peptides have shown to be potent antioxidants without important side effects (Sarmadi & Ismail, 2010), with milk proteins being one of the most frequently studied sources. To date, many antioxidant peptides derived from both CN and whey proteins have been characterised by *in vitro* chemical assays (Pihlanto, 2006; Power, Jakeman, & Fitzgerald, 2013). These assays are restricted because of their limited similarity to physiological conditions, and further studies are needed to confirm the demonstrated effects. In the last few years, cell cultures have being used to assess the potential health effects of antioxidant milk-derived hydrolysates and/or peptides. These models are useful because they allow evaluation of additional factors such as peptide bioavailability and metabolism besides biological activity. However, to our knowledge, no cell culture experiments demonstrating the antioxidant activity on the cardiovascular system have been conducted.

The results of animal experiments and human trials suggest that fermented milk products may exert antioxidant effects associated to cardiovascular benefits. Zommara, Tougo, Sakanao, and Imaizumi (1998) found that fermented milk produces an anti-peroxidative action on rats fed a vitamin-E deficient diet. Moreover, the consumption of fermented goat milk by healthy individuals improved the total plasma antioxidant activity, lowered the levels of oxidised low density lipoprotein, isoprostanes and the glutathione redox ratio, and prolonged the resistance of the lipoprotein fraction to oxidation, resulting in the improvement of the anti-atherogenicity (Kullisaar et al., 2003). Although the compounds responsible for these effects have not been identified yet, peptides released during milk fermentation might have a key function. Consequently, further studies elucidating the role of antioxidant peptides in the protective activity of fermented milks on the cardiovascular system are needed.

3.1.3. Hypocholesterolaemic peptides

Diet plays a key role in plasma lipids profile, and consequently it is used as a strategy to prevent or decrease the incidence of cardiovascular diseases (Erdmann et al., 2008). It was observed that whey protein displayed a hypocholesterolaemic effect in rats after the ingestion of cholesterol free or cholesterol-enriched diets compared to CN or soybean proteins enriched diets (Nagaoka, Kanamaru, & Kuzuya, 1991; Nagaoka, Kanamaru, Kuzuya, Kojima, and Kuwata, 1992). This effect was confirmed when a tryptic hydrolysate of β-lactoglobulin (β-Lg) was administered to rats fed a diet rich in cholesterol. A notable reduction in the cholesterol level as well as an increase of the high density lipoproteins–cholesterol content, and of the excretion of fecal steroid was observed (Nagaoka et al., 2001).

A peptide called lactostatin, IIAEK, corresponding to fragment f(71–75) of β-Lg, obtained from its tryptic hydrolysate, showed hypocholesterolaemic activity in rats, being this effect higher than that showed by β-sitosterol (Nagaoka et al., 2001). Another β-Lg derived peptide, β-lactotensin (HIRL), obtained by hydrolysis with chymotrypsin, decreased total cholesterol and low density lipoproteins plus very low density lipoproteins–cholesterol content in mice fed a cholesterol-enriched diet (Yamauchi, Ohinata, & Yoshikawa, 2003). The exact mechanisms involved in the hypocholesterolaemic activity of these peptides have not been elucidated yet, but the preliminary results suggested that the amino acid composition may play a key influence (Erdmann et al., 2008). However, more studies are clearly needed to confirm the results, especially in humans determining the exact mechanism of this action.

3.2. Effects on intestinal health

The intestine is an organ responsible for nutrient absorption, barrier functions, signal recognition, and the production of endogenous active molecules. Many physiological actions are known to be regulated by hormones or cytokines, but substances contained in the diet are also thought to play a role as modulators of intestinal functions. Peptides derived from milk proteins are a group of substances with potential effects at gastrointestinal level through different mechanisms of action. They have been shown to exert protective actions on intestinal mucus, modulatory effects of mineral absorption, and anti-inflammatory, antidiabetic, and satiating activities.

3.2.1. Protective effect on the gut mucosa

The protective properties of the gastrointestinal mucus gel are attributed to its principal component, mucins, high molecular weight glycoproteins secreted by goblet cells. Disruption of the structure of this mucus has important physiological implications (Corfield et al., 2000; Linden, Sutton, Karlsson, Korolik, & McGuckin, 2008), and strong evidence was reported when spontaneous colitis and colon cancer were developed in mice genetically deficient in the mucin gene *Muc2* (Van der Sluis et al., 2006; Velcich et al., 2002).

Although intestinal epithelial cells functions are usually controlled by endogenous agents, it is worth mentioning that these cells come into continuous contact with high concentrations of food components and substances along the gut luminal surface; hence, they might be also regulated by external factors, such as nutrients (Gibson & Muir, 2005; Moughan et al., 2007). Interestingly, some dietary compounds have proven capacity to positively influence the protective properties of the mucus gel layer (Montagne, Piel, & Lalles, 2004). Dairy proteins, hydrolysates, and peptides have been demonstrated to modify the dynamics of mucus mainly via influencing the mucin secretion and expression, and the number of goblet cells. The published studies showing evidence of these bioactivities in *ex-vivo*, *in vitro* and/or *in vivo* assays are summarised in Table 2.

In the first studies carried out using an *ex-vivo* preparation of rat jejunum, CN and α-lactalbumin (α-La) hydrolysates evoked an increase in the mucin secretion, while native CN did not produce any effect (Claustre et al., 2002; Trompette et al., 2003). The peptide β-casomorphin 7, derived from β-CN, showed a strong rise in the mucin glycoprotein secretion. Since the observed effects were abolished by pre-treatment with opioid antagonist naloxone, it has been suggested that the activity might be mediated by interaction with opioid receptors. Additionally, Zoghbi et al. (2006) demonstrated that β-casomorphin 7 significantly enhanced the mucin secretion and stimulated the expression of mucin *Muc2* and *Muc3* genes in rat intestinal cells DHE, and *MUC5AC* gene in human intestinal cells HT29-MTX. Recently, a whey protein hydrolysate rich in β-Lg-derived peptides was found to induce mucin secretion and *MUC5AC* gene expression in HT29-MTX cells (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013b). Among the peptides contained in this hydrolysate, sequence β-lactorphin was identified as the major responsible for the observed effects. Cells exposed to β-lactorphin increased their mucin synthesis, whereas no significant differences were observed in the *MUC5AC* expression analysis (Martínez-Maqueda et al., 2013b). The stimulatory effect of a CN hydrolysate has been also assessed in HT29-MTX cells by Martínez-Maqueda, Miralles, Cruz-Huerta, and Recio (2013a). The hydrolysate and two α_{S1}-CN-peptides contained in it, promoted the mucin output via inducing mucin-like glycoprotein secretion and *MUC5AC* over-expression. Similarly, Plaisancié et al. (2013) have evaluated the impact of a total peptide pool from commercial yoghurt in

Table 2
Dairy proteins, hydrolysates and peptides carrying biological activities related to gastrointestinal mucosal protection.

Ex-vivo/in vitro/in vivo assays	Protein fragment/ hydrolysate[a]	Peptide sequence	Dose[b]	Mechanisms of action/effects	% of controls[c]	Reference
Preparation of isolated vasculary perfused rat jejunum	CN hydrolysate	–	0.5[d]	Induction of jejunal mucin secretion	417	Claustre et al. (2002)
	α-La hydrolysate	–	5.0[d]	Induction of jejunal mucin secretion	335	Claustre et al. (2002)
	β-CN f(60–63)	YPFP	0.001	Induction of jejunal mucin secretion	N.R.	Trompette et al. (2003)
	β-CN f(60–63)	YPFP-NH_2	0.001	Induction of jejunal mucin secretion	445	Trompette et al. (2003)
	β-CN f(60–66)	YPFPGPI	0.120	Induction of jejunal mucin secretion	563	Claustre et al. (2002)
	β-CN f(60–66)	YPFPGPI	0.120	Induction of jejunal mucin secretion	555	Trompette et al. (2003)
Rat intestinal mucin-producing cells (DHE)	β-CN f(60–66)	YPFPGPI	0.100	Induction of mucinlike glycoprotein secretion Mucin genes *Muc2* and *Muc3* overexpression	227 225/208	Zoghbi et al. (2006)
Human intestinal mucin-producing cells (HT29-MTX)	CN hydrolysate	–	0.1[d]	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	210 180	Martínez-Maqueda et al. (2013a)
	WPC hydrolysate	–	0.1[d] 1.0[d]	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	152 153	Martínez-Maqueda et al. (2013b)
	α_{S1}-CN f(143–149)	AYFYPEL	0.050 0.100	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	162 174	Martínez-Maqueda et al. (2013a)
	α_{S1}-CN f(144–149)	YFYPEL	0.050 0.500	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	166 179	Martínez-Maqueda et al. (2013a)
	β-CN f(51–55)	YPFVE	0.100	Induction of mucinlike glycoprotein secretion	234	Martínez-Maqueda et al. (2012a)
	β-CN f(60–66)	YPFPGPI	0.100	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	163 176	Zoghbi et al. (2006)
	β-CN f(94–123)	GVSKVKEAM APKHKEMPF PKYPVEPFTESQ	0.10[e]	Induction of mucinlike glycoprotein secretion Mucin genes *MUC2* and *MUC4* overexpression	N.R. N.R.	Plaisancié et al. (2013)
	α-La f(50–53)	YGLF-NH_2	0.10 0.50	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	201 160	Martínez-Maqueda et al. (2013a)
	β-Lg f(102–105)	YLLF	0.50	Induction of mucinlike glycoprotein secretion	174	Martínez-Maqueda et al. (2013b)
	β-Lg f(102–105)	YLLF-NH_2	0.10 0.50	Induction of mucinlike glycoprotein secretion Mucin gene *MUC5AC* overexpression	453 222	Martínez-Maqueda et al., 2012a Martínez-Maqueda et al., 2013b
Rat gastric epitelial cells (RGM1)	α-La protein	–	3.0[f]	Stimulate mucin synthesis, mucin secretion and prostaglandin E_2 synthesis	123/119/140	Ushida, Shimokawa, Toida, Matsui, and Takase (2007)
Supplementation in the diet of rats	CN hydrolysate	–	114[g]	Average daily gain, average daily food intake, gain:feed ratio and ileal endogenous nitrogen flow enhanced Upregulation of mucin genes *Muc3* in the small intestine and *Muc4* in the colon	410/149/270 277/325	Han et al. (2008)
	Cheese whey protein	–	160[g]	Protection against DSS-induced colitis: decrease in mucosal and luminal markers of colitis, and faecal mucin excretion and faecal microbiota enhanced	N.R.	Sprong et al. (2010)
Intragastric gavage in rats	WPI	–	0.75[g]	Protection against ethanol-induced ulcerative lesions: reduction of ulcerative lesion index and plasma gastrin, and stimulation of gastric mucus production	−74/−38 168	Castro, Carvalho, Tinti, Possenti, and Sgarbieri (2010)
	α-La protein	–	0.30[g]	Increase the thickness of the mucus gel layer in corpus and antrum gastric mucosa	163/164	Ushida et al. (2007)
	α-La hydrolysate	–	0.20[g]	Protection against indomethacin-induced ulcerative lesions: induction of gastric mucus production and prostaglandin E2 biosynthesis	134/536	Mezzaroba et al. (2006)
	β-CN f(94–123)	GVSKVKEAMA PKHKEMPFPK YPVEPFTESQ	0.01[e] 0.10[e] 0.100	Upregulation of duodenal and ileal *Muc4* mRNA expression Increase in the duodenal goblet cells recount Upregulation of jejunal *Muc2* mRNA expression Increase in the jejunal and ileal goblet cells recounts Upregulation of jejunal *Muc4* mRNA expression	156/157 133 152 143/134 161	Plaisancié et al. (2013)

[a] WPC, whey protein concentrate.
[b] Dose given in mM except where otherwise indicated.
[c] Maximum effect within the different treatment conditions assessed; N.R., non-reported.
[d] Dose is weight/volume.
[e] Dose is μM.
[f] Dose is mg mL^{-1}.
[g] Dose is g kg^{-1}.

HT29-MTX cells, observing an increase of the secreted mucin, and the mRNA levels of *MUC2* and *MUC4* genes. The β-CN fragment f(94–123) was suggested to be responsible for the reported bioactivities after evaluating its effects in intestinal cells and in rats. Oral administration of this peptide to rats yielded an increase in the goblet cells counts, modified *Muc2* and *Muc4* mRNA expression, and ultimately improved gut mucosa protection by enhancing crypts containing Paneth cells and increasing the number of rat defensin 5 and lysozyme (LZ) mRNA transcripts in the rat small intestine (Plaisancié et al., 2013).

Other *in vivo* studies have also pointed to the regulation of the protective mucus layer by dairy proteins and their degradation products. A diet containing a CN hydrolysate as the exclusive source of nitrogen was given to rats in which enhancements in the ileal endogenous nitrogen flow and in the expression of *Muc3* in the small intestine, and Muc4 in the colon were observed (Han, Deglaire, Sengupta, & Moughan, 2008). In the study of Sprong, Schonewille, and van der Meer (2010), the protective effect of cheese whey protein was examined in the dextran sulphate sodium (DSS)-induced model of rat colitis. After seven days of DSS consumption, rats fed the cheese whey protein diet showed lower levels for both mucosal and luminal markers of colitis. Although faecal mucin excretion was higher, expression of *Muc2* in the colon was not statistically different from controls. Incorporation of cheese whey protein to the diet also resulted in an increase of the counts of protective microbiota species. Interestingly, one group of rats fed a diet consisting in CN with threonine/cysteine supplementation was included in the study, and the observed protection against DSS-induced colitis was similar to the rats fed the cheese whey protein diet (Sprong et al., 2010). It was therefore suggested that reported beneficial effects of cheese whey protein might be due to its high threonine and cysteine content. In this context, availability of specific amino acids like threonine, cysteine, proline and serine has been shown to be important to increase the number of *Muc2*-containing goblet cells, up-regulate the mucin synthesis, and restore the bowel microbiota, thus helping colonic defences and mucosal healing in DSS-treated rats (Faure et al., 2006).

3.2.2. *Modulatory peptides of mineral absorption*

Mineral deficiencies are the most important nutritional problems worldwide, with the iron deficiency being the most common. This disorder happens when the mineral ingestion from diet does not meet a daily need of minerals. In this context, the mineral fortification is one of the best and most common strategies to prevent this deficiency (Zimmermann & Hurrell, 2007). It has been proposed that the phosphorylated regions released from CN during digestion, CPPs, could increase mineral solubility at intestinal pH, modulating its bioavailability (Meisel & Fitzgerald, 2003). Several authors have demonstrated the release of CPPs in rats and minipigs after the ingestion of CN (Kasai, Iwasaki, Tanaka, & Kiriyama, 1995; Meisel & Frister, 1988, 1989; Naito & Suzuki, 1972, 1974; Sato, Noguchi, & Naito, 1986; Sato, Shindo, Gunshin, Noguchi, & Naito, 1991). In humans, the liberation of CPPs has been reported after consumption of milk, yogurt, and CN (Boutrou et al., 2013; Chabance et al., 1998; Meisel et al., 2003). Also, CPPs have been demonstrated to resist further degradation by digestive enzymes and enteric bacteria in both rats and humans (Brommage, Juillerat, & Jost, 1991; Hirayama, Toyota, Hidaka, & Naito, 1992; Kasai et al., 1995; Meisel et al., 2003). Regarding the *in vivo* studies with CPP preparations, several authors have reported an increase of the soluble calcium concentration in the intestine of rats due to CPPs released from CN contained in the diet, which should improve the absorption of this mineral (Kitts, Yuan, Nagasawa, & Moriyama, 1992; Lee, Noguchi, & Naito, 1979, 1980, 1983; Sato, Noguchi, & Naito, 1983; Sato et al., 1986). However, the results of studies on the influence of CPPs upon calcium absorption in humans and animal models are controversial.

The addition of a pool of CPPs to aqueous solutions and infant foods containing phytate increased calcium absorption in rat pups after gastric intubation, being able to overcome the negative effect of phytate on its absorption (Hansen, Sandström, & Lönnerdal, 1996). In agreement with this result, in rats fed an isolated soy protein-based diet supplemented with CPPs and calcium, an increase of calcium absorption was reported, although the effect was not dose dependent (Saito, Lee, & Kimura, 1998). In contrast, diet supplementation with CPPs and calcium did not improve calcium absorption in growing pigs fed a soy-based diet (Pointillart & Guéguen, 1989), in rats fed a whey protein-based diet (Brommage et al., 1991; Kopra, Scholz-Ahrens, & Barth, 1992; Scholz-Ahrens, Kopra, & Barth, 1990), or in rats fed an experimental diet containing mainly egg albumin, corn starch, and glucose (Bennett et al., 2000; Tsuchita, Goto, Yonehara, & Kuwata, 1995). In ovariectomised rats, used as a postmenopausal bone loss model, calcium absorption was enhanced in the first three days when CPPs derived from β-CN and calcium (at higher doses) were supplemented (Tsuchita, Sekiguchi, Kuwata, Igarashi, & Ezawa, 1993). No effects of CPPs upon calcium absorption was observed in rats after administration by gastric intubation of non-fortified milk while an increase was observed when administered milk was fortified with calcium (Mora-Gutierrez, Farrell, Attaie, McWhinney, & Wang, 2007; Tsuchita, Suzuki, & Kuwata, 2001). Using a ligated duodenal loop technique, CPPs increased calcium absorption in rachitic and normal chicks (Mykkänen & Wasserman, 1980), and rats (Kitts et al., 1992; Sato et al., 1986). These results are in disagreement with the observations of Yuan and Kitts (1991) in the same model, who did not observe any difference on calcium absorption after addition of CPPs to CN or soybean enriched diets. The controversial results obtained in the above mentioned studies could be caused by several factors influencing the effect of CPPs, such as the animal model, physiological status of the individuals, type of diet and its calcium or vitamin D content, source and composition of CPPs, and the length of the assay, among others.

In humans, an increase of calcium absorption has been shown due to the addition of CPPs to rice-based cereal, although the effect was not observed after addition to whole-grain cereal or to three different meals with different calcium and phytate contents (Hansen, Sandström, Jensen, & Sorensen, 1997a,b). It has been not observed influence of CPPs upon calcium bioavailability after their ingestion with milk (López-Huertas et al., 2006; Narva, Kärkkäinen, Poussa, Lamberg-Allardt, & Korpela, 2003) or with a drink of calcium lactate (Teucher et al., 2006). In contrast, Heany, Saito, and Orimo (1994) showed a positive role of CPPs in postmenopausal women with low calcium absorption capacity, despite the fact that this effect was not observed in women with normal absorption capacity of this mineral.

CPPs bound to calcium salts have been also found to be useful to remineralise the teeth of mammals, displaying anticariogenic effects when added to dentifrices, oral care products, chewing gum or other confectionery foods (Luo & Wong, 2004). In human trials, synthetic nanocomplexes of CPPs and calcium salts incorporated in mouth rinses and sugar-free chewing gums have been proven to be potential anticariogenic agents (Reynolds, Cai, Shen, & Walker, 2003).

Concerning the iron bioavailability, a positive influence of CPPs on this parameter has been reported, reflected in an increase in the haemoglobin and haematocrit levels, as well as in the iron liver storage when iron deficient rats were supplemented with iron and hydrolysed β-CN or β-CN fragment f(1–25)4P (Aït-Oukhatar et al., 1997, 1999). Likewise, the blood iron levels were increased in rats after ingestion of iron bound to hydrolysed CN (Chaud et al., 2002).

These results were confirmed using a perfused rat intestinal model loop system, in which the iron bound to β-CN f(1–25)4P increased the absorption of this mineral in iron deficient and non-iron deficient rats (Aït-Oukhatar et al., 2002; Ani-Kibangou et al., 2005; Bouhallab et al., 2002; Pérès et al., 1997; Pérès, Bouhallab, Bureau, Neuville et al., 1999; Pérès, Bouhallab, Bureau, Maubois et al., 1999). In addition, diverse fractions of CPPs exerted different effects on iron absorption, being this effect higher when the iron was bound to β-CN fractions than to α_S-CN fractions (Bouhallab et al., 2002; Kibangou et al., 2005), suggesting the influence of the structural characteristics and conformations of CPPs on their capacity to improve the iron bioavailability.

In humans, the effect of CPPs on iron bioavailability is controversial. Whereas Hurrell, Lynch, Trinidad, Dassenko, and Cook (1989) reported an improvement of iron absorption after the intake of hydrolysed CN, Aït-Oukhatar et al. (2002) did not observe any effect after the ingestion of iron bound to β-CN f(1–25). *In vivo* studies with other minerals, such as zinc, have also found variable results. Hansen et al. (1996) reported an increased zinc absorption in rat pups after the addition of CPPs to aqueous solutions and infant foods containing phytate, avoiding the negative effect of this compound on zinc bioavailability. However, no effect was reported from soybean protein based diet containing CPPs in rats (Matsui, Okumura, & Yano, 2002). As observed for calcium, zinc absorption was reported to increase when CPPs were added to rice-based cereal. However, no effects were observed when CPPs were added to whole-grain cereal or three different meals with different calcium and phytate contents (Hansen et al., 1997a,b). These results indicate that the effects of CPPs can be influenced by factors, such as the food matrix, i.e., liquid or solid diet, as well as the phytate content (Miquel & Farré, 2007).

3.2.3. *Anti-inflammatory peptides at gastrointestinal level*

Inflammatory bowel disease is a chronic and degenerative condition comprising a group of disorders, being the ulcerative colitis and Crohn's disease the most relevant both in incidence and prevalence. Although the aetiology of this disease remains unknown, it has been demonstrated that genetic, immunological, environmental, and lifestyle factors are implied (Bernstein et al., 2009). Currently, inflammatory bowel disease is mainly treated with drugs that suppress the immune system. However, the multiple adverse effects shown by these drugs have made that their use is limited and selective to a small number of patients. To avoid these limitations, new and natural treatments without side effects are being searched and developed (Sánchez de Medina et al., 2010). The most extensively studied peptide for its activity against intestinal inflammation is the bovine caseinomacropeptide (CMP). In recent years, different animal models of intestinal inflammation have been used to provide evidence on the anti-inflammatory effect of CMP at gastrointestinal level. The activity of this peptide in mice with colitis and ileitis induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS), a model that shares important similarities with human Crohn's disease has been established (Daddaoua et al., 2005; Requena et al., 2008). Recently, the effects have been assessed in the DSS-induced model of rat colitis (López Posadas et al., 2010). These studies also evaluated the mechanism of action of CMP indicating that it mainly acts on the inflammatory/immune response by the activation of macrophages, favouring the differentiation of regulatory T cells and limiting the activation of T helper 1 cells (Sánchez de Medina et al., 2010).

Preliminary studies have shown the anti-inflammatory effect of β-CN hydrolysates by *Lactobacillus delbrueckii* ssp. *lactis* CRL581 in a murine TNBS-induced colitis model (Espeche Turbay, de Moreno de LeBlanc, Perdigón, Savoy de Giori, & Hebert, 2012). Thus, these results suggest that other milk peptides in addition to CMP might act as protective agents on intestinal inflammation, and further studies focused on the identification of potentially responsible sequences, and the evaluation of their mechanism of action should be designed.

3.2.4. *Peptides against type 2 diabetes*

Ingestion of a meal provokes the secretion, at gastrointestinal level, of incretins hormones, such as glucagon-like peptide-1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP). These hormones are involved in the modulation of gut motility, secretion of gastric and pancreatic enzymes, nutrient absorption, and stimulation of the insulin secretion from the pancreas that allows the disposal of the absorbed glucose (Drucker & Nauck, 2006). Type 2 diabetes, representing 90–95% of the diagnosed cases of diabetes, is characterised by multiple pathophysiologic defects including progressive dysfunction of pancreatic cells, insulin resistance, and increased production of hepatic glucose (Bharatam, Patel, Adane, Mittal, & Sundriyal, 2007). It has been demonstrated that continuous intravenous administration of GLP-1 normalises blood glucose levels in diabetic patients (Nauck et al., 1993). However, the rapid degradation of this hormone by the enzyme dipeptidyl-peptidase IV (DPP-IV) makes this strategy for the type 2 diabetes treatment unfeasible. Currently, incretin-based therapies include a combination of GLP-1 analogues resistant to DPP-IV action, and orally bioavailable DPP-IV inhibitors (Fadini & Avogaro, 2011).

Diet supplementation with whey protein has been demonstrated, by preclinical and clinical studies, to potentiate insulin release and ameliorate postprandial glucose control in both healthy and type 2 diabetic subjects (Sousa et al., 2012). Initially, it was suggested that these effects were due to its rapid digestion and the consequent fast increase of the levels of different amino acids in plasma (Luhovyy, Akhavan, & Anderson, 2007). However, the intake of free amino acid mixtures did not produce the same effects (Nilsson, Holst, & Bjorck, 2007). Recently, it has been hypothesised that peptides released from whey proteins during their transit through gastrointestinal tract might be responsible for the post-meal glycaemic response produced after whey intake (Akhavan, Luhovyy, Brown, Cho, & Anderson, 2010). Even the acute ingestion of a whey protein hydrolysate by rats resulted in a higher transient leucine response with a sequential increase in insulin (Toedebusch et al., 2012). A recent study has demonstrated the dose-dependent insulinotropic effect of whey protein hydrolysates in a cell-based co-culture approach using pancreatic BRIN-BD11 beta cells and Caco-2 cells monolayers (Gaudel et al., 2013). These authors confirmed these effects in obese (ob/ob) mice, observing, after the oral administration of those hydrolysates, an improvement of blood glucose clearance, reduction of hyperinsulinemia, and restoration of the pancreatic islet capacity to secrete insulin in response to glucose.

In addition, a significant reduction of DPP-IV activity was observed in the proximal small bowel of rodents fed whey proteins (Gunnarsson et al., 2006), suggesting that peptides released from these proteins might exert DPP-IV inhibitory effects at intestinal level. Recent studies have demonstrated the *in vitro* DPP-IV inhibitory activity of hydrolysates derived from whey proteins, and peptides contained in these hydrolysates, and potentially responsible for the observed effects have been identified (Nongonierma & Fitzgerald, 2013). Among these peptides, sequences IPA (β-lactosin) and IPAVF, derived from β-Lg are amongst the most effective DPP-IV inhibitory peptides described to date (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Tulipano, Sibilia, Caroli, & Cocchi, 2011). A β-Lg-derived peptide, with the sequence VAGTWY, presented hypoglycemic effects in the oral glucose tolerance test in mice (Uchida, Ohshiba, & Mogami, 2011). This *in vivo* effect has been also demonstrated for the peptide LPQNIPPL,

a CN-derived fragment described as DPP-IV inhibitor (Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). In fact, a recent in silico study has shown that both CN and whey proteins serve as precursors of DPP-IV inhibitory peptides because of the high number of fragments contained within them that match DPP-IV inhibitory sequences described in the literature (Lacroix & Li-Chan, 2012).

3.2.5. Effect on satiety

It is recognised that protein is the most satiating macronutrient of food (Fromentin et al., 2012). Although it is not clear if the source of protein can influence this satiating effect, the consumption of dairy foods and calcium has been recommended in several diets aiming the regulation of the body weight. Several clinical studies have proposed a certain satiating effect for dairy products. For instance, in a 6-month trial with an energy-restricted diet, the group receiving milk supplementation showed a decreased desire to eat and hunger versus the placebo group, although no differences in the level of ghrelin or leptin were found (Gilbert et al., 2011). In a recent study with 49 overweight and obese adults, the high-dairy group had similar weight loss than the low-dairy group, but high-dairy group showed slightly higher peptide YY concentrations in plasma and enhanced sensation of satisfaction (Jones et al., 2013).

The mechanisms of action of different protein types on satiety are still under investigation, but it has been proposed that it could be related to a slowdown of the gastric emptying, an increase in brain amino acids, or the presence of specific peptides or amino acids. In this respect, it has been highlighted that in animal or human trials, diet has to be well equilibrated in its essential amino acid content in order to prevent depression in food intake due to conditioned food aversion (Fromentin et al., 2012). In particular, it has been proposed that whey proteins contribute to short term appetite suppression, with this effect being higher than that obtained with CN, soy protein and egg albumin (Anderson, Tecimer, Shah, & Zafar, 2004). This effect can be attributed to the high content in branched-chain amino acids (mainly, L-leucine), the presence of certain peptides, such as CMP, or it can be mediated by the release of satiety hormones (cholecystokinin, GLP-1, GIP, peptide YY or ghrelin). A review that summarises the evidence built in animal and human trials on the effect of whey protein consumption on satiety and intake regulatory mechanisms has been previously published (Luhovy et al., 2007). Later reports have compared the effect of whey protein and a whey protein hydrolysate, when consumed before a meal, on glucose and insulin levels and pre- and post-meal satiety. Interestingly, it was found that the whey protein, but not the whey protein hydrolysate, reduced post-meal blood glucose and insulin concentrations in a dose-dependent manner, and diminished food intake (Akhavan et al., 2010). Therefore, it seems relevant the form in which the protein is ingested, or in other words, the peptides that reach the gut during gastrointestinal digestion of whey proteins. Peptide composition and characterisation of hydrolysates is definitive in this kind of studies to extract cause-effect conclusions. Other studies have confirmed that whey proteins enhance satiety in humans in a short time period of time compared with carbohydrates but the effect could not be attributed to CMP since same effect was achieved with a WPI without CMP (Lam, Moughan, Awati, & Morton, 2009). Similarly, in a double-blind acute study with 20 overweight/obese males, no differences were found on plasma cholecystokinin levels or subjective satiety after ingestion of CMP with different glycosylation levels (Keogh et al., 2010). However, a positive effect was shown in a study with individuals with phenylketonuria where the consumption of CMP at breakfast was compared with an amino acid-based formula. Lower postprandial ghrelin levels after breakfast containing CMP associated with a greater feeling of fullness were found (MacLeod, Clayton, van Calcar, & Ney, 2010). Consequently, more work is needed to confirm the effect or certain whey components on satiety, and to elucidate the mechanism of action implicated in the activity of the whey protein fraction. Finally, it has been pointed out that the difference in satiety by various proteins can be also influenced by the physical properties of food in the gut, and factors, such as, viscosity, or clotting of proteins in the gut play an important role. For instance, in a comparative study with whey protein drinks and alginate-based drinks with different viscosities and protein contents, it was found that subjects reported reduced hunger with protein drinks with higher viscosity independently of the protein content, suggesting that viscosity can exert a higher effect than protein concentration (Solah et al., 2010).

Concerning the CN fraction of milk, it is known that peripheral opioid and cholecystokinin receptors are activated by ingestion of CN, and blocking receptors with antagonists reduces their effect on food intake (Froetschel, Azain, Edwards, Barb, & Amos, 2001; Pupovac & Anderson, 2002). Therefore, the presence of peptides able to interact with these receptors will determine the activity of CN hydrolysates. Because the composition of the CN hydrolysates varies with the hydrolysis process, peptidomic characterisation of the products is essential.

In a comparative study of CN and whey on gastrointestinal hormone secretion and appetite, it was found a greater subjective satiety after the whey load. Whey also produced a higher increase in postprandial plasma amino acid concentration, and higher levels of cholecystokinin, GLP-1 and GIP than CN. These results emphasise the importance of the type of protein, and confirm that post-absorptive increases in plasma amino acids and gastrointestinal hormones are involved in the appetite response (Hall, Millward, Long, & Morgan, 2003).

3.3. Effects on body defences

Apart from their nutritional value, milk proteins are able to exert physiological activities aiming to protect the newborn mammal. Furthermore, throughout the course of digestion of milk, bioactive peptides are released from consumed proteins. These peptides may possess not only immunomodulatory, but also antimicrobial and antiproliferative properties that help improving the defence system of the neonate.

3.3.1. Antimicrobial peptides

The antimicrobial activities of milk protein-derived peptides are very diverse, ranging from those with a prebiotic effect, peptides with the ability to prevent the attachment or invasion of pathogen microorganisms, to peptides killing or inhibiting the growth of microorganisms. This section will refer to this latter group of peptides with special emphasis on those peptides with antibacterial activity. The antiviral activity of dairy proteins, modifications thereof, and derived peptides has been reviewed by different authors (Berlutti et al., 2011; Pan et al., 2006).

Milk-derived antimicrobial peptides are characterised by a highly positively charged sequence and certain amphiphilic character. It has been proposed that the net positive charge will aid binding to negatively charged bacterial membranes and the amphiphilic character will help membrane disruption. Some peptides, like LFcin and lactoferrampin, contain also a hydrophobic region with a tryptophan residue, which is involved in membrane insertion (Schiffer, Chang, & Stevens, 1992). However, recent reports have also described the activity of anionic peptides-enriched extracts from Cheddar cheese against *Listeria monocytogenes* (Demers-Mathieu et al., 2013a). The origin and activity of some of

the most relevant antibacterial peptides derived from milk proteins are summarised in Table 3.

The antimicrobial peptides that have received most attention in the last two decades are derived from lactoferrin (LF). Hydrolysis of bovine LF with pepsin generates several protein fragments with antibacterial activity. Among them, fragment f(17–41/42), known as LFcin (Bellamy et al., 1992), is one of the most potent with a broad spectrum against Gram-positive and Gram-negative bacteria; but also other active fragments from the N-lobe of the protein, i.e., f(267–285), f(267–288), and f(277–288), are released by the action of this enzyme (Recio & Visser, 1999b). A peptide from this region of the protein, f(268–288), called lactoferrampin, was later chemically synthesised, and it has demonstrated activity against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Van der Kraan et al., 2004). Recently, it has been shown that chimerisation of LFcin and lactoferrampin increases activity against various microorganisms, such as *Candida albicans*, and *Burkholderia pseudomallei* (Bolscher, Nazmi, van Marle, van 't Hof, & Veerman, 2012; Puknun et al., 2013).

Digestion of the major bovine whey proteins, β-Lg and α-La, with trypsin and chymotrypsin, respectively, releases peptides with moderate activity against Gram-positive bacteria (Pellegrini, Dettling, Thomas, & Hunziker, 2001; Pellegrini, Thomas, Bramaz, Hunziker, & Von Fellenberg, 1999). Recently, Demers-Mathieu et al. (2013b) obtained an antibacterial fraction rich in anionic peptides of about 8 amino acids long by nanofiltration of a tryptic hydrolysate of a WPI. The same research group has recently demonstrated the antibacterial activity of a peptic hydrolysate of a

Table 3
Fragments, origin, antibacterial, and other biological activities of the most relevant antibacterial peptides.[a]

Fragment[b]	Isolation	Antibacterial activity	Other activities[c]	References
α_{S1}-CN f(1–23)	Bovine CN digested with chymosin	Gram-positive bacteria, fungi and yeast	Immunomodulatory	Lahov and Regelson (1996)
α_{S1}-CN f(99–109)	Bovine sodium caseinate digested with pepsin	Several Gram-positive and Gram-negative bacteria	N.R.	McCann et al. (2006)
α_{S1}-CN f(21–29) α_{S1}-CN f(30–38)	Bovine sodium caseinate fermented with *Lactobacillus acidophilus* DPC6026	Several Gram-positive and Gram-negative bacteria	N.R.	Hayes, Ross, Fitzgerald, Hill, and Stanton (2006)
α_{S2}-CN f(164–169) α_{S2}-CN f(183–207)	Bovine α_{S2}-CN digested with pepsin	Several Gram-positive and Gram-negative bacteria	Growth promoter	Recio and Visser (1999b) Smith, Wilkinson, and Liu (1997)
$^{O}\alpha_{S2}$-CN f(165–170) $^{O}\alpha_{S2}$-CN f(165–181) $^{O}\alpha_{S2}$-CN f(184–208) $^{O}\alpha_{S2}$-CN f(203–208)	Ovine α_{S2}-CN digested with pepsin	Several Gram-positive and Gram-negative bacteria	Antihypertensive Antioxidant	López-Expósito, Gómez-Ruiz, Amigo, and Recio (2006), Recio et al. (2006)
^{H}κ-CN f(43–97)	Human milk digested with pepsin	Several Gram-positive and Gram-negative bacteria, yeasts	N.R.	Liepke, Zucht, Frossmann, and Stöndker (2001)
κ-CN f(106–169)	Bovine CN digested with chymosin	*S. mutans* *P. gingivalis* *E. coli*	Bifidogenic Immunomodulatory	Malkoski et al. (2001) Prouxl, Gauthier, and Roy (1992) Brody (2000)
κ-CN f(18–24) κ-CN f(30–32) κ-CN f(139–146)	Bovine κ-CN digested with pepsin	Several Gram-positive and Gram-negative bacteria	N.R.	López-Expósito, Minervini, Amigo, and Recio (2006)
^{H}β-CN f(184–210)	Human β-CN digested with a proteinase of *Lactobacillus helveticus* PR4	Several Gram-positive and Gram-negative bacteria	N.R.	Minervini et al. (2003)
LF f(17-41/42)	Bovine LF digested with pepsin or chymosin	Several Gram-positive and Gram-negative bacteria, viruses, fungi, parasites	Antitumoral Antiinflamatory	Bellamy et al. (1992) Hoek, Milne, Grieve, Dionoysius, and Smith (1997) Iigo et al. (1999) Levay and Viljoen (1995)
^{H}LF f(1–47)	Human LF digested with pepsin	Several Gram-positive and Gram-negative bacteria	N.R.	Bellamy et al. (1992)
LF f(1–48) LF f(1–47) LF f(277–288) LF f(267–285) LF f(267–288)	Bovine LF digested with pepsin	*Micrococcus flavus*	N.R.	Recio and Visser (1999b)
^{C}LF f(14–42)	Caprine LF digested with pepsin	*Micrococcus flavus* *Escherichia coli*	N.R.	Recio and Visser (2000)
α-La f(1–5) α-La f(17–31)S-S(109–114) α-La f(61–68)S-S(75–80)	Bovine α-La digested with chymotrypsin	Several Gram-positive bacteria	N.R.	Pellegrini et al. (1999)
β-Lg f(15–20) β-Lg f(25–40) β-Lg f(78–83) β-Lg f(92–100)	Bovine β-Lg digested with trypsin	Several Gram-positive bacteria	N.R.	Pellegrini et al. (2001)
LZ f(98–112)	Hen egg white LZ digested with clostripain	Several Gram-positive and Gram-negative bacteria	N.R.	Pellegrini et al. (1997) Ibrahim, Thomas, and Pellegrini (2001)
LZ f(98–108) LZ f(15–21)	Hen egg white LZ digested with pepsin and trypsin	Gram-positive and Gram-negative bacteria	N.R.	Mine, Ma, and Lauriau (2004)
LZ (46–61)	Hen egg white LZ digested with papain and trypsin	Gram-positive and Gram-negative bacteria	N.R.	Memarpoor-Yazdi, Assodeh, and Chamani (2012)

[a] Fragments are bovine unless otherwise indicated; peptides obtained by chemical synthesis are not included. Adapted from López-Expósito and Recio (2008).
[b] Upper case superscripts define the origin of the precursor protein if not bovine: ^{O}, ovine; ^{H}, human; ^{C}, caprine origin.
[c] N.R., Non-reported.

WPI, and identified various active sequences derived from β-Lg and α-La (Theolier, Hammami, Labelle, Fliss, & Jean, 2013).

Although CN has been traditionally considered important from the nutritional point of view, it is also source of antibacterial peptides. Most relevant peptides from the CN fraction correspond to the N-terminal region of α_{S1}-CN, several fragments from the C-terminal domain of α_{S2}-CN and some peptides from κ-CN (Table 3). All of them are active against Gram-positive and -negative bacteria, and most of them have a net positive charge. More details about the formation and the *in vitro* activity of these sequences have been reviewed by López-Expósito & Recio (2008).

Because evidence of the activity of these antibacterial fragments should be based on *in vivo* studies, several animal assays and few clinical trials with some of these sequences or hydrolysates have been conducted. Most of the *in vivo* studies have used the entire protein LF, but the observed activity can be attributed to the protein or some of the generated peptides during digestion, such as LFcin-containing fragments (Kuwata, Yip, Tomita, & Hutchens, 1998; Kuwata, Yip, Yamauchi et al., 1998). *In vivo* studies with bovine LF orally administered to mice have demonstrated protective effects against infections or septic shock, although it is not clear whether these effects can be attributed to the antibacterial activity of the protein or to immunity modulation (reviewed by Legrand, 2012; Van Hooijdonk, Kussendrager, & Steijns, 2000). It has been demonstrated that orally administered LF or its hydrolysate can play a role on gastrointestinal health and intestinal flora of newborns (animals or humans). For instance, a recent clinical trial with 472 infants in neonatal care showed that LF reduced fungal late-onset sepsis, especially in low weight infants (Manzoni et al., 2012). Several clinical studies on the role of LF in children and neonates have been recently reviewed (Embleton, Berrington, McGuire, Stewart, & Cummings, 2013; Ochoa, Pezo, Cruz, Chea-Woo, & Cleary, 2012). Other studies with LF or hydrolysates thereof have shown their *in vivo* protective effect in urinary tract infection (Håversen et al., 2000), and hepatic colonisation of *L. monocytogenes* in mice (Lee et al., 2005).

The *in vivo* antibacterial activity of a α_{S1}-CN derived antibacterial fragment, isracidin, against *L. monocytogenes*, *Streptococcus pyogenes*, and *Staphylococcus aureus*, has been demonstrated in mice. Protection of other mammals (rabbits, guinea pigs and sheeps) against this latter microorganism has been also confirmed. Later on, a tryptic CN hydrolysate that exerts antimicrobial activity through stimulation of the bacterial autolytic system was assayed in newborn calves affected with colibacillosis. The hydrolysate acted as antimicrobial but also as immunostimulant and growth promoter (Biziulevičius, Žukaité, Normantienè, Biziulevičienè, & Arestov, 2003).

3.3.2. Immunomodulatory peptides

Milk contains several components, essential during neonatal development, that exhibit an important protective role against infection by modulating the immune system of the newborn. Among these components, it is found that CN, its fractions, whey proteins, LF, and their different hydrolysates show modulatory effects on the immune system in both *in vitro* and *in vivo* studies (Gauthier, Pouliot, & Saint-Sauveur, 2006; Gill, Doull, Rutherfurd, & Cross, 2000). However, most of these studies have been conducted by *in vitro* assays, focussing on the specific immune system, which requires an antigen-specific response (Cross & Gill, 2000).

It has been reported that whey protein and its peptide fractions, obtained from hydrolysis with trypsin and chymotrypsin, modulated the immune system when they were administered to mice (Saint-Sauveur, Gauthier, Boutin, Montoni, & Fliss, 2009). These authors showed an increase in serum immunoglobulin (Ig) A levels in non-infected and *E. coli* O157:H7 infected mice. In relation to whey proteins, it has been shown that α-La enhanced humoural immune response in mice, with this effect being higher than that shown for CN, soy or wheat diet (Bounous & Kongshavn, 1985; Bounous, Létourneau & Kongshavn, 1983; Bounous, Shenouda, Kongshavn, & Osmond, 1985). These results were confirmed when mice were fed α-La hydrolysate (Bounous, 1981; Bounous & Kongshavn, 1982).

LF is considered one of the main host defences against infections, displaying different biological activities including immunomodulation (González-Chávez, Erévalo-Gallegos, & Rascón-Cruz, 2009; Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002; Wakabayashi, Yamauchi, & Takase, 2006). Thus, the ingestion of LF accelerated the improvement of immune system in immunocompromised mice (Artym, Zimecki, Paprocka, & Kruzel, 2003; Takahura et al., 2004). In this sense, it was observed a higher IgA level in the bile and intestine of mice orally immunised with cholera toxin and fed a pepsin LF hydrolysate (Miyauchi, Kaino, Shinoda, Fukuwatari, & Hayasawa, 1997), indicating that this hydrolysate improved mucosal immunity. It has to be pointed out that IgA production is the main humoural immune response given by gut-associated lymphoid tissue, which prevents the entry of potentially harmful antigens that could enter through the oral route. Wang et al. (2000) showed that the administration of LF and its pepsin hydrolysate increased the mucosal immune response in mice, and this increase was higher in tumour bearing mice, suggesting an anti-metastasis and anti-carcinogenesis activity mediated by interleukine (IL)-18 production. In piglets, the ingestion of a LFcin-lactoferrampin mixture increased serum IgA, IgG and IgM levels, thus improving the immune function and gut health (Tang et al., 2009).

Several studies have shown that peptides released from milk fermented with *L. helveticus* R389 are able to improve the mucosal immune system in non-infected (LeBlanc, Matar, Valdéz, LeBlanc, & Perdigón, 2002; Vinderola, Matar, Palacios, & Perdigón, 2007; Vinderola, Matar, & Perdigón, 2007) or *E. coli* O157:H7 infected mice (LeBlanc, Fliss, & Matar, 2004), as well as to decrease the development of induced fibrosarcoma in mice (LeBlanc et al., 2002). On the other hand, a tryptic CN hydrolysate has also shown an immunostimulatory activity in newborn calf partly due to the capacity of this hydrolysate to stimulate the autolytic microbial system (Biziulevičius et al., 2003). Likewise, an immunomodulatory activity of CPPs was shown in mice fed ovalbumin-based diet supplemented with these peptides that was mediated by an increase of IgA levels in serum, intestine, and faeces towards β-Lg injected peritoneally or bacterial LPS of *Salmonella typhimurium* orally administered (Otani, Kihara, & Park, 2000; Otani, Nakano, & Kawahara, 2003). These authors attributed these effects to an increase in IL-5 and IL-6 levels (Otani et al., 2003). A previous study reported increased IgA level in human faeces after the ingestion of cake supplemented with CPPs (Kitamura & Otani, 2002), with this effect being higher in people that ingested dairy products with low frequency.

Despite all these studies where an immunomodulant activity of different hydrolysates obtained from milk proteins was observed, the peptides responsible for this activity remain unidentified. Thus, further studies intended to identify these sequences, elucidate their mechanism of action, and discover new bioactive sequences are needed.

3.3.3. Antiproliferative peptides

Currently, cancer is the second leading cause of mortality worldwide. It is well known that 90–95% of cancer cases are attributed to environment and lifestyle factors, including tobacco and alcohol consumption, diet, sun exposure, pollutants, infections, stress, obesity, and physical inactivity (Anand et al., 2008). Among these factors, dietary patterns and food components are closely

associated with several types of cancer, being responsible for the 35% of cancer deaths (Manson, 2003). However, in vitro and in vivo studies have revealed that a large number of food compounds could lower cancer risk and even sensitise tumour cells to anti-cancer therapies (De Kok, van Breda, & Manson, 2008).

In the last few years, food protein-derived peptides have become a group of nutraceuticals with demonstrated activity preventing the different stages of cancer, including initiation, promotion, and progression (De Mejia & Dia, 2010). Among the milk derived peptides, LFcin B is the most extensively studied. As shown in Table 4, LFcin has demonstrated, by cell culture experiments, a potent activity against different types of cancer cell lines, including breast, colon, fibrosarcoma, leukaemia, oral, and ovarian cancer cells, without harming normal lymphocytes, fibroblasts, endothelial, or epithelial cells (Furlong, Mader, & Hoskin, 2010). The selectivity of action of this peptide seems to be due to its strongly cationic character that allows it to interact with negatively charged structures on cancer cells, resulting in the destabilisation of cancer cell membranes (Hoskin & Ramamoorthy, 2008). However, the net neutral charge of healthy non-transformed cells keeps them spared. In addition to this mechanism of action related to its cationic nature, LFcin has been shown *in vitro* to act inducing apoptosis, arresting cell cycle, modulating gene expression, and preventing the angiogenesis (De Mejia & Dia, 2010).

In vivo evidence has revealed that subcutaneous administration of LFcin B resulted in a significant inhibition of tumour growth and liver and lung metastases of lymphoma and melanoma cells in spontaneous and experimental metastasis mice models (Yoo, Watanabe, Koike et al., 1997; Yoo, Watanabe, Watanabe et al., 1997). Moreover, these authors demonstrated the capacity of this peptide to reduce the number of tumour-induced blood vessels inhibiting tumour angiogenesis. Subcutaneous treatment of Meth A fibrosarcoma mice xenografts with LFcin B significantly inhibited tumour growth (Eliassen et al., 2002). Same effect was observed after treating established neuroblastoma xenografts with repeated injections of this peptide (Eliassen et al., 2006).

The ability of CPPs to bind calcium has been demonstrated to be responsible for the protective effect of these peptides from toxicity caused by calcium overload in differentiated intestinal cells, preventing their apoptosis (Perego et al., 2013). In addition, CPPs act against intestinal tumour HT-29 and AZ-97 cells inhibiting their proliferation and inducing programmed cell death through activation of voltage-activated calcium channels, which mediate the calcium flood according to the depolarisation state of the cell (Perego, Cosentino, Fiorilli, Tettamanti, & Ferraretto, 2012).

Opioid CN-derived peptides, β-casomorphin 7 and β-casomorphin 5, have also shown antiproliferative and cell cycle arresting activity on breast cancer cells (Hatzglou, Bakogeorgou, Hatzoglou, Martin, & Castanas, 1996; Maneckjee, Biswas, & Vonderhaar, 1990). It has been suggested that these effects are mediated by interaction with opioid receptors. Similarly, the interaction with specific opioid and somatostatin receptors present in the intestinal

Table 4
Effects of peptide lactoferricin against cancer demonstrated by cell culture experiments and animals models.

Type of cancer	Cell line/animal model	Effects/mechanisms of action	Reference
Breast cancer	MCF-7, T-47D, MDA-MB-435 cells	Cytotoxic activity/induction of apoptosis	Mader, Salsman, Conrad, and Hoskin (2005)
Colon carcinoma	C26 cells	Cytotoxic activity	Eliassen et al. (2002)
	Caco2 cells	Reduction of cell proliferation/cell cycle arrest at S phase by down regulation of cyclin E1	Freiburghaus, Janicke, Lindmark-Månsson, Oredsson, and Paulsson (2009)
	Ultraviolet-irradiated Caco2 cells	Reduction of DNA damage	
	Colo-35 and HT-29 cells	Cytotoxic activity/induction of apoptosis	Freiburghaus, Lindmark-Månsson, Oredsson, and Paulsson (2012)
			Mader et al. (2005)
Fibrosarcoma	Meth A cells	Cytotoxic activity/tumour cell membrane disruption	Eliassen et al. (2002)
Leukaemia	THP-1 human monocytic leukaemic cells	Induction of apoptosis mediated by production of intracellular reactive oxygen species (ROS) and activation of Ca^{2+}/Mg^{2+}-dependent endonucleases	Yoo, Watanabe, Koike et al. (1997)
	Jurkat T leukaemia cells		Mader et al. (2005, 2007)
			Zhang and Liu (2010)
		Induction of apoptosis mediated by production of ROS, activation of caspases-3 and -9, and sequential permeabilisation of the cell membrane	
		Reduction of DNA methyltransferases expression	
Lymphoma	Subcutaneous inoculation of A20 cell lymphomas in syngeneic mice	Tumour necrosis and regression of the tumors/induction of long-term specific cellular immunity against the A20 lymphoma	Berge et al. (2010)
	Spontaneous metastasis models (L5178Y-ML25) in syngeneic mice	Inhibition of tumour metastasis	Yoo, Watanabe, Watanabe et al. (1997)
	Raji and Ramos Burkitt's B-lymphoma cells	Induction of apoptosis/DNA fragmentation, chromatin condensation, and nuclear disintegration	Furlong et al. (2010)
	Deficient mice bearing B-lymphoma xenografts	Extension of survival of mice	
Melanoma	B16F10 cells	Cytotoxic activity	Eliassen et al. (2002)
	Spontaneous metastasis models (B16-BL6 cells) in syngeneic mice	Inhibition of tumour metastasis	Yoo, Watanabe, Watanabe et al. (1997)
Neuroblastoma	Human MYCN-amplified and non-MYCN amplified neuroblastoma cell lines	Cytotoxic activity through destabilisation of the cytoplasmic membrane, and activation of caspases-6, -7 and -9	Eliassen et al. (2006)
	Neuroblastoma xenografts in nude rats	Reduction of the growth of neuroblastoma xenografts	
Oral cancer	Oral squamous cell carcinoma cell line SAS	Induction of apoptosis by cleavage of caspase-3 and poly-ADP ribose polymerase. Phosphorylation of extracellular signal-regulated kinase, and c-Jun N-terminal kinase/stress activated protein kinase	Sakai, Banno, Kato, Nozawa, and Kawaguchi (2005)
Ovarian cancer	Skov3 and Caov3	Cytotoxic activity/induction of apoptosis	Mader et al. (2005)

tract of mammals might be responsible for the antiproliferative effects on colon cancer cells demonstrated for these β-casomorphins and other milk-derived opioid peptides (Pepe, Tenore, Mastrocinque, Stusio, & Campiglia, 2013).

3.4. Effects on the nervous system

3.4.1. Relaxing peptides

The popular sedative and calming properties of milk can be attributed to various compounds including proteins and peptides released during digestion. In fact, α-casozepine (YLGYLEQLLR), a peptide derived from tryptic hydrolysis of α_{S1}-CN, with benzodiazepine-like activity has shown anticonvulsant and anxiolytic activities in rats (Miclo et al., 2001). However, the mechanism of action of the tryptic hydrolysate differs from that of diazepam. While diazepam induced a disinhibition state in rats, the tryptic hydrolysate did not display such a side effect (Violle et al., 2006) despite its affinity for gamma-aminobutyric acid (GABA) type A receptors. Specific linking of bovine α_{S1}-CN tryptic hydrolysate-derived peptides on GABA receptor is involved in anxiolysis, but not on that implied in memory-impairing effects (Messaoudi, Lalonde, Schroeder, & Desor, 2009). A fragment, corresponding to sequence α_{S1}-CN f(91–97) could be the responsible for the *in vivo* activity of α-casozepine (Cakir-Kiefer et al., 2011). Oral intake of an encapsulated α_{S1}-CN tryptic hydrolysate, containing this peptide, before a stressing situation, decreases the blood pressure increase induced by the stress. Moreover, the plasma cortisol concentration decreased in the treated subjects compared with those who have taken a placebo (Messaoudi, Lefranc-Millot, Desor, Demagny, & Bourdon, 2005). In another study, the oral intake of the encapsulated hydrolysate by female volunteers significantly reduced their digestive, cardiovascular, intellectual, emotional and social stress-related symptoms (Kim et al, 2007).

Moreover, it has been reported an anti-stress effect of LF in the neonatal maternal separation model (Takeuchi et al., 2003), and in an elevated plus-maze test combined with an electric foot-shock in adult rats (Kamemori, Takeuchi, Hayashida, & Harada, 2004).

3.4.2. Antinociceptive peptides

Pain receptors or nociceptors are nerve endings found in the tissues of various organs and systems, characterised by distinguishing securely and efficiently a harmless event from another. It has been reported that milk proteins such as LF reduces nociceptive activity mediated by μ-opioid receptor in several models of pain in rats (Hayashida, Takeuchi, Shimizu, Ando, & Harada, 2003a). Moreover, these authors have demonstrated that the analgesia induced by spinal administration of morphine was greatly potentiated by coadministration of LF (Hayashida, Takeuchi, Shimizu, Ando, & Harada, 2003b), and that LF may block the development of tolerance to morphine in mice, possibly via the selective activation of NO synthase (Tsuchiya et al., 2006). Raju, Kumar, Arutselvan, Thejomoorthy, and Puvanakrishan (2005) have found that PEEP1261, a tetrapeptide corresponding to f(39–42) of human LF possesses antinoceptive activity with optimal effect at 40 mg kg^{-1} body weight in both tail-flick model and acetic acid induced writhing in rats. Furthermore, it was observed that this peptide exhibited also antipyretic activity.

4. Future prospects

Beyond their well-known nutritional value, milk proteins may exhibit a plethora of biological activities that influence the growth, development and function of specific organs, metabolic responses to absorbed nutrients, and defence systems, among others. Many of these activities could be exerted by peptides released from parent protein during gastrointestinal digestion or food processing. The research area of bioactive peptides is only at its beginning and more sequences along with additional physiological effects will be discovered in the future. Most of the properties of milk bioactive peptides have been demonstrated by *in vitro* assays and/or animal model systems. However, data obtained from these studies are insufficient to demonstrate efficacy of those peptides in humans. Evaluation of the peptide doses and treatment durations, and elucidation of the molecular mechanisms of actions are still required for many bioactive peptides. So far, evidence for such effect exists only for a few proteins and peptides, and once the beneficial effect has been demonstrated in acute human intervention studies the results cannot be always translated to longitudinal effects. To generate evidence of these beneficial effects, further long-term human trials using sufficient number of subjects, controlled doses and formulations are needed. It has been proposed that discrepancies found in some clinical trials could be due to human diversity and phenotypic differences between individuals. In this context, future clinical trials of food bioactives could benefit from data on genotype, metabolomic profiles and proteomics or transcriptomics data of the volunteers. In these studies, there is also a need to identify and validate biomarkers that conclusively be related to a certain health benefit. It is expected that new biomarkers will be developed using novel approaches including functional genomics, food metabolomics, microbiomics and epigenetics, and by exploring markers in human studies based on foods and diets, and not just their individual ingredients. Bioavailability of food peptides is also crucial in these studies, since it is dependent of numerous factors such as the food matrix, food composition but also it may vary between individuals. Finally, it has to be taken into account that the pharmacological approach is not always applicable to food bioactives where the physiological effects are smaller. Scientific progress in the field must be targeted at a better understanding of how these food-derived peptides interact with the human body and can prevent the initiation, development or progression of risk factors for diet-related chronic diseases.

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