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## A mini-review on health and nutritional aspects of cheese with a focus on bioactive peptides

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**Abstract** This paper is a mini-review on the nutritional value of cheese with a focus on the identification of different biologically active peptides in cheese and the evidence built about their health benefits. From a nutritional point of view, cheese is a rich source of essential nutrients such as proteins, vitamins, minerals, and also short chain fatty acids that are important as part of a healthy diet. In addition, during cheese ripening, casein is hydrolyzed into a large variety of peptides by proteases and peptidases from milk, rennet, starter culture, and secondary microbial flora. Some of these peptides are structurally similar to endogenous peptides that play a crucial role in the organism as hormones, neurotransmitters, or antibiotics. Some of them can also survive gastrointestinal digestion or serve as precursors of the final peptide form. Furthermore, some of these cheese-derived peptides can interact with the same receptors than endogenous peptides and exert agonistic or antagonistic effects in the organism. This paper reviews the identification of different biologically active peptides in cheese and the evidence built about their health benefits. Activities have been mainly proven by using *in vitro* assays and in cell cultures, but in some cases the activity has been also assessed in animal models. In any case, there is still a long way to demonstrate the “hidden” health benefits of cheese in humans.

### 干酪中生物活性肽的健康和营养-综述

**摘要：**从营养角度来看，干酪富含非常重要的营养物质，如蛋白质、维生素、矿物质和一些短链脂肪酸，这些物质是健康饮食中很重要的一部分。另外，干酪在成熟期间，酪蛋白被来自牛乳、凝乳酶、发酵剂和次级微生物菌群的蛋白酶和肽酶水解成各种各样的肽。这些肽中有的与干酪中内源肽如激素、神经递质、激素的结构相似，在有机体合成中起着关键的作用。有些肽能够以胃肠消化的形式存在或作为最后肽的前体物。另外，一些干酪衍生肽能够与结构相似的内源肽受体一起相互作用在机体中发挥竞争或拮抗的作用。本文综述了干酪中不同生物活性肽的鉴定以及对人体健康的评价。肽的生物活性主要通过采用体外细胞培养方法进行评价，但是，大多数情况下，肽的生物活性通过动物模型来评价。干酪对人类营养和健康的作用还有很多未知，有待进一步挖掘。

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**Keywords** Cheese · Composition · Health · Nutrition · Bioactive peptides

**关键词** 干酪 · 组成 · 健康 · 营养 · 生物活性肽

## 1 Introduction

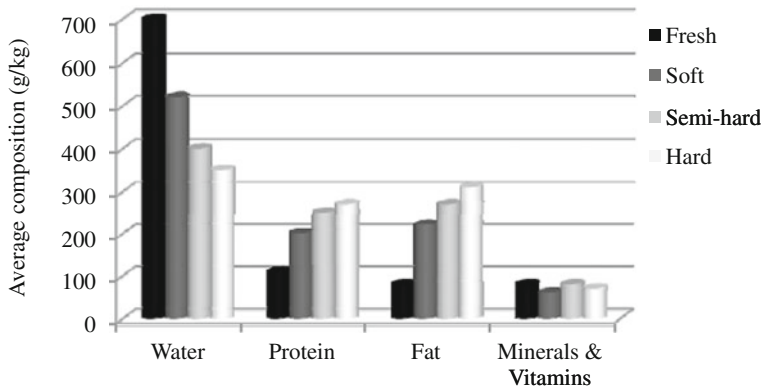
Cheese is a nutritious, versatile dairy food. A wide variety of cheese types are available to meet specific consumer requirements and allow convenience use. European production (EU 27) during 2009 was 8,297 kt, with Germany being the most productive country with 1,852 kt. Per capita consumption of cheese in EU 27 has been reported to be 16.6 kg during 2009. From the integrant countries, Greece, France, Germany, Netherlands, Italy, and Finland were the biggest consumers with more than 20 kg/capita during 2009 (IDF 2010).

Cheese has had an adverse nutritional image due to the association between saturated fatty acids, cholesterol, and the salt content with cardiovascular disease. However, different types of fats have different health effects, and not all saturated fat within foods, such as cheese, elevate plasma cholesterol to the same extent. In addition, cheese is a rich source of other essential nutrients such as proteins, vitamins, minerals, and also short chain fatty acids and certain *trans*-fatty acids that can be considered as part of a healthy diet. During cheese ripening, casein is hydrolyzed into a large variety of peptides by proteases and peptidases from milk, rennet, starter culture, and secondary microbial flora. Some of these peptides are structurally similar to endogenous peptides that play a crucial role in the organism as hormones or antibiotics. These peptides generated during ripening can survive gastrointestinal digestion or serve as precursors from the final peptide form that could interact with the same receptors than endogenous peptides and exert agonistic or antagonistic effects in the organism.

This review briefly summarizes some nutritional aspects of cheese and focus on the formation of bioactive peptides during cheese ripening. The degree of evidence reached, i.e., activity demonstrated by *in vitro* or *in vivo* assays, will be discussed in each case.

## 2 Nutritional value of cheese

Cheese contains a high concentration of essential nutrients relative to its energy level. Its precise nutrient content is influenced by the type of milk used, the manufacture process, and the ripening time. Figure 1 shows the average cheese composition in fresh, soft, semi-hard, and hard cheese. The water-insoluble nutrients are retained in the cheese curd whereas the water-soluble milk constituents remain in the whey (Walther et al. 2008). Most of the lactose from milk is lost in whey during cheese manufacture and the residual lactose in cheese curd is usually fermented to lactic acid by the starter bacteria. With the exception of fresh cheeses, most cheeses are lactose-free or contain only trace amounts. Thus, cheeses can be consumed without ill effects by lactose-intolerant individuals.



**Fig. 1** Average composition of fresh, soft, semi-hard and hard cheeses ( $\text{g}\cdot\text{kg}^{-1}$ ). Adapted from Walther et al. (2008)

## 2.1 Fat and cholesterol

The fat content of cheese varies considerably, depending on the milk used and the method of manufacture. From a nutritional point of view, the digestibility of fat in different varieties of cheese is in the range 88–94 % (Renner 1987). Cheese fat generally contains approximately 66 % saturated (SFA) (57.4 % palmitic, 21.6 % myristic, and 17.6 % stearic), 30 % monounsaturated (MUFA), and 4 % polyunsaturated fatty acids (PUFA). Thus, cheese represents a significant dietary source of both total fat and SFA. SFAs have a poor image because there is some evidence of a negative influence on blood lipids. However, individual SFAs influence blood cholesterol level differently (Legrand and Rioux 2010). Total plasma cholesterol raising effects of SFAs are generally greater with medium chain lengths (lauric C12:0, myristic C14:0, and palmitic C16:0) than for those with longer chain lengths (stearic acid C18:0) (German and Dillard 2006). In addition, stearic acid, which is an important component of the SFAs in cheese, is rapidly converted to the MUFA oleic acid C18:1, which is considered to be one of the healthier sources of fat in the diet and is not related with cardiovascular risk (Jakobsen et al. 2009). It is important to note that some SFA play an important role in cell regulation by protein modification (acetylation), in gene expression as well as in the modulation of genetic regulation, in the regulation of the bioavailability of PUFA, and in fat deposition (Rioux et al. 2003, 2005; Rioux and Legrand 2007; Astrup et al. 2011). In addition, it is known that butyric acid may play a role in cancer prevention (German 1999); caprylic and capric acids have antiviral activities (Thormar et al. 1994) and lauric acid may have antiviral, antibacterial, and anti-cariogenic properties (Thormar and Hilmarsson 2007; Schuster et al. 1980).

The cholesterol content of cheese is a function of its fat content and ranges from approximately 10 to 100 mg/100 g depending on the variety. Despite considerable consumer confusion, dietary cholesterol has much less influence on blood cholesterol level than dietary saturated fat (Keys 1984). Dietary cholesterol does not serve as a metabolic fuel. Instead, it is used by the body as a precursor for cell membranes, bile salts, and steroid hormones that are essential for life. Studies show that healthy people response to  $100 \text{ mg}\cdot\text{day}^{-1}$  of dietary cholesterol is minimal and elevates total plasma cholesterol very slightly (Parodi 2004). Furthermore, as it happens with the SFAs,

dietary cholesterol not only raises atherogenic LDL but also raises the anti-atherogenic HDL cholesterol (Mc Namara 2000).

Conjugated linoleic acid (CLA) is a potentially beneficial component of cheese fat. CLA is a mixture of positional and geometric isomers of linoleic acid (C18:2) that contain conjugated unsaturated double bonds. The principal isomer is *cis*-9, *trans*-11-octadecanoic acid which accounts for more than 82 % of total CLA in dairy products (Chin et al. 1992). CLA has been reported to have several beneficial effects in health-related disorders when tested in vitro and in animal models. These include anti-carcinogenic (Belury 2002), anti-adipogenic (Pariza et al. 2001), anti-atherogenic (Koba et al. 2002), anti-diabetogenic, and anti-inflammatory properties (Ryder et al. 2001; Yang and Cook 2003). However, the majority of studies supporting CLA as a component with beneficial health properties have yet to be substantiated through human clinical trials (Wahle et al. 2004).

## 2.2 Vitamins and minerals

Most of the fat-soluble vitamins in milk are retained in cheese fat. The concentration of water-soluble vitamins in cheese is generally lower than in milk due to the losses in the whey. However, some water-soluble vitamins such as riboflavin, vitamin B<sub>12</sub>, niacin, and folate remain in sufficient quantities to have a significant effect on human nutrition. The loss of some of the B vitamins is offset, to a certain extent, by microbial synthesis during cheese ripening. In particular, propionic acid bacteria synthesize significant levels of vitamin B<sub>12</sub> in hard cheeses such as Emmental (Renner 1987). Cheeses are also good sources of vitamin A. A 50-g portion of Cheddar provides 28 % (male) and 32 % (female) of the recommended daily intake for adults (19–50 years) (Ash and Wilbey 2010).

Cheese is also an important source of several minerals, in particular calcium (Ca), zinc, phosphorus (P), and magnesium. Ca and P levels in cheese are much higher than in milk: four to five times in soft cheeses, seven to eight times in semi-hard cheeses, and up to 10 times in a hard cheese (de la Fuente and Juárez 2001). Actually, a 50-g serving of hard cheese provides approximately 400 mg Ca which covers almost 100 % of the recommended daily intake of Ca in children between 1 and 10 years old. Furthermore, the Ca/P ratio is particularly useful to the body, as it is digested in a form that is highly bio-available because of the complexes that are formed between Ca and the casein peptides within cheese. Such complexes maintain Ca in a soluble form and protect the Ca against precipitation in the intestine, facilitating Ca absorption (Ebringer et al. 2008). As cheese is a concentrated source of bio-available Ca, increasing the amount consumed in the daily diet together with a good source of vitamin D has the potential to safeguard against osteoporosis in future, particularly in those that consume inadequate quantities of Ca at a young age (Ash and Wilbey 2010). In addition, it may aid in losing weight when combined with low-energy diets (Barba and Russo 2006). Dietary Ca could affect the body fat mass by increasing fecal fat excretion as well as by stimulating lipolysis and inhibiting lipogenesis (Zemel and Miller 2004). Furthermore, it has been demonstrated that calcium has hypolipidemic mechanisms via (1) the inhibition of fat absorption and increased fecal fat excretion, (2) inhibition of the absorption of bile acids, and (iii) a Ca-induced increase in the conversion of cholesterol to bile acids (Tholstrup 2006).

Cheese contributes very little to dietary iron; however, a wide range of sodium (Na) levels are found in cheese due to different amounts of salt added during cheesemaking. Although there is considerable awareness about the fact that Na intake contributes to hypertension, cheese adds only about 5–8 % of total Na intake (Renner 1987).

### 2.3 Proteins

Cheese contains a high content of biologically valuable protein which ranges between 4 % (cream cheese) and 40 % (Parmesan) depending upon the variety. The nutritional value of cheese proteins does not change during cheese manufacturing and the content tends to vary inversely with the fat content (O'Brien and O'Connor 2004).

Cheese protein is almost 100 % digestible, as the ripening phase of cheese manufacture involves a progressive breakdown of casein to water-soluble peptides and free amino acids. Between those peptides, a number of bioactive peptides have been found. These peptides are only active after they have been released from their parent protein by proteolysis and can exert a wide range of activities such as hypertensive, antioxidant, antimicrobial, and immunomodulant between others (Tidona et al. 2009). Among the amino acids content in cheese, it is important to highlight the high lysine content. Lysine has a high bioavailability in cheese due to the absence of Maillard reactions (de la Fuente and Juárez 2001).

## 3 Bioactive peptides in cheese

### 3.1 Antihypertensive peptides

Hypertension affects up to an average of 30 % of the adult population in the developed countries, and the relationship between hypertension and coronary heart disease is well established. The treatment of hypertension is no longer limited to the simple prescription of pharmaceuticals. Dietary efforts to decrease saturated fat and sodium and increase potassium, calcium, and soluble fiber intake affect positively blood pressure. In addition, several proteins from different foods, such as, eggs, tuna, and soya sauce, have been identified as precursors of antihypertensive peptides. To date, milk proteins are the main source of this kind of bioactive peptides (Phelan and Kerins 2011; Hernández-Ledesma et al. 2011). At this respect, antihypertensive peptides have been found in processed dairy products, including several types of cheese, without any intentional functional role.

The identification of antihypertensive peptides was initially focused on the search for angiotensin-converting enzyme (ACE, EC 3.4.15.1) inhibitors. This enzyme plays a role in the renin–angiotensin system by converting angiotensin I to a potent vasoconstrictor, angiotensin II, which also induces the release of aldosterone and therefore increases the sodium concentration and blood pressure. ACE also takes part of the kinin–kallikrein system as it hydrolyzes bradykinin, which has a vasodilator action. However, several of the food-derived antihypertensive peptides may act by different mechanisms other than inhibiting ACE such as direct vasodilator effects, antioxidant activity, or by interaction with opioid receptors (Martínez-Maqueda et al.

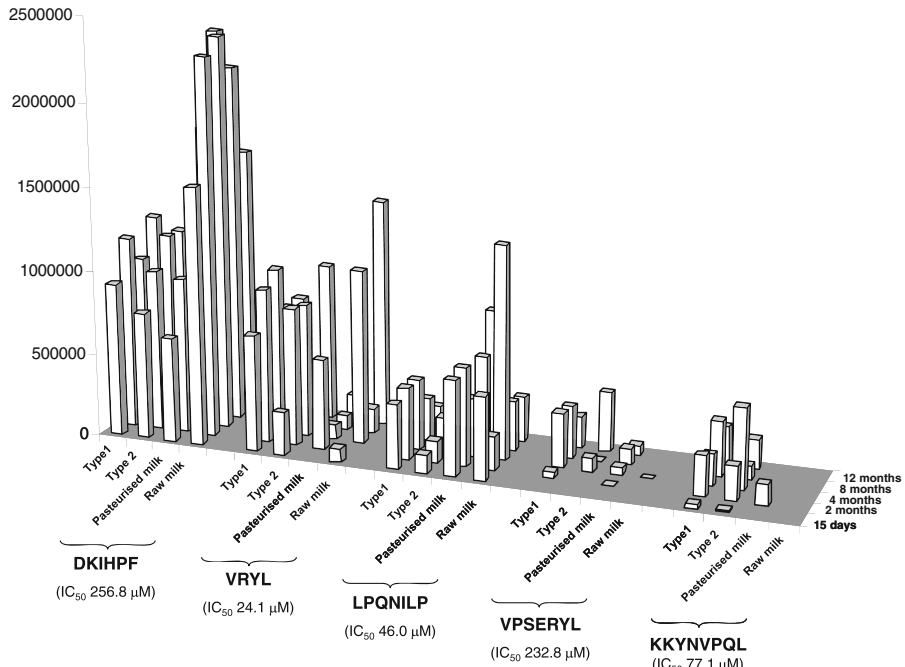
2012). It is also important to highlight the lack of correlation found for some peptides between the *in vitro* ACE-inhibitory activity and the antihypertensive effect. This discrepancy can be due to their further degradation during gastrointestinal digestion, the impossibility to reach the target organ in the organism in a sufficient amount, or because other mechanisms different than ACE inhibition may be involved. This fact has provided doubts regarding the use of the *in vitro* ACE-inhibitory activity as the exclusive criteria to identify potential antihypertensive peptides (Hernández-Ledesma et al. 2011).

Cheese is a complex food matrix containing a large number of different peptides which change with the ripening time. Due to this complexity, many of the studies performed in cheese are aimed at the evaluation of the *in vitro* ACE-inhibitory activity of cheese extracts (Paul and van Hekken 2010). The influence of certain probiotic strains (Ong and Shah 2008; Wang et al. 2011) or simulated gastrointestinal digestion (Parrot et al. 2003) on the ACE-inhibitory activity of different kind of cheeses has also been studied. The application of tandem mass spectrometry techniques to follow the formation of peptides in cheese during ripening has been an important step forward. The identification of novel fragments with ACE-inhibitory activity has been performed by several fractionation steps and consequent evaluation of the activity. This strategy was applied to identify peptides in Manchego cheese (Gómez-Ruiz et al. 2002), and the ACE-inhibitory activity of the identified fragments was confirmed by chemical synthesis and evaluation of the activity of the synthetic peptides (Gómez-Ruiz et al. 2004a). The formation of selected peptides could be followed in the different cheese types and during ripening. Figure 2 shows the estimated amount of some of the most active peptides in four batches of Manchego cheese during ripening. The five investigated peptides were detected in almost all types of Manchego cheese although their concentration strongly varied with the type of cheese and the maturation time (Gómez-Ruiz et al. 2004b). The antihypertensive activity of some of these peptides was confirmed in spontaneously hypertensive rats (Miguel et al. 2010). Peptides identified in different cheese types with ACE-inhibitory activity or antihypertensive effects in spontaneously hypertensive rats are summarized in Table 1. In addition, the application of mass spectrometry has allowed the quantification of the antihypertensive peptides IPP and VPP in different cheeses of Swiss origin (Bütikofer et al. 2007, 2008; Meyer et al. 2009). The assessment of ACE-inhibitory activity of cheese extracts and peptides has been recently reviewed by Sieber et al. (2010). Interestingly, some cheese varieties contain similar concentrations of VPP and IPP than fermented milk products with blood-pressure-lowering capacity. At this respect, the confirmation of the antihypertensive effect of cheeses containing significant amounts of antihypertensive peptides in animal models or humans is still needed.

### 3.2 Phosphopeptides

Casein phosphopeptides (CPPs) are peptides with various degrees of phosphorylation which are released *in vitro* and *in vivo* by enzymatic hydrolysis of the different casein fractions (Adamson and Reynolds 1995; Tirelli et al. 1997; Clare and Swaisgood 2000; Phelan et al. 2009). Most CPPs contain clusters of three phosphorylated serine residues followed by two glutamic acid residues named the “phosphoserine cluster”.





**Fig. 2** Estimated amount of peptide (in arbitrary units) after ion extraction of their corresponding characteristic ions (molecular ion and doubly charged ion when present) in the Mr 3000 permeates of the four batches of Manchego cheese considered during cheese ripening. *Type 1* and *Type 2* correspond to two different experimental Manchego cheeses manufactured from pasteurized ovine milk; *Pasteurised milk* corresponds to Manchego cheese manufactured from pasteurized ovine milk and inoculated with a commercial mixed-strain starter; *Raw milk* corresponds to Manchego cheese prepared with raw ewes' milk without addition of bacterial starters. Reproduced with permission from Gómez-Ruiz et al. (2004b) and Journal of Chromatography A

The presence of this anionic triplet (SerP-SerP-SerP-Glu-Glu) is a distinctive feature for all functional CPPs. As these peptides have a high net negative charge, they efficiently bind divalent cations with the formation of soluble complexes (Hansen et al. 1997a; Meisel 1998; Pérès et al. 1999; Scholtz-Ahrens and Schrezenmeier 2000). Gravaghi et al. (2007) have demonstrated that the ability of CPPs to elicit the optimal biological effects relies on the presence of Ca–CPP aggregates in the appropriated conformation and concentration. Calcium divalent counterions may facilitate the organization of peptides in the aggregates. Bouhallabb et al. (2002) have reported that the mechanism involved in the differences of iron absorption could be partly explained by differences in protein composition that affect the accessibility of iron–peptide complexes. Moreover, other non-phosphorylated residues can be also involved in the binding and stability of the complexes formed (Ferraretto et al. 2003; Boutrou et al. 2010).

It has been reported that CPPs are released in the gut and accumulate in the lower part of the small intestine (ileum) where mineral absorption takes place (Naito and Suzuki 1974; Lee et al. 1980). Several CPPs have been recovered from the stomach and duodenum of adult humans after milk digestion (Chabance et al. 1998) and in the intestinal digests of miniature pigs (Meisel and Frister 1988). Several in vivo studies





Table 1 (continued)

Cheese	$\alpha_s1$ -Casein	$\beta$ -Casein	Reference
Mahón	RL (various fragments) <sup>18</sup> ENLL <sup>21</sup> <sup>14</sup> EVLN <sup>17</sup> <sup>12</sup> PQEV <sup>16</sup> <sup>17</sup> NENLL <sup>21</sup> <sup>19</sup> NLLRF <sup>23</sup>	PQ, RI, PK (various fragments)	Gómez-Ruiz et al. (2006)
	<sup>68</sup> EEL <sup>70*</sup> <sup>1</sup> RPK <sup>3</sup>	<sup>4</sup> EEL <sup>7</sup> QP, PP (various fragments) <sup>94</sup> GVPK <sup>97</sup> <sup>76</sup> PLTQTP <sup>81</sup> <sup>82</sup> VVVP <sup>86</sup> <sup>128</sup> TDVEK <sup>132</sup> <sup>201</sup> VRGP <sup>204</sup> (120.9 $\mu$ M) <sup>47</sup> DKHP <sup>51</sup> (113.1 $\mu$ M) <sup>47</sup> DKHPF <sup>52</sup> (256.8 $\mu$ M) PP (various fragments) MPI (various fragments) <sup>64</sup> GPIH <sup>67</sup> <sup>11.5</sup> PEVP <sup>118</sup> <sup>63</sup> PGPIH <sup>67</sup> <sup>11.5</sup> PVEPF <sup>119</sup> <sup>47</sup> DKHP <sup>51</sup> (113.1 $\mu$ M) <sup>47</sup> DKHPF <sup>52</sup> (256.8 $\mu$ M) <sup>1</sup> REL <sup>3</sup> <sup>62</sup> FPGPIH <sup>67</sup>	
Goat cheese			Gómez-Ruiz et al. (2006)
Cabrales	MPL (various fragments)		Gómez-Ruiz et al. (2006)

**Table 1** (continued)

Cheese	$\alpha_s1$ -Casein	$\beta$ -Casein	Reference
Cheddar	<sup>1</sup> RPKHPIKHQ <sup>9</sup> (13.4 $\mu$ M)	<sup>47</sup> DKIHPF <sup>52</sup> (256.8 $\mu$ M)	Ong et al. (2007)
	<sup>1</sup> RPKHPI <sup>6</sup> (>1,000 $\mu$ M)	<sup>193</sup> YQEPVLGPVRGPFPIV <sup>209</sup> (550 $\mu$ M)	Ong and Shah (2008)
	<sup>1</sup> RPKHPIK <sup>7</sup>	<sup>58</sup> LYYPFGPIHNSLPQ <sup>72</sup> (320 $\mu$ M)	Stepaniak et al. (2001)
	<sup>24</sup> FVAPFPEVF <sup>32</sup>		
	<sup>27</sup> PFPE <sup>30</sup>		
Swiss Cheeses	<sup>102</sup> KKYNNVQLE <sup>110</sup>		
		<sup>84</sup> Vpp <sup>86</sup> (9.0 $\mu$ M) (−32.1 mmHg)	Bütikofer et al. (2007)
Asiago d'allevo		<sup>74</sup> Ipp <sup>76</sup> (5.0 $\mu$ M) (−28.3 mmHg)	Bütikofer et al. (2008)
	<sup>27</sup> PFPE <sup>30</sup> (>1,000 $\mu$ M)	<sup>47</sup> DKIHPF <sup>52</sup> (256.8 $\mu$ M)	Lignitto et al. (2010)
	<sup>24</sup> FVAPFPEVF <sup>32</sup>	<sup>193</sup> YQEPVLGPVRGPFPIV <sup>209</sup> (550 $\mu$ M)	
	<sup>24</sup> FVAPFPE <sup>30</sup>	<sup>1</sup> RELEEL <sup>6</sup>	
	<sup>1</sup> RPKHPIKHQGLPQE <sup>14</sup>	<sup>2</sup> ELEEL <sup>6</sup>	
Norvegia	<sup>1</sup> RPKHPIKHQGLPQ <sup>13</sup>		
		<sup>58</sup> LYYPFGPIHNSLPQ <sup>72</sup> (320 $\mu$ M)	Stepaniak et al. (2001)
Blue		<sup>193</sup> YQEPVLGPVRGPFPIV <sup>209</sup> (550 $\mu$ M)	
		<sup>58</sup> LYYPFGPIHNSLPQ <sup>72</sup> (320 $\mu$ M)	Stepaniak et al. (2001)
Jarlsberg		<sup>193</sup> YQEPVLGPVRGPFPIV <sup>209</sup> (550 $\mu$ M)	
		<sup>58</sup> LYYPFGPIHNSLPQ <sup>72</sup> (320 $\mu$ M)	Stepaniak et al. (2001)
Mexican Fresco		<sup>193</sup> YQEPVLGPVRGPFPIV <sup>209</sup> (550 $\mu$ M)	
	<sup>1</sup> RPKHPIKHQGLPQEV <sup>15</sup>	<sup>193</sup> YQEPVLGPVRGPFPIV <sup>209</sup> (550 $\mu$ M)	Torres-Llanez et al. (2011)
	<sup>1</sup> RPKHPIKHQGLPQEVLENLLR <sup>22</sup>	<sup>193</sup> YQEPVLGPVRGPFPI <sup>207</sup>	
		<sup>193</sup> YQEPVLGPVRGPF <sup>205</sup>	
	<sup>24</sup> FVAPFPEVFGK <sup>34</sup>		
	<sup>14</sup> EVLNENLLRF <sup>23</sup>		

For those available in vitro angiotensin inhibitory activity (IC<sub>50</sub> value,  $\mu$ M) or decrease of the systolic blood pressure (mmHg) in spontaneously hypertensive rats is indicated. Each peptide sequence is described by the one letter code, superscript numbers are referred to the amino acid position into the protein sequence

have investigated if CPPs could increase mineral absorption, especially  $\text{Ca}^{2+}$  absorption. Although these studies provide considerable evidence for a potential effect of CPPs to improve mineral absorption, results are still controversial (Scholtz-Ahrens and Schrezenmeir 2000; Meisel and FitzGerald 2003; Mills et al. 2011). This variability can be due to the diversity of the experimental approaches used as well as variations in the methodologies used to assess mineral bioavailability (Korhonen and Pihlanto 2006). Moreover, it seems that results may depend on the food matrix (Hansen et al. 1997b). On the other hand, the effect of CPPs may also be influenced by the physical status of the population (Heaney 1996; Teucher et al. 2006), the levels of CPPs (Bennett et al. 2000), and the ratio of Ca/Pi (Erba et al. 2001, 2002).

In the last years, several studies have isolated, characterized, and identified CPPs from different types of cheese such as Parmigiano Reggiano (Addeo et al. 1992; Lund and Ardö 2004), Comté (Roudot-Algaron et al. 1994), Grana Padano (Ferranti et al. 1997; Sforza et al. 2003), Cheddar (Singh et al. 1997), Emmental (Gagnaire et al. 2001), Herrgård (Lund and Ardö 2004; Ardö et al. 2007), and Beaufort cheese (Dupas et al. 2009) between other types of cheeses. The presence of CPPs in these different cheese types has been investigated by analyzing the total water-soluble extracts (Sforza et al. 2003; Gagnaire et al. 2001; Roudot-Algaron et al. 1994; Ardö et al. 2007) or using an enrichment step by precipitation (Ferranti et al. 1997; Lund and Ardö 2004; Dupas et al. 2009). Although a number of enzymes are common to many cheese varieties, the peptide composition is unique for each cheese type and reflects a characteristic ripening process. Therefore, CPPs could be regarded as transient intermediate components in the cheese; they either accumulate or are degraded by cheese enzymes to shorter peptides and free amino acids, including SerP.

Apart from the metal binding and anti-cariogenic properties (Cochrane and Reynolds 2009), CPPs have other bioactive properties such as antioxidant (Kitts 2005), immunostimulatory (Kitts and Nakamura 2006), and influence on growth and differentiation of osteoblastic cells (Tulipano et al. 2010).

### 3.3 Opioid peptides

Opioid peptides exert their activity by binding to specific receptors of the target cell in an agonistic or antagonistic fashion. Milk-derived opioid peptides are characterized by the presence of a tyrosine residue at the N-terminal and another aromatic amino acid at third or fourth position which is an important structural motif that fits into the binding site of opioid receptors (Nagpal et al. 2011). The most studied opioid receptor ligands are those termed  $\beta$ -casomorphins derived from  $\beta$ -casein. The role of proteolytic systems in the release of  $\beta$ -casomorphins during simulated gastrointestinal digestion or in vivo digestion has not been fully clarified yet. No current studies report quantitative values for the formation of  $\beta$ -casomorphins during in vivo digestion of dairy products. However, there are indications that the sequential action of several digestive enzymes may be involved in the formation of  $\beta$ -casomorphins (European Food Safety Authority 2009). Animal data clearly indicate that  $\beta$ -casomorphins can act as opioid receptor agonists, probably acting via  $\mu$ -type receptors. However, in vivo opioid effects for  $\beta$ -casomorphins have only been observed following intra-peritoneal administration (Taira et al. 1990) or intra-cerebroventricular administration (Brantl et al. 1981; Grecksch et al. 1981). Relatively little

is known about the bioavailability of this kind of peptides. There are only two studies reporting the presence of  $\beta$ -casomorphins immunoreactive material in blood. Singh et al. (1999) found  $\beta$ -casomorphins immunoreactive material in plasma in 2- and 4-week-old pups fed with a bovine casein-based formula but not in adult dogs. Similarly, Umbach et al. (1985) demonstrated the presence of  $\beta$ -casomorphins immunoreactive material in plasma of newborn calves after milk intake. Authors speculated that the intestinal mucosa of the newborn is more permeable to the relatively large peptides due to their immature tight junction through which peptides cross, thereby escaping hydrolysis. In addition to their effects on the central nervous system,  $\beta$ -casomorphins have been also shown to modulate intestinal transport of amino acids (Brandsch et al. 1994), to modulate mucus secretion by intestinal mucus producing cells (Claustre et al. 2002), and to influence postprandial metabolism by stimulating insulin and somatostatin secretion (Froetschel 1996). They also have been demonstrated to prolong gastrointestinal transit time and to exert an anti-diarrheal action in both animals and humans (Daniel et al. 1990).

The formation and fate of  $\beta$ -casomorphins have been studied in different cheese varieties. Only few studies report on the presence and levels of  $\beta$ -casomorphins in cheeses. Jarmolowska et al. (1999) using amino acid composition analysis reported  $\beta$ -casomorphin-7 [ $\beta$ -CN (60-66)] in extracts from Brie cheese. The same group also identified  $\beta$ -casomorphin-5 [ $\beta$ -CN (60-64)] in Gouda Cheese extract (Kostyra et al. 2004). Later on, Sienkiewicz-Szlapka et al. (2009) evaluated the content of agonistic ( $\beta$ -casomorphin-5 and  $\beta$ -casomorphin-7) and antagonistic [casoxin-6, casoxin-C (derived from bovine  $\kappa$ -casein) and lactoferroxin A] opioid peptides in three semi-hard (Edamski, Gouda, and Kasztelan) and in two ripening mold cheeses (Brie and Rokpol) varieties. They found that mold cheeses contained higher amounts of  $\beta$ -casomorphins than the semi-hard cheeses which contained a fairly high amount of casoxin-6 and a low quantity of  $\beta$ -casomorphins. All the extracts, except that from Gouda, were found to affect the intestinal mobility in isolated rabbit ileum similarly to morphine, and this effect was reversed by naloxone. Recently, De Noni and Cattaneo (2010) investigated the occurrence of  $\beta$ -casomorphin-5 and  $\beta$ -casomorphin-7 in 10 commercial cheeses (Gorgonzola, Caprino, Brie, Taleggio, Gouda, Fontina, Cheddar, and Grana Padano) and their digests.  $\beta$ -Casomorphin-5 was not detected in any of the evaluated cheeses; however,  $\beta$ -casomorphin-7 was found in all the cheeses evaluated with the exception of Taleggio, Caprino, and Grana Padano samples. Peptide amount was in the range of 0.01–0.15 mg.kg<sup>-1</sup> cheese, the highest level being recovered in Brie sample. Following a simulated gastrointestinal digestion,  $\beta$ -casomorphin-7 levels increased up to 21.77 mg.kg<sup>-1</sup> cheese. This work provided evidence that release of  $\beta$ -casomorphin-7 is mainly promoted by the action of gastrointestinal proteases during in vitro digestion. More research needs to be done to evaluate the absorption of these peptides in a complex food matrix as cheese.

There are also several studies indicating that different cheeses such as Parmigiano Reggiano (Addeo et al. 1992), Crescenza (Smacchi and Gobbetti 1998), Cheddar, and Jarlsberg (Stepaniak et al. 1995) contain peptides incorporating the  $\beta$ -casomorphin-7 sequence, which could act as precursors during further digestion processes.

$\beta$ -Casomorphin-9 [ $\beta$ -CN (60-68)] and several peptides including the  $\beta$ -casomorphin-7 sequence were found in Cheddar cheese by Singh et al. (1995, 1997).  $\beta$ -Casomorphin-9 was also found in Gouda by Saito et al. (2000). This finding

was later on confirmed by Toelstede and Hofmann (2008) who identified  $\beta$ -casomorphin-10 [ $\beta$ -CN (60-69)] in the water-soluble extract of a matured Gouda cheese. The caprine analog of  $\beta$ -casomorphin-9 was also found in the water-soluble extracts of Caprino de Piemonte, an Italian goat cheese (Rizzello et al. 2005).

### 3.4 Other bioactivities

Contrary to the bioactivities described above, there are only a few reported studies dealing with antiproliferative, antimicrobial, or antioxidant bioactivities of peptides isolated from cheese.

With regard to cancer prevention, a considerable number of studies have indicated that dairy products may possess anticancer activity (Parodi 2007). By serving as an apoptotic inducer for tumor development, bovine milk proteins such as lactoferrin and lactoferricin have been demonstrated to suppress the growth of cancer cells in which DNA damage is involved in vitro and in vivo (Roy et al. 2002; De Moreno de LeBlanc et al. 2005; Mader et al. 2005). It seems that in addition to a certain net positive charge and hydrophobicity, the ability to adopt an amphipathic conformation is critical for antitumor activity (Yang et al. 2004).

On the contrary, peptides naturally occurring in cheese with antiproliferative properties have not been yet characterized. To date, the antiproliferative effects of cheese extracts are being evaluated and all the described results are based on either cheese extracts or cheese whey. Yasuda et al. (2010) investigated the potential role of commercial cheese products on the cell growth and induction of DNA fragmentation in HL-60 human promyelocytic leukemia cells as a cancer model. Among 12 cheese extracts tested, six (Montagnard, Pont-l'Eveque, Brie, Camembert, Danablu, and Blue) revealed strong growth inhibition activity and induction of DNA fragmentation in HL-60 cells. They also found a positive correlation between the ripeness of various cheeses and their antiproliferative activity. It remains to be elucidated whether these active molecules from cheeses may be present at an enough concentration to exert an activity, whether the bioavailability of these compounds is sufficient and if the activity observed is specific not to normal cells but to cancer cells.

De Simone et al. (2009) evaluated the protective effects of peptides derived from Mozzarella whey samples against peroxide-induced damage in CaCo-2 cell lines. They observed that the exposure of the cells to the waste whey resulted in a 43 % reduction in cell proliferation and decreased mitochondrial superoxide anion production analyzed by flow cytometry. The absence of this effect in the peptide extract from original milk suggested that the production of specific bioactive compounds occurs specifically during the production process of Mozzarella cheese. Later on, the same group identified a partially RP-HPLC purified peptide sub-fraction as responsible for the observed activity (De Simone et al. 2011). On the mentioned sub-fraction, they identified by MALDI-TOF/MS a number of peptides mainly derived from  $\kappa$ -CN, glyco-caseinmacropeptide, and  $\beta$ -CN.

The activity of antibacterial peptides is defined as a membrane-lytic activity, where they tend to assemble to form channels, with specificity for prokaryotic cell membranes (Floris et al. 2003). An amphiphilic and a positive net charge are recognized as major structural motifs determining the interaction with bacterial membranes, which has been accepted as a common target in their mechanism of action. However, it has

been demonstrated that some antibacterial peptides can reach intracellular targets and other peptides lacking a net positive charge can exhibit antibacterial activity (López-Expósito and Recio 2008). The majority of antimicrobial peptides derived from food sources have been identified in milk, milk hydrolysates, and fermented milks (López-Expósito and Recio 2006). However, there is almost no information considering the potential of cheese as a source of antibacterial peptides, in which antibacterial peptides can be generated by hydrolysis of milk proteins and can then even interact with microorganisms involved in cheese processing. Rizzello et al. (2005) and Losito et al. (2006) undertook an investigation where nine different Italian cheese varieties, differing mainly in the type of milk, starter, technology, and time of ripening were screened for their antibacterial activity. Water-soluble extracts (WSE) of each cheese was firstly fractionated by FPLC and the antibacterial properties towards *Lactobacillus sakei* A15 of each fraction were tested. The most active fractions were then further tested against more resistant microorganisms (*Escherichia coli*, *Salmonella*, *Yersinia enterocolitica*, *Staphylococcus aureus*) and analyzed by HPLC–ESI–MS and MS/MS in order to identify the peptide sequences contained in the fractions. The most active fractions corresponded to the varieties Pecorino Romano, Canestrato Pugliese, Crescenza, Caprino del Piemonte, and Caciocavallo. Authors identified more than 30 peptides with a high level of homology with N-terminal, C-terminal, or whole fragments of previously reported milk-derived antimicrobial sequences such as isracidin (Hill et al. 1974) and kappacin (Malkoski et al. 2001). Later on, Pritchard et al. (2010) examined the antibacterial activity in the WSE of three different Australian Cheddar cheeses against *E. coli*, *B. cereus*, and *S. aureus*. Results showed that peptides greater than 10 kDa were able to significantly inhibit *E. coli* and *B. cereus* growth in a moderate manner. However, the peptides responsible for the activity were not identified.

So far, Cheddar is the only cheese variety which has been screened for antioxidant activity. Gupta et al. (2009) evaluated the antioxidant properties of Cheddar cheeses prepared with *Lactobacillus casei* ssp. *casei* 300, *Lactobacillus paracasei* ssp. *paracasei* 22, and without adjunct cultures by three different methods: ABTS (Hernández-Ledesma et al. 2005), DPPH radical scavenging activity (RSA) (Shimada et al. 1992), and superoxide anion scavenging activity (SOSA) (Liu et al. 1997). The results obtained by the three methods employed indicated that antioxidant activity of the WSE was dependent of the ripening period. They observed a gradual increase of the antioxidant activity up to the 4 months of ripening, after which the antioxidant activity decreased and retained similar values from the seventh to the ninth month of ripening. The authors observed that changes in the antioxidant activity were very similar to the rate of formation of soluble peptides (proteolysis) in all the cheese samples. Thus, to a certain extent, a higher degree of early proteolysis was related with a higher antioxidant activity, although it was also dependent on the starter culture employed. Comparing the antioxidant activity and sensory scores, the cheese with the higher antioxidant activity as well as the highest sensory score after 4 months of ripening was the cheese prepared with *L. casei* ssp. *casei* 300. The same authors (Gupta et al. 2010) identified the main peptides responsible for the antioxidant activity observed in the cheese by HPLC–MS/MS. The peptides matched the fragments f(98–105) from bovine  $\beta$ -CN (VKEAMAPK) and f(80–90) from bovine  $\alpha_{s1}$ -CN (HIQKEDVPSER). Experiments with chemically synthesized peptides showed



that the DPPH radical scavenging activity of both peptides was comparable to that from commercial chemical antioxidants such as BHA, t-BHQ, or ferulic acid. In the same line, Pritchard et al. (2010) have recently evaluated the inhibition of DPPH radicals by the WSE of three different Australian Cheddar cheeses. They found a moderate antioxidant activity in those fractions containing peptides with a molecular weight greater than 10 kDa, although peptide identification was not performed.

### 3.5 Concluding remarks

Cheese can be considered an important component of an equilibrated diet from a nutritional point of view. It is an excellent source of protein and minerals, especially calcium in a highly bioavailable form. Although cheese suffers from an adverse image due to its fat and salt content, the variety of cheeses with a reduced fat or salt content available on the market make possible to include this food in all kind of diets. In addition to these nutritional benefits, the protein fraction of cheese can act as precursor of biologically active molecules. During cheese ripening and food digestion, a large variety of peptides are released from milk caseins. Some of these peptides are structurally similar to endogenous peptides and, therefore, they can interact with receptors at the gastrointestinal tract (for instance, opioid receptors), facilitate mineral absorption (CPPs), or being absorbed and reach the blood stream. Although the amount of food-derived peptides absorbed after oral ingestion can be low, there is increasing evidence being built in clinical studies of several biological effects related with the ingestion of some of these sequences. These effects may not be as clear as those observed for pharmaceuticals (food is not a drug) and, therefore, special care should be taken when performing these intervention studies. There are many aspects to be taken in account when evaluating the bioactivity of food components. This includes quantitative determination of the active molecules, ensuring resistance to technological processes and during product shelf life, interaction with other food components, genotypic differences of the target population, etc. In addition, certain aspects, as the identification of the active form in the organism, and the elucidation of the mechanism of action are also required to support health claims based on clinical studies.

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