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minireview

Cheese as Functional Food: The Example of Parmigiano Reggiano and Grana Padano

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Summary

Italian hard cooked types of cheese, like Parmigiano Reggiano and Grana Padano, are characterised by positive nutritional qualities. In fact, they contain substances that have particular biological activities, and therefore they can be fully considered, according to the definition given by the European Unit, as 'functional' foods. This short review concisely describes these components and the beneficial effects related to their activities. The description of the biologically active components has been organised in the following paragraphs: protein and peptides, fat and lipids, carbohydrates and prebiotics, probiotic bacteria, vitamins, mineral salts, and components of dairy products active in disease prevention. In particular, several known bioactive peptides were found in Parmigiano Reggiano cheese samples: for example, phosphopeptides, which are known for their mineral-binding capacity and vehiculation activity, peptides with immunomodulatory activity, and angiotensin-converting enzyme-inhibitory peptides with anti-hypertensive effects. Among lipids, the role of conjugated linoleic acid and other fatty acids present in these cheese types was taken into consideration. The presence of oligosaccharides with prebiotic properties and probiotic bacteria was also described. Finally, particular emphasis was given to highly available calcium and its impact on bone health.

Key words: Italian hard cooked cheese, functional properties, protein and peptides, fat and lipids, carbohydrates and mineral salts, disease prevention

Introduction

A study conducted at the INRAN (National Research Institute for Food and Nutrition, Rome, Italy) has led to the conclusion that it is simplistic to consider milk and dairy products exclusively as sources of essential nutrients (protein, calcium and vitamins) since it has been reported that milk and dairy products contain about 2000 molecules, some of which possess a specific biological activity (*1*). Among dairy foods, Italian hard cooked types of cheese, such as Parmigiano Reggiano and Grana Padano, are characterised by positive nutritional qualities. In fact, these cheese types contain substances that have particular biological activities, and therefore they can be fully considered, according to the definition given by the EU, as 'functional' foods, which are defined as 'foods that contain biologically active components able to improve human health or reduce the risk of disease, or otherwise produce beneficial effects on the health and welfare of the consumer' (*2*). In this short review, these components and the beneficial effects related to their activities are succinctly described.

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Protein and Peptides

Proteins constitute about 33 % of Italian hard cooked types of cheese, presenting, from a quantitative perspective, the most important components of Parmigiano Reggiano and Grana Padano cheese. The proteins of these types of cheese are characterised by a high biological value. In fact, they contain high concentrations of all the essential amino acids. Amino acids such as isoleucine, leucine, lysine, threonine, tryptophan, valine, methionine, phenylalanine, tyrosine and histidine are present in optimal amounts and in a particularly bioavailable form (*2*). In particular, the high content of lysine in cheese protein is noticeable, especially if compared with that of wheat, whose protein lacks this amino acid (*3*).

Besides this nutritional aspect, Italian hard cooked cheese proteins also have a capacity to be assimilated relatively fast, since during ripening, a great percentage of them are hydrolysed into peptones, peptides and free amino acids by proteolytic enzymes, deriving both from milk and from the lactic acid bacteria that develop in the curd. This is similar to a predigestion, which leads to the formation of compounds with a progressively lower molecular mass until free amino acids are formed. At the end of this process, free amino acids constitute approx. 25 % of total nitrogen. Moreover, these low-molecular-mass peptides and free amino acids themselves play a role in the stimulation of both acid and pepsin gastric secretion, which further increases the digestion rate of proteins (*4*).

Overall, Italian hard cooked types of cheese are characterised by a very varied composition of nitrogen components. Apart from a variable amount of intact casein, they contain peptides of various length and free amino acids. During digestion, these three nitrogen components undergo a different absorption (slow, accelerated and fast, respectively), allowing for a modulation in the utilisation of the protein substrate and its more efficient assimilation from the diet (*5*).

Bioactive peptides: general description

The primary function of milk and dairy proteins is to supply the body with fundamental amino acids and organic nitrogen adequately. However, in addition to a nutritional role, milk proteins have a physiological importance, being a source of biologically active peptides. This aspect has been studied since 1979, and numerous peptides derived from milk proteins (α_{s1}-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, immunoglobulins, lactoferrin, phosphoglycoproteins, transferrin and serum albumin), their sequence and biological activity have been determined. Some reviews are specifically devoted to this subject (*6–11*). Bioactive peptides (consisting of 2–30 amino acid residues), encrypted in major milk proteins, are latent and inactive until they are released and activated by enzymatic proteolysis, *e.g.* during gastrointestinal digestion or food processing (*12*). After activation, they can act within the body by influencing physiological processes and modulating various biological functions (*11*).

As previously reported, during cheese ripening, milk caseins are first hydrolysed by the action of milk proteases, rennet proteases (only to a lesser extent in Italian hard cooked types of cheese, due to the almost complete denaturation of chymosin during the cooking phase) and the proteases and proteinases of lactic acid bacteria used as starters. The large fragments formed are then further degraded by proteolytic enzymes from the microbiota of both the whey starter and the milk. Lactic acid bacteria proteinases which, after lysis of the cell, are associated with the cell wall and intracellular peptidases are released in the curd and are responsible for the formation of small peptides and free amino acids. Digestive enzymes in the gastrointestinal tract (stomach and intestinal proteases and brush border peptidases) further hydrolyse oligopeptides, contributing to the formation of bioactive peptides (*4*). Thus, the enzyme-modified cheese process, mainly designed to produce flavour ingredients, may simultaneously produce bioactive peptides which are considered physiologically important (*13*).

Most of these studies on bioactive peptides were performed on hydrolysed and digested casein or by administering milk or generic dairy products to mice. However, as stated by Korhonen and Pihlanto (*14*), during cheese ripening, a great variety of peptides are formed, many of which have been shown to exert biological activities. So, it is reasonable to think that many of these bioactive peptides can be produced also during the ripening of Parmigiano Reggiano and Grana Padano, mainly by lactic acid bacteria starters (*15,16*). In fact, one of the very specific species of whey starters used for Grana Padano and Parmigiano Reggiano, *Lactobacillus helveticus*, is one of the species mainly responsible for the formation of these bioactive compounds (*17,18*). Some studies (*19,20*) have investigated the formation of peptides in Parmigiano Reggiano and Grana Padano cheese and/or their digested products *in vitro*; however, this topic needs to be studied further, with particular emphasis on biopeptide formation. As attested by Sforza *et al.* (*20*), several known bioactive peptides were found to be present in Parmigiano Reggiano cheese samples; for example, phosphopeptides derived from the N-terminal part of β-casein, known for their mineral binding capacity and vehiculation activity (*21*), are present in Parmigiano Reggiano cheese aged for more than 16 months.

Bioactive peptides act in the body of mammals according to mechanisms similar to hormones. The peptides introduced with dietary cheese or produced by intestinal hydrolysis can reach the target sites in the luminal side of the gastrointestinal tract or, after being absorbed into the systemic circulation, the peripheral organs (*9,10, 12,22*). These peptides may exert different beneficial physiological functions: opioid peptides are opioid receptor ligands which can modulate absorption processes in the intestinal tract; ACE-inhibitory peptides are blood pressure regulators and exert an antihypertensive effect; immunomodulating casein peptides stimulate the activities of the cells of the immune system; caseinophosphopeptides may function as carriers for different minerals, especially calcium. Many of these peptides can have two or more different biological activities (*12*). In the following paragraphs, the principal peptides derived from dairy protein and their biological functions are summarised, referring for a more detailed description to the specific reviews.

Opioid peptides

A number of milk protein peptides have been shown to behave like opioid receptor ligands able to address opioidergic reactions in adults or in neonates. With respect to the proteins from which they are derived, these peptides have been named α-casein exorphins or casoxin D (α-casein), β-casomorphins or β-casorphin (β-casein), casoxin or casoxin A, B or C (κ-casein), and other coming from whey proteins that can be present in cheese only in a negligible amount (α-lactorphins from α-lactalbumin, β-lactorphin from β-lactoglobulin or lactoferroxins from lactoferrin). Only casoxins and lactoferroxins display antagonistic properties; the other peptides behave like opioid receptor agonists (*23*).

β-Casomorphins were the first milk protein-derived opioid receptor ligands whose amino acid sequences were identified. It is thus conceivable that more information about this peptide group has been collected than about any other group of milk opioids. They were called β-casomorphins due to their morphine-like behaviour.

Teschemacher *et al.* (*24*) found that β-casomorphins arrive to the cardiovascular system after gastrointestinal digestion of milk or milk products in more than negligible amounts – at least in adult animals or humans; enzymatic degradation in the intestinal wall and in blood appears to prevent it. The authors have also described that these peptides can have a regulatory influence as 'food hormones', participating in the control of gastrointestinal functions in adults and neonates.

Immunomodulatory peptides

There is a substantial body of evidence according to which several of the biologically active peptides released by enzymatic hydrolysis of milk proteins are very potent immunoregulatory peptides (*25*), affecting cells of the immune system, and consequently the downstream immunological responses and cellular functions (*25,26*).

It was demonstrated that peptides from α_{s1}-casein (residues 194–199 and 23–34) and β-casein (residues 63–68 and 191–193) stimulate phagocytosis by murine peritoneal macrophages and consequently exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous treatment (*25,27–31*). The peptide α_{s1}-casein (residues 23–34) was demonstrated to be produced in Parmigiano Reggiano during ripening (*19*). Quantification of this peptide is not given in the paper, but its presence is attested at 6 months of ripening, probably produced by a chymosin cleavage.

The peptide isracidin (N-terminal sequence 1–23 of α_{s1}-casein) has been found to protect mice against infections by *Staphylococcus aureus* and stimulate a phagocytic response in mice infected with *Candida albicans* (*32*). This peptide was found by Sforza *et al.* (*20*) in samples of Parmigiano Reggiano cheese. The authors do not give a specific quantity of this peptide, but they write that it is probably produced by a chymosin cleavage and it is the most abundant in the group of peptides whose amount is decreased during cheese ripening. A figure in the paper attests that the peptides of this group are still present, in low amounts, at 12–25 months of ripening.

β-Casomorphin-7 derived from β-casein was used as a possible immunostimulating substrate, since it is a ligand of opioid receptors that have also been found on the surface of human T-lymphocytes (*33–35*).

β-Casokinin-10 (residues 193–202 of β-casein) and another heptapeptide from β-casein (residues 177–183), such as residues 23–34 of bovine α_{s1}-casein, have been found to inhibit angiotensin-converting enzyme (ACE), which may act on the immune system, preventing the breakdown of bradykinin (*25,36*). The latter acts as a mediator of the acute inflammatory process and is thus able to stimulate macrophages and enhance lymphocyte migration.

Regazzo *et al.* (*37*) found that β-casein (residues 193–209), a long and hydrophobic peptide composed of 17 amino acid residues, has immunomodulatory activity. This peptide was demonstrated by Sforza *et al.* (*20*) to be formed during Parmigiano Reggiano ripening and present in the cheese samples. The authors do not give a specific quantity of this peptide, but they write that it is probably produced by lactic acid bacteria proteases and that chymosin itself is responsible for its production. This peptide is not formed during the early stage of cheese production, but its amount increases during ageing and it is present in a small amount from the start to the end of the ageing period, indicating that its production continued throughout the ripening period.

Dipeptide (Tyr-Gly), which corresponds to a partial sequence in the primary structure of bovine κ-casein (residues 38–39), was used for immunotherapy of human immunodeficiency virus infection (*35,38*).

Caseinophosphopeptides

Casein phosphopeptides or caseinophosphopeptides (CPPs) are a mixture of phosphorylated peptides of different molecular mass formed *in vivo* when casein is degraded by proteolytic enzymes in the digestive tract (*39*). Phosphate residues, represented as serine esters, constitute the anionic binding site for minerals such as calcium, iron and zinc and form with them soluble salts, increasing their intestinal bioavailability. Bouhallab and Bouglè (*40*) showed that a purified phosphopeptide (β(1–25)) exhibits a positive effect on iron bioavailability *in vivo*, reporting its efficiency in the absorption and availability of iron and the mechanism involved.

Numerous studies show that caseinophosphopeptides, by means of a bond with calcium, make the latter more soluble, improving its absorption and increasing its availability (*41,42*). This system of passive transport is the main way of absorption of the calcium requested for bone calcification. Erba *et al.* (*41*) state that the positive effect of CPPs on passive calcium absorption seems to depend on the relative amounts of both CPPs and calcium in intestinal lumen. In that study, the ratio CPPs/Ca=15 was identified as the most efficient to increase mineral transport.

Moreover, Aimutis (*43*) demonstrated that CCPs can possess an anticariogenic effect, *i.e.* have a role in the prevention of dental caries. Tooth enamel is a polymeric substance consisting of crystalline calcium phosphate embedded in a protein matrix. Dental caries develops as a result of acidic demineralisation (calcium and phosphorus solubilisation) of tooth enamel. CPP can form nanoclusters with amorphous calcium phosphate (ACP) at the tooth surface to provide a reservoir of calcium and phosphate ions to maintain a state of supersaturation on the surface of tooth enamel. This would buffer the pH of the plaque, and also provide ions for tooth enamel remineralisation.

Recently, Cattaneo *et al.* (*44*) have found that casein phosphopeptides were released during *in vitro* gastrointestinal digestion of Grana Padano cheese samples aged 13, 19 and 26 months.

The ACE-inhibitory peptides

ACE-inhibitory peptides deserve special mention. They are generated by the activity of specific enzymes that cut proteins like β-casein and κ-casein (*45*). The angiotensin-converting enzyme, called ACE, cuts the chain of angiotensin I, which is then converted into angiotensin II. Angiotensin II acts on several mechanisms that regulate blood pressure, such as vasoconstriction, sodium reabsorption, and aldosterone release. Moreover, angiotensin II acts by degrading bradykinin, which exerts a regulatory role on vasodilation. All this leads to an increase in blood pressure. These biopeptides inhibit the function of the ACE, inducing a drop in both systolic and diastolic pressure; the same mechanism is activated by drugs belonging to the class of ACE-inhibitors.

Basiricò *et al.* (*46*) demonstrated for the first time the presence of eight different potent ACE-inhibitory peptides in Parmigiano Reggiano cheese samples and in their relative intestinal digestate. In particular, VPP, IPP, LHLPLP and HLPLP were revealed in a water-soluble extract, and their total amount was in the range of 8.46 to 21.55 mg per kg of cheese. The mass fraction of ACE-inhibitory peptides in the total peptide content of undigested water-soluble extract was 0.10 to 0.38 %. After *in vitro* gastrointestinal digestion of this water extract, the same ACE-inhibitory peptides, along with the newly formed AYFYPEL and AYFYPE, were found. The total amount of ACE-inhibitory peptides in digested Parmigiano Reggiano samples was in the range of 1959.33 to 3122.52 mg/kg, and the mass fraction of ACE-inhibitory peptides in the total peptides in digested water-soluble extract was 13.68 to 21.81 %. At the end of the digestive process, the authors found relevant amounts of LHLPLP (2483.95 mg/kg) and HLPLP (58.97 mg/kg). Other detected peptides included the newly formed AYFYPEL (6.46 mg/kg) and AYFYPE (7.34 mg/kg). Two of these peptides are Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), in the amounts of 3.97 and 2.32 mg/kg, respectively: these peptides were found by Hata *et al.* (*47*) to be responsible for a marked decrease in blood diastolic and systolic pressure in patients with blood pressure problems.

Crippa *et al.* (*48*) conducted a randomised, double-blind, placebo-controlled study on 30 subjects suffering from hypertension, who presented pathological pressure values (maximum >140 and/or minimum >90 mm Hg). All patients received, at random, a dietary supplementation with Grana Padano (30 g daily) and placebo (obtained with flavoured bread mixed with fat and salt in the amounts equal to those of the cheese). Supplementing the diet with Grana Padano cheese resulted in a significant reduction in systolic and diastolic blood pressure.

Recently, Bernabucci *et al.* (*49*) found ACE-inhibitory activity and antihypertensive effect of naturally formed bioactive peptides in a lyophilised water-soluble extract from 32-month-aged Parmigiano Reggiano and Grana Padano. They were evaluated in undigested and *in vitro* digested water-soluble extracts and tested in 20 spontaneously hypertensive rats. Both Parmigiano Reggiano and Grana Padano water-soluble extracts showed a consistent ACE-inhibitory activity and IC_{50} value of Grana Padano water-soluble extract was lower than that of Parmigiano Reggiano. The *in vitro* enzymatic digestion of these water-soluble extracts did not significantly affect IC_{50} values.

The presence of ACE-inhibitory peptides in undigested (and also in *in vitro* digested in the stomach and intestine) Grana Padano samples was recently demonstrated also by Stuknytė *et al.* (*50*). The authors found the following peptides in the undigested Grana Padano samples (in mg/kg): VPP 2.76, IPP 2.49, RYLG 0.07, HLPLP 0.23, LHLPLP 0.38, in total mass fraction of ACE-inhibitory peptides of 5.93 mg/kg. After gastric digestion, the following peptides were found (in mg/kg): VPP 2.85, IPP 1.22, RYLG 1.72, HLPLP 0.71, in total mass fraction of ACE-inhibitory peptides of 6.50 mg/kg. After intestinal digestion the following peptides were found (in mg/kg): HLPLP 4.64 and LHLPLP 306, in total mass fraction of ACE-inhibitory peptides of 311 mg/kg. The authors affirm that, together with plasmin, proteinases and peptidases of *Lactobacillus helveticus* spp., the main lactic acid bacterium present in the whey starter added to raw milk prior to renneting, are mainly responsible for this extensive proteolysis in Grana Padano cheese.

Parmigiano Reggiano protein and glucose tolerance

In a study published in Diabetologia, Tricò *et al.* (*51*) showed that the administration of a meal composed of 50 g of Parmigiano Reggiano, a boiled egg, and 300 mL of water, 30 minutes prior the ingestion of a strong glucose load, has a hypoglycaemic effect in comparison with a control group that did not receive this meal. The authors observed that proteins co-ingested with carbohydrates exert a hypoglycaemic effect on both normal subjects and subjects with diabetes, through the enhancement of insulin secretion and a minor delay in gastric emptying.

Fat and Lipids

Milk and dairy fat is the most complex fat in the human diet, consisting of more than 400 distinct fatty acid species (*52*). In dairy fat, short-, medium- and long-chain fatty acids, odd-chain fatty acids, branched chain fatty acids, conjugated linoleic acids (CLA), ruminal *trans* fatty acids (vaccenic acid), n-3 and n-6 fatty acids are represented. Many of these fatty acids are present in our diets in significant amounts only from dairy products (*53*). In particular, dairy fat is a rich source of butyric acid (C4:0), CLA, *cis-* and *trans*-palmitoleic acid (C16:1), and the branched chain fatty acid phytanic acid (C20:0). Some of these are present in dairy fat only in a small percentage, but these small amounts may still be biologically relevant, alone or within the context of other fatty acids (*53*).

Fat is present in Italian hard cooked types of cheese from a minimum of 25.5 % to a maximum of 31.4 %, with a mean value of 28.4 %, compatible with the definition of semi-fat cheese (*4*). The content of cholesterol is relatively low, amounting on average to 83–91 mg per 100 g of cheese.

Triglycerides present in Italian hard cooked types of cheese are characterised by more than 25 % of medium- and short-chain fatty acids (from C4 to C10). These compounds are naturally more easily absorbed than long-chain fatty acids (they do not need lipoprotein transportation) and provide very quick energy to the organism, as they follow different ways of assimilation from long-chain fatty acids (*4,5*).

Moreover, during the ripening of Italian hard cooked types of cheese, a partial lipolysis of fats occurs, which makes available a certain amount of fatty acids in a free form, facilitating their absorption (*4*), in analogy with the process of protein fractionation to amino acids previously described.

In a review regarding the effects of foods rich in fat on human health, Kratz *et al.* (*53*) summarised various studies (*54–58*) demonstrating that dairy fat consumption is associated with markers of better metabolic health and that dairy fat may protect against metabolic dysfunction. Kratz *et al.* (*53*) suggested that a potential mechanism by which dairy fat may exert beneficial effects on cardiometabolic risk is the reduction of chronic inflammation and lipid peroxidation, as suggested by Wang *et al.* (*59*) in a study showing that dairy fat intake is inversely related to inflammation and oxidative stress in overweight adolescents.

White *et al.* (*60*) demonstrated that medium-chain fatty acids (and in particular capric acid) can induce the phenomenon of vasodilation *in vitro* and *in vivo*. Moreover, these compounds, along with short-chain fatty acids, can help in increasing the body antioxidant defences (*61*). Even phospholipids can have antioxidant properties: Franson *et al.* (*62*) reported that sphingosine directly inhibits phospholipases A2 and D. A research by Yoshida *et al.* (*63*) demonstrated an inhibitory effect of phosphatidylserine on iron-dependent lipid peroxidation. Gordon *et al.* (*64*) found that phosphorylethanoline inhibited superoxide anion, with a mechanism that was not due to inhibition of cellular superoxide generation but to scavenging of generated superoxide anion.

In the following paragraphs, some dairy fatty acids of fats or groups of dairy fatty acids that have shown particular benefits for human health are described.

Conjugated linoleic acids

The term conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid characterised by the presence of two conjugated double bonds. The CLA are present in abundance in animal products, especially in milk and cheese.

The main isomer in dairy fat is rumenic acid, *cis*-9, *trans*-11 CLA, which can represent from 79 to 94 % of the total CLA of dairy fat. The other isomer frequently found in dairy fat is the *trans*-10, *cis*-12 CLA. Numerous studies have highlighted the potential health beneficial effects of these two fatty acids.

There is increasing evidence that individual isomers of CLA may have unique biological or biochemical effects. In particular, some beneficial effects of the *trans*-10, *cis*-12 CLA isomer in rodents were described. Body composition changes (reduced body fat, enhanced body water, enhanced body protein, and enhanced body ash contents) were associated with feeding the *trans*-10, *cis*-12 CLA isomer (*65*). The *trans*-10, *cis*-12 CLA isomer also exerts specific effects on adipocytes, in particular reducing the uptake of lipid by inhibiting the activities of lipoprotein lipase and stearoyl-CoA desaturase (*66*). These effects on body composition appear to be due in part to reduced fat deposition and increased lipolysis in adipocytes, possibly coupled with enhanced fatty acid oxidation in both muscle cells and adipocytes (*67*). Moreover, *trans*-10, *cis*-12 CLA inhibits preadipocyte differentiation, decreasing body fat accumulation (*68*). Park *et al.* (*69*) indicate that CLA prevents body fat accumulation and mass gain and that their dietary administration may be more effective in protecting against fat mass regain following mass loss than a mass loss treatment.

Other beneficial effects are due to the presence of the other CLA isomer, the *cis*-9, *trans*-11 CLA. Pariza *et al.* (*66*) demonstrated that this isomer enhances growth and probably feeds efficiency in young rodents. Some other beneficial effects are due to the presence of both isomers or possibly to a synergy between them. Pariza *et al.* (*66*) showed that both the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers appear to be active in inhibiting carcinogenesis in animal models. Ip *et al.* (*70*) showed that the anticancer efficacies of the two isomers (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) were very similar. With the administration of both CLA, 6 weeks after carcinogen administration in rats, the total number of premalignant lesions was reduced by 33–36 %; at 24 weeks, the total number of mammary carcinomas was reduced by 35–40 %. Also Masso-Welch *et al.* (*71*) found an inhibition of angiogenesis by CLA, defined as 'cancer chemopreventive agent'.

Antiatherogenic activity, antidiabetogenic effect and antiaggregant action, such as the capacity to stimulate bone mineralisation and immune response, regulate the allergenic responses and decrease hypertension, have also been suggested for these molecules (*2*). A retrospective case-control study conducted in Costa Rica demonstrated a strong inverse relationship between *cis*-9, *trans*-11 CLA in adipose tissue and the risk of myocardial infarction (*72*). In this study, the presence of the *cis*-9, *trans*-11 CLA acid in the adipose tissue was strongly connected to the consumption of dairy products. This suggested that CLA may play the role of protective factor when ingested by dairy products.

Prandini *et al.* (*73*) reported a content of 183 mg of *cis*-9, *trans*-11 CLA (+ *trans*-9, *cis*-11 CLA) per 100 g of Parmigiano Reggiano cheese (corresponding to 6.18 mg per g of fat) and 145 mg per 100 g of Grana Padano cheese (corresponding to 5.22 mg per g of fat), while the same authors reported a content of 37.7 mg of *trans*-9, *trans*-11 CLA (+ *trans*-10, *trans*-12 CLA) per 100 g of Parmigiano Reggiano cheese (corresponding to 1.27 mg per g of fat) and 27.2 mg per 100 g of Grana Padano cheese (corresponding to 0.98 mg per g of fat).

Prandini *et al.* (*74*) also reported a total CLA value of 385 mg per 100 g of fat in the same types of cheese, corresponding to 1.06 mg per g of cheese (for a fat content of

27.43 g/100 g). CLA content markedly depends on animal feed; in fact, in conventional Grana Padano, its content is 0.55 g per 100 g of total fatty acids, while in organic Grana Padano it is higher, 0.86 g per 100 g of total fatty acids (*75*).

Other fatty acids potentially beneficial for health

Kratz *et al.* (*53*) suggested that the butyric acid at the mass fraction typical of dairy product fat (4 %) can have relevant clinical effects on body mass and metabolic health. It also seems possible that butyrate consumption can have beneficial effects on chronic inflammation of the gastrointestinal tract. There is also experimental evidence (*76*) that butyric acid may lead to an increased synthesis of melatonin and metallothionein, one of the major antioxidant proteins of plasma. It can also stimulate the secretion of apolipoprotein A–I and apolipoprotein B-100 (*77*), improving the physiological homeostasis of cholesterol and plasma triglycerides. The content of butyric acid in Parmigiano Reggiano cheese was reported by Sandri *et al.* (*78*), who found a value of 59 mg per 100 g of fat at 12 months of ripening, while at 24 months of ripening the value increased to 144 mg per 100 g of fat, due to lipolysis. Malacarne *et al.* (*79*) reported a butyric acid content in Parmigiano Reggiano of 3.4 (1 month), 17.5 (6 months), 29.9 (12 months), 38.8 (18 months) and 71.0 (24 months) mg per 100 g of fat in the inner part of the wheel, while in the outer part of the wheel, where lipolysis is more accentuated, from 6 months onwards values were higher: 3.8 (1 month), 25.2 (6 months), 50.4 (12 months), 83.3 (18 months) and 123.9 (24 months) mg per 100 g of fat.

Trans-palmitoleic acid was associated with a lower incidence of diabetes (*56*). In particular, it should be noted that the consumption of full-fat dairy products has been directly and strongly associated with *trans*-palmitoleic acid levels in plasma phospholipids (*56*). Palmitoleic acid, in fact, plays an important role in the regulation of hepatic lipogenesis and in the adipocyte lipogenesis. Therefore, the consumption of dietary fats rich with this fatty acid can positively affect both energy homeostasis and metabolic health. These considerations may be relevant, since full-fat dairy products are one of the few dietary sources of palmitoleic acid (*53*). The mass fraction of *trans*-palmitoleic acid in Parmigiano Reggiano was reported by Castagnetti *et al.* (*80*), which was 0.08 g per 100 g of fat when cattle feed was fresh or preserved forage, while it was 0.11 g per 100 g of fat when cows were fed fresh forage with integrated extruded whole linseed flour.

Another fatty acid of potential interest is phytanic acid, a branched chain fatty acid (C20:0). This acid is characterised by a C16 chain with four methyl groups as side chains attached in positions 3, 7, 11 and 15 (*81*). There are a series of *in vitro* experiments suggesting that phytanic acid from fat of dairy products may be relevant for energy and glucose homeostasis (*53*). Unfortunately, so far, to our knowledge, amounts of phytanic acid in Parmigiano Reggiano or Grana Padano cheese have not been detected. A study (*82*) has been done on other types of cheese, less hard than Italian hard cooked cheese, as Emmental (167–303 mg per 100 g of lipids), Edam (288 mg per 100 g of lipids) and Gouda (256–265 mg per 100 g of lipids). Other studies have reported the content of phytanic acid in milk that is rather variable: Capuano *et al.* (*83*) reported an average content of 146 mg per 100 g of fat, but with oscillations between 65 and 328 mg per 100 g of fat; Schröder *et al.* (*84*) reported an average content of 116 mg per 100 g of lipids, but in organic milk the content was higher (153 mg per 100 g of lipids); Che *et al.* (*85*) registered the highest value in September (121 mg per 100 g of fat) and the lowest in May (96 mg per 100 g of fat), depending also, to a great extent, on cattle feed, being higher with pasture (21–76 mg per 100 g of fat) than with maize silage (0–48 mg per 100 g of fat) and concentrate (10–34 mg per 100 g of fat).

Carbohydrates and Prebiotics

Another important characteristic of Italian hard cooked cheese is the total absence of lactose, the main carbohydrate in milk. Lactose disappears in the early hours after the cheese making process. In fact, the fermentation of lactose into lactic acid and the subsequent acidification of the curd is one of the most important processes in the production technology of Parmigiano Reggiano DOP and Grana Padano DOP. Based on the standards of the European Commission (April 2003), Parmigiano Reggiano and Grana Padano can be defined as 'lactose-free' products because they contain lactose in amounts not higher than 0.10 mg per 100 kcal of product (*86*). The absence of lactose in ripened cheese which is ready for consumption is a very important fact for subjects who are intolerant to this sugar or for individuals who, due to an insufficiency of lactase enzyme, are unable to digest lactose. Lactose intolerance is a very common disease among Asian populations, who historically do not consume milk and dairy products and consequently suffer from lactase deficiency; Western populations suffer more often from some types of lactose malabsorption, and only occasionally of intolerance. Lactose intolerance induces a reduction in calcium ingestion, sometimes below the requirements, as a consequence of the elimination of milk and dairy products from the diet. Increasing evidence suggests that individuals with lactase deficiency can reach an adequate level of calcium intake through Italian hard cooked cheese consumption, thereby improving bone health and preventing osteoporosis (*2*).

Oligosaccharides with prebiotic properties

In the carbohydrate fraction of Italian hard cooked cheese, it is important to emphasise the presence of certain oligosaccharides, *i.e.* short-chain non-digestible carbohydrates with a possible prebiotic effect. In particular, these carbohydrates could stimulate the growth and/or activity of one or more bacterial populations in the colon, with specific health benefits. Prebiotics are defined as those food components which, resisting the acid environment of the stomach and the action of bile salts and digestive enzymes in the small intestine, reach the colon, where they selectively stimulate the multiplication of existing beneficial strains (bifidobacteria and lactobacilli) and induce local and systemic effects advantageous for the host (*87,88*).

Prebiotics act as a selective substrate for fermentations, influencing the microbial activity in the intestine and the absorption of minerals, as well as stimulating the immune system (*86*). The oligosaccharides are also involved in many cellular recognition processes and have numerous biological activities including immunostimulatory, anti-inflammatory, antiviral and immunological function (*89*).

In a study carried out on a large number of samples of Parmigiano Reggiano, Coppa (*87,90*) demonstrated for the first time the presence of a significant proportion of oligosaccharides, compounds which can act as prebiotics able to favour the development of intestinal 'bifidogenic microbiota'. The analysis of the characterisation of the oligosaccharides showed the presence of several peaks (over 50), whose sequence was found to be very constant in all the examined samples (*87*). These peaks are considerably more numerous and substantially different from those present in the cow's milk employed for cheese production; consequently, their presence in the final product must be linked to the action of digestive-fermentative processes that, taking place in various stages of the production, could lead to the synthesis of new oligosaccharide molecules. The mass fraction of oligosaccharides in Parmigiano Reggiano was approx. 2.5 g per 100 g.

The presence of glycosaminoglycans was also found in Parmigiano Reggiano cheese. This denomination identifies a class of natural molecules active in the regulation of primary biological activities such as, among the many, cell interaction with growth factors and regulation of blood coagulation functions, showing at the same time antithrombotic, antiviral and anti-inflammatory activity (*91*).

Probiotic Bacteria

To be considered a probiotic, a bacterial strain must be a normal component of human intestinal microbiota, be absolutely safe for use in human beings, be active and vital under the conditions present in the intestine, be resistant to gastrointestinal secretions (gastric juice, bile and pancreatic juice), and be able to persist, at least temporarily, in the human gut. There is experimental evidence that some of the bacteria in Italian hard cooked cheese can have probiotic functions. In particular, it has been shown that, at 12 months of ripening, in Parmigiano Reggiano, the presence of *Lactobacillus rhamnosus* is still detectable, even if at low levels (10^3–10^4 CFU/g), having the actual characteristics of probiotic (*92*). Also, Pancaldi *et al.* (*86*) emphasise the probiotic nature of Parmigiano Reggiano and its possible use in the prevention of intestinal and extraintestinal diseases at all ages, from infants to the elderly.

Vitamins

Milk and cheese belong to food that in the daily ration covers only a small proportion of the vitamin requirements. However, with reference to the values indicated by the Recommended Levels of Nutrient Intake (LARN) for adults, 100 g of Parmigiano Reggiano completely cover the need for vitamin B12 and biotin, a third of the requirement of vitamin A and B2, and from 1/5 to 1/10 of the requirements of other vitamins (*4,5*).

Mineral Salts

Italian hard cooked types of cheese also contain a large amount of macrominerals (namely calcium, phosphorus, sodium and chloride) and trace elements (particularly zinc and selenium). In particular, these types of cheese are a very important source of calcium, because of both their high content of this element (1159 mg per 100 g) and its particular bioavailability. The content of calcium in these types of cheese depends also on cattle breed, the milk of Italian Brown cows being richer in calcium than that of Italian Friesian cows (*93*).

Calcium, vitamin D and proteins are the three main nutrients that sustain the development and maintenance of bone structure. Many reports relating to the intake of these nutrients have emphasised their importance in the prevention of bone loss and thus in the reduction of the risk of fractures in the elderly (*94*). Being a source of calcium and protein, dairy products could play a positive role on bone health (*42*). Perego *et al.* (*95*) have shown that casein phosphopeptides (produced by hydrolysis of Italian hard cooked cheese proteins, see Protein and Peptides section) can activate calcium uptake by intestinal cells. From this point of view, Italian hard cooked types of cheese can be very important foods for the skeleton bone growth: they represent an excellent source of many essential nutrients for the skeletal development and health. The presence of proteins of high biological value and highly bioavailable calcium makes these types of cheese really 'functional foods' for treating bone homeostasis and for the prevention of osteoporosis (*42*).

Recently, De Luca *et al.* (*96*) have demonstrated that gastrointestinal digestates of Grana Padano and Trentingrana cheese promote intestinal calcium uptake and extracellular bone matrix formation *in vitro*.

Among the trace elements, the presence of selenium and zinc in milk and Italian hard cooked cheese is of particular interest, since Se is the cofactor of glutathione peroxidase and Zn is the cofactor of the enzyme superoxide dismutase. Therefore, Se and Zn indirectly inhibit the activity of the prooxidant iron and free radical in general. Moreover, they induce the synthesis of metallothionein and activate the ornithine decarboxylase for the synthesis of polyamines (*61*).

Components of Dairy Products and Disease Prevention

The last part of the review focuses on the components of Italian hard cooked cheese that can have a role in the prevention of some diseases. In the following paragraphs, the most important experimental evidence of this is succinctly exposed.

Dairy products can reduce the occurrence of type 2 diabetes

Numerous studies have demonstrated a relationship between the consumption of dairy products, in particular of some of their constituents, and a reduction of diabetes occurrence.

Statistical studies have found that subjects who received greater amounts of dairy products within their diet decreased the risk of developing type 2 diabetes (DM2) by an average of 14 % (*97,98*). Also, Sluijs *et al.* (*99*) suggest that increased consumption of cheese tends to be inversely associated with the risk of diabetes.

In a large prospective study, Malik *et al.* (*100*) showed that an increased consumption of dairy products during adolescence is significantly associated with a reduced risk of type 2 diabetes in adulthood. This finding is in agreement with the results from previous studies (*101,102*) performed on adults, which showed inverse associations between the intake of dairy products and type 2 diabetes. Overall, there is evidence of the beneficial effect of daily intake of dairy products on glucose homeostasis, indicating a possible beneficial effect of dairy consumption in the prevention of the development of type 2 diabetes (*97*).

Recent literature suggests that the fatty acids contained in dairy products can exert a potential role in preventing diabetes (*56,99,103*). In addition, dairy products are rich in calcium, vitamin D and magnesium, all constituents that may exert a protective function against diabetes (*102,104*).

Some scientific studies have shown that the incidence of diabetes is inversely associated with dairy products with high fat content (*56,100,103*). Many researchers have suggested an association between the quality of fat, rather than the quantity, and diabetes risk. For example, cheese is a source of saturated fatty acids; Hu *et al.* (*105*) suggested that these fatty acids may be involved in reducing the risk of diabetes. However, based on Krachler *et al.* (*103*), the higher amount of odd-chain fatty acids in dairy products too can lead to a diabetes risk reduction. Recently, Ericson *et al.* (*106*) detected an association between the presence of short-chain saturated fatty acids, typical of dairy products, and the onset of diabetes protection. Dairy products are also the best sources of lauric acid (12:0) and myristic acid (14:0).

Dairy products can reduce symptoms of intestinal problems

Pancaldi *et al.* (*86*) presented three cases of infants suffering from various forms of intestinal problems, subjected to a special diet therapy, in order to resolve situations that would be difficult to manage using special varieties of infant formula on the market. These children were given a mixture consisting of 40 g of Parmigiano Reggiano aged for at least 36 months, 40 g of rice or corn and tapioca, 40 g of sugar, 10 g of corn oil, all added to 1 litre of water. This compound, known as NO (New Olivi, from the researcher who invented this composition), is able to provide about 60 kcal per 100 mL.

After the introduction of the food based on Parmigiano Reggiano cheese, all cases showed a rapid and progressive relief of symptoms. In fact, Italian hard cooked types of cheese have a high concentration of easily absorbable amino acids and oligopeptides, similar to that of protein hydrolysates. As for the lipid component, medium- and short-chain fatty acids are directly absorbed in the intestine and immediately employed as a significant source of energy. The use of Italian hard cooked cheese as food therapy is appropriate not only for their high nutritional value, but also for their characteristics as functional food, which produce beneficial effects on the gastrointestinal tract. Moreover, their efficacy in pathological conditions is further increased by the prebiotic and probiotic effects resulting from oligosaccharides and by the natural bacterial microbiota present in the Italian hard cooked cheese mass.

Dairy products can have a beneficial role in metabolic syndrome

Dairy products as Italian hard cooked cheese may have a beneficial role also in the alleviation of metabolic syndrome (*98*). This syndrome is a cluster of risk factors for increased mortality, including obesity, altered glucose homeostasis, hypertension and atherogenic dyslipidaemia. Individuals with metabolic syndrome often suffer from a chronic inflammatory state.

Dairy products can regulate blood pressure

Epidemiological studies suggest that the consumption of dairy products may be associated with a 13 % reduction of the risk of high blood pressure (*98,107*). The content of calcium and vitamin D, as well as some peptides of milk (see the ACE-inhibitory peptides section), may exert a beneficial effect on blood pressure by inhibiting ACE, which modulates the endothelial function (*108, 109*).

Dairy products can prevent cardiovascular diseases

Warensjö *et al.* (*55*) reported an inverse association between the intake of fat from dairy products and the risk factors for cardiovascular diseases including triglycerides, total cholesterol, and insulin in the serum during fasting. A possible explanation may be that the intake of dairy fat reduces the risk of myocardial infarction through a mechanism which involves the reduction of fat and triglycerides, and improves metabolic health.

Dairy products can prevent insurgence of colon cancer

Perego *et al.* (*110*) showed that casein phosphopeptides derived from dairy foods can prevent insurgence of colon cancer, protecting differentiated intestinal cells from calcium overload toxicity and prevent their apoptosis, favouring proliferation while inducing apoptosis in undifferentiated tumour cells.

Conclusions

In this review, the functional properties of Italian hard cooked types of cheese were described, highlighting the effects of each component on human health. It appears that there are many important benefits related to the dietary intake of the components of these types of cheese, making these products truly valuable functional foods.

In particular, proteins in Italian hard cooked types of cheese possess biological properties attributable to potentially bioactive peptide sequences; these peptides, hidden and inactive in the primary protein structure, may be released and activated by proteolytic processes during the technological treatment or during gastrointestinal digestion and act in the body by influencing physiological processes and modulating various biological functions. In particular, several ACE-inhibitory peptides were found in Parmigiano Reggiano cheese samples and in their relative intestinal digestate. In particular, VPP, IPP, LHLPLP and HLPLP were revealed in water-soluble extract, while after *in vitro* gastrointestinal digestion of this water extract, the same ACE-inhibitory peptides along with the newly formed AYFYPEL and AYFYPE were found. Some of these peptides are responsible for a marked decrease in blood diastolic and systolic pressure in patients with blood pressure problems.

As far as lipid fraction is concerned, Italian hard cooked cheese can be an important source of fatty acids, which appear to be particularly beneficial to human health. Many of them are present in significant amounts only in dairy products. In particular, Italian hard cooked cheese fat is a source of butyric acid (C4:0) (30–140 mg per 100 g of fat, depending on cheese ripening), CLA (385 mg per 100 g of fat, corresponding to 1.06 mg per g of cheese, but variable according to cattle feed), *trans*-palmitoleic acid (tC16:1) (0.08–0.11 mg per 100 g of fat, depending on cattle feed), and possibly also the branched chain fatty acid phytanic acid (C20:0), but in this case the presence in Italian hard cooked cheese, attested for other types of cheese, needs confirmation. CLA may have unique and precious biological or biochemical effects: in particular, body composition changes (reduced body fat, enhanced body water, enhanced body protein, and enhanced body ash contents) were associated with CLA. They exert specific effects on adipocytes too, in particular reducing the uptake of lipid by inhibiting the activities of lipoprotein lipase and stearoyl-CoA desaturase; moreover, CLA can prevent body fat accumulation and mass gain. Butyric acid can have relevant clinical effects on body mass and metabolic health and can have beneficial effects on chronic inflammatory conditions of the gastrointestinal tract. *Trans*-palmitoleic acid was associated with a lower incidence of diabetes, and phytanic acid may be relevant for energy and glucose homeostasis.

Italian hard cooked types of cheese are also characterised by the presence of certain oligosaccharides, *i.e.* short-chain non-digestible carbohydrates, with a possible prebiotic effect, which could stimulate the growth and/or the activity of one or more bacteria in the colon, with specific health benefits and numerous biological activities such as immunostimulant, anti-inflammatory, antiviral and immunological.

There is experimental evidence that some of the bacteria in the Parmigiano Reggiano cheese ripened for 12 months are still present, even if at low levels, and can have probiotic functions. Vitamins are also present in Italian hard cooked cheese, even if some of them do not completely cover the recommended levels of nutrients (LARN) indicated by Italian Society of Human Nutrition (SINU).

Italian hard cooked cheese also contains a large amount of macrominerals (calcium, phosphorus, sodium, chloride) and trace elements (particularly zinc and selenium). The particularly high content and bioavailability of calcium, together with the presence of vitamin D and specific proteins, make these types of cheese really 'functional foods' for bone homeostasis and for the prevention of osteoporosis.

Among all these properties, two in particular seem to be of great interest: the ability to keep blood pressure regulated, thanks to ACE-inhibitory peptides, and the possibility of these types of cheese to act as foods rich in bioavailable calcium, especially for countries such as Asia, where osteoporosis in menopause is an increasingly important issue. This evidence needs to be highlighted to counteract the negative impact of the smear campaign against animal foods that are publicised to exert negative influence on human health.

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