Section I
Biochemical and technological aspects

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Whey proteins. From supplementation to physiological modulation

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Summary
Results from the experimental and clinical studies started around 30 years ago due to the pioneering work of Bounous and to the parallel incredible progress made by biochemistry and food technologies, make currently available to nutritionists and health professionals in general a series of highly purified whey protein formulations – extremely similar to the native ones. Further adjustments of the production technique consisting of delactosing and elimination of fats have allowed us to achieve highly purified preparations that are also safer from an allergen point of view and also more suitable in hypolipidic diet regimens. Research continues to look for biochemical processes that are at the basis of the positive clinical effects produced by whey proteins but at least one consideration seems to emerge from the numerous past works mentioned in this first section. The acknowledgement of a series of effects linked to the dosage that is in some way proportional to body weight - correlated with the nutritional quality of the raw material that is the source of the macromolecules indispensable for anabolic processes – should be accompanied by others, linked to the intake of more modest quantities – likely related to a particular aminoacid composition or, in any case, to small peptide sequences – compatible with the modulation of important cell functions, including the control of the oxidative balance mediated by the synthesis of endogenous glutathione.

Riassunto
I risultati degli studi sperimentali e clinici iniziati circa 30 anni fa grazie all'opera pionieristica di Bounous da una parte ed i passi da gigante compiuti, in parallelo, dalla biochimica e dalle tecnologie alimentari rendono oggi disponibili al nutrizionista ed ai professionisti della salute in genere formulazioni altamente purificate – in condizioni molto simili a quelle native – di proteine da siero di latte. Ulteriori affinamenti della tecnica di produzione, attraverso l'allontanamento del lattosio e dei grassi, consentono di ottenere, accanto all'elevata resa, preparati sempre più sicuri sotto il profilo dell'allergenità e, rispettivamente, maneggevoli, anche in regimi dietetici ipolipidici. La ricerca è continuamente impegnata a dare un volto ai processi biochimici che sono alla base dei favorevoli ri-

scontri clinici esibiti dalle proteine da siero di latte ma almeno una considerazione sembra emergere dai molteplici lavori passati in rassegna in questa prima sezione. Accanto ad una serie di effetti legati ad un dosaggio in qualche modo proporzionale al peso corporeo – correlati con la qualità nutrizionale della materia prima quale fonte di macromolecole indispensabili per i processi anabolici – bisogna riconoscerne altri, legati all'assunzione di più modeste quantità – verosimilmente correlati con la particolare composizione amminoacidica o, comunque, a piccole sequenze peptidiche – compatibili con la modulazione di importanti funzioni cellulari, tra cui il controllo del bilancio ossidativo attraverso la sintesi endogena di glutatione

Whey proteins. Biochemical aspects

Overview

For millenniums milk (unless otherwise indicated to be intended here as cow's milk) – as such or in the form of derivatives – has been an important source of nutrients in human nutrition (1). Over the past decades, the development of biochemical techniques has allowed us to separate from milk and identify a series of components that, given a series of *in vitro* and *in vivo* studies, have been considered as being "nutraceuticals", in other words "healthy" foods, the specific nutritional properties of which are responsible for a series of proven and favourable biological effects potentially useful for curative purposes (2, 3). This is the case of whey proteins that have seen an increase in their use in clinical nutrition due to studies by the Italian-Canadian Bounous (4).

As is well-known, milk is composed mainly of water (86-87%), in which carbohydrates (5.0%), lipids (3.75%), proteins (3.38%), mineral elements (0.9%) and traces of 'minor' constituents (vitamins, nucleotides, pigments, gas, etc.) are dissolved or dispersed (2) (Fig. 1).

In turn, the proteins present in milk are divided into two main categories, caseins (approx. 26 g/kg) and whey proteins (approx. 6.5 g/kg) (2) (Fig. 2). This distinction derives directly from the production technology of common dairy products, such as "mozzarella" and "ricotta" cheeses (5). In fact, by adding 'rennet' (a natural mix of proteases that normally are of animal origin) to heated milk, milk "coagulates" some of the proteins which are initially suspended in the milk's aque-

Figure 1 - Schematic representation of the chemical composition of milk.

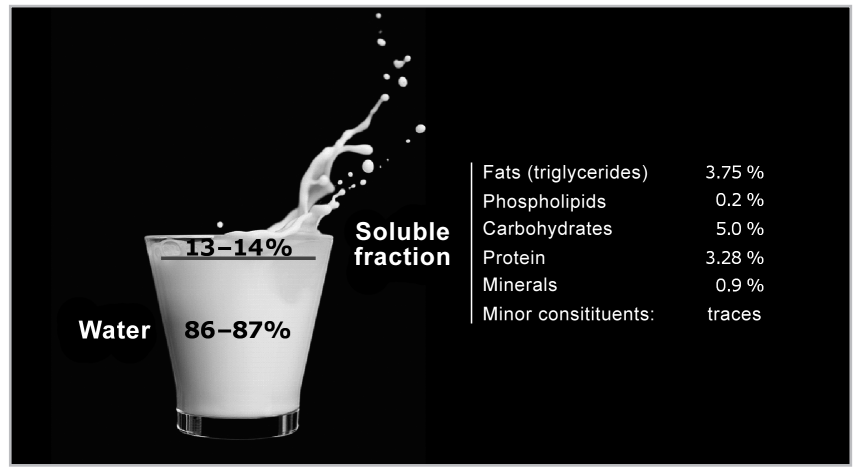
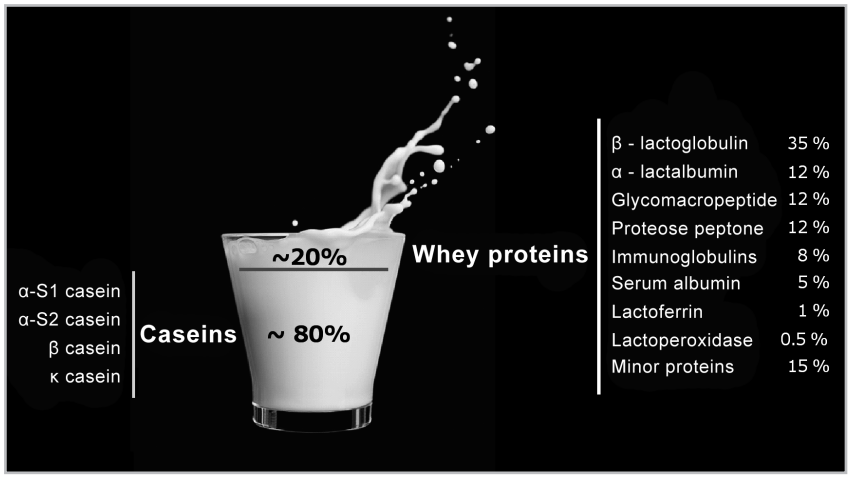


Figure 2 - Schematic representation of the protein composition of milk.



ous phase and that then precipitate to the bottom forming a pasty mass called 'curd'. Caseins remain trapped within this mass that is used as a raw material in the production of "mozzarella" cheese. As the 'curd' organises itself, it squeezes out of its three-dimensional reticulate a liquid called "whey"; whey proteins collect in this liquid, that is used as a raw material in the production of ricotta cheese.

There are marked differences between caseins and whey proteins and this justifies their different use in clinical nutrition (2).

Composition and general biological properties

Limiting our attention to whey proteins, essentially these include β–lactoglobulin, α-lactalbumin, immunoglobulins, glycomacropeptide, serum albumin, lactoferrin and lactoperoxidase, and these have a series of distinct biological properties (see below) (6) (Tab. 1).

From a nutritional point of view, they represent the protein fraction of milk with the greatest biologi-

Table 1 - Composition* and main properties of whey proteins.

Fraction	Molecular weight	AA	-S-S-/mol + cys**	Concentration***	Percentage****	Biological properties
β–lactoglobulin°	18 kDa	162	2 + 1	3.2 g/L	55 – 50%	Source of essential and branched aminoacids
α–lactalbumin°	14 kDa	123	4	1.2 g/L	25 – 20%	Source of essential and branched aminoacids
Immunoglobulins°°	150–420 kDa	variable	variable	0.7 g/L	15 – 10%	Immunomodulatory action (maximum in colostrum)
Glycomacropeptide	8 kDa	64	0.1°°°	-	15 – 10%	Source of essential and branched aminoacids. No phe.
Serum albumin	66 kDa	582	17 + 1	0.4 g/L	10 – 5%	Source of essential aminoacids
Lactoferrin	80 kDa	708	16	≤ 0.1 g/L	2 – 1%	Antioxidant, anti-infective, probiotic action
Lactoperoxidase°°	78 kDa	595	15		0.5%	Inibition of bacterial growth

*Possible variations according to the various physiological states. **Composition in cystine and cysteine. ***As referred to 1 liter of whole milk. ****As referred to total whey proteins.
°Synthesised in the mammary gland. °°Of plasmatic origin. °°°Expressed as g of cystein per 100 g of a protein formula.

cal value as they contain all essential aminoacids, nearly always in concentrations markedly superior to those of vegetable origin, including soy (1). Furthermore, unlike caseins, they are particularly rich in sulphur aminoacids such as methionine and especially cysteine, a precursor of compounds with elevated biological activity, such as glutathione (see below) (6). Moreover, whey proteins show high levels of lateral branched-chain aminoacids, such as leucine, isoleucine and valine, fundamental for tissue growth and repair, but also well-known for their effects on muscle performance thanks to their ability to store glycogen (6). In particular, leucine plays a crucial role in protein biosynthesis (7). Finally, the content of tryptophan is also significant.

β–lactoglobulin

β–lactoglobulin, with a concentration between 2 and 4 g/L depending on the mammalian species considered (8), represents around half of the total whey proteins (9).
The interest of biochemists and food technicians in the transformation and preservation of milk has long been focused on bovine β–lactoglobulin not only because it offers an interesting model for molecular modelling studies but also because it is a widely used ingredient in many food products (10).
In physiological conditions it is present as a monomer that can balance its dimeric form; the latter prevails in bovine species whereas the monomeric form is prevalent in horses.
Each monomer - of which at least 2 variants or isoforms are known, A (Asp_{64} and Val_{118}) and B (Gly_{64} and Ala_{118}) different due to as many punctiform mutations – is composed of 162 aminoacid residues, among which cysteine (5) and tryptophan (2) are of particular importance due to their physical-chemical, biological and technological-food properties. Four of the cysteine residues give rise to two intrachain disulphide bridges (Cys_{106}-Cys_{119} e Cys_{66}-Cys_{160}) whilst the remaining residue (Cys_{121}) is free (11); this latter, which in its native form cannot be reached by the solvent, following structural changes induced by a physical treatment can be exposed to the solvent, enhancing therefore the formation of protein polymers stabilised by interchain disulphide bridges. Of the two tryptophan residues, Trp_{19} is inside the hydrophobic core, whilst Trp_{61} occupies a more superficial position. The secondary structure is composed mainly of β sheets. Nine antiparallel β–filaments have been identified (classified using letters from A to I) that alone cover 54% of the structure and 2 α–helixes that represent 27%, whilst the remaining sequence is structured as a random coil (12).
β-lactoglobulin synthesis takes place in the mammary gland (13) starting from a precursor (pre β-lactoglobulin) that is then differentiated into its mature form after losing a peptide signal that, depending on the species, is composed of 16-18 aminoacids. An enzyme (disulphide isomerase) ensures its correct structuring before being transferred to the Golgi apparatus and then accumulated in the secretory vesicles.
From a nutritional point of view, β-lactoglobulin, like other whey proteins, is important as it represents a precious source of cysteine and essential lateral branched-chain aminoacids (14). Despite β-lactoglobulin has been attributed with allergizing properties due to its absence in human milk and despite it belonging to the lipocalin protein family (proteins that are thought to be resistant to certain proteases) (15-18), it also seems to play an important biological role in modulating the bioavailability of important lipophilic micronutrients, such as retinoids (14). This property is not only linked to the strong structural analogy with the Retinol Binding Proteins but also to the presence of a real retinol binding site (19, 20). Finally, a possible involvement of β-lactoglobulin in the regulation of lymphatic function has also been described (14).

α–lactalbumin

α-lactalbumin constitutes approximately 20-35% of whey proteins (9) and it is a monomeric globular protein lighter than β-lactoglobulin and is composed of a bigger fraction without carbohydrates and 3 minor fractions of which 2 are glycosylated. In many animal species it is extremely heterogeneous due to a genetic polymorphism (21). Three variants have been described in bovines - A, B, and C - of which only the B form is present in European breeds (22, 23).

From a structural point of view, worthy of mention is the presence of 8 cysteine residues that give rise to 4 intrachain disulphide bridges and to some specific negatively charged carboxylic residues that are able to complex calcium and are fundamental elements that stabilise the protein in aqueous solution.

From a functional point of view α-lactalbumin is an integral part of galactosyltransferase's enzymatic system which is responsible for lactose synthesis (24).

The protein has a nutritional profile which is practically superimposable with that of β-lactoglobulin with which it shares high levels of lateral branched-chain aminoacids however unlike β-lactoglobulin, α-lactalbumin has double the number of cystine residues that are considered precursors of glutathione synthesis (9). Both in its native form as well as after hydrolysis, it is able to boost the immune system in murine models through modulation of lymphocytic function (4, 25). In its purified form, due to its similarity with human α-lactalbumin, it is used in baby formulas (9).

Immunoglobulins

The immunoglobulin fraction, that constitutes approximately 10-15% of whey proteins, contains molecules of plasma glycoprotein nature with antibody function and belonging to all 5 classes (G, M, A, D, E) that have specific immunomodulatory (26), antiviral and anti-bacterial (against strains of *E. coli, Salmonella enteriditis, S. typhimurium,* and *Shigella flexneri*) properties (13, 27, 28).

Glycomacropeptide

Glycomacropeptide constitutes 10-15% of whey proteins and is normally released during the process transforming milk into cheese, due to the action of chymosin on κ–casein (9). κ–casein is released as para–κ–casein (residues 1-105), that remains with the curd, whilst glycomacropeptide (residues 106-169), for this reason also called caseinmacropeptide, is removed together with the serum (29).

There are numerous reasons that make this protein fraction particularly interesting from a nutritional point of view.

In fact, it provides a great quantity of branched aminoacids but lacks aromatic aminoacids such as tryptophan, tyrosine and phenylalanine (9). The absence of phenylalanine makes glycomacropeptide particularly useful in the diets of patients affected by phenylketonuria (9, 30); however, the lack of other essential aminoacids in glycomacropeptide (arginine, cysteine, histidine, tryptophan and tyrosine) does not permit its use as a unique source of protein in these subjects (31).

Furthermore, numerous studies carried out over the last decade have suggested a possible role of glycomacropeptide, especially in its non-glycosylated form (CMP), in the control of intestinal function (31-33). In particular, it has been observed that CMP inhibits gastric secretion and slows down stomach contractions but favours the release of cholecystokinin, the satiety hormone involved in the absorption and digestion of food in the duodenum of animals and of man (34). This effect seems to be linked to the direct stimulation of intestinal receptors by the integral peptide, that among other things, has the unique property not only of resisting gastric digestion (35) but also of being entirely absorbed and then partially hydrolysed in the circulation as demonstrated in studies carried out on adults after the ingestion of

milk or yoghurt (36). It is on the basis of these data that glycomacropeptide based products have been recently put onto the market with the purpose of controlling appetite and therefore, body weight; their clinical efficacy, however, remains to be established. In fact, a short-term clinical trial revealed that CMP has no effect on food as a source of energy or on objective satiety indicators in adults (37).

Also serine phosphorylated peptides can be released from glycomacropeptide through targeted hydrolysis and these are able to bind calcium, thus reducing its precipitation as phosphate salt. In this respect, numerous studies have shown an increased calcium absorption in rats after the ingestion of caseins, by correlating it to the presence of CPPs in the small intestine that would, therefore, act as metal carriers (38-42). This would explain, at least in part, the favourable effect of CPPs that has often been seen on bone mineralisation (43, 44). The singular "chelating" action and therefore, transporting effect of these peptides seem not to be limited to calcium but can also be extended to other metals of nutritional importance, such as iron and zinc (45).

Apart from this, in any case, glycomacropeptide seems to perform a modulating action on the development and maintenance of the natural microflora of the digestive tract, where it seems to promote, thanks to its high carbohydrate levels, the growth of bifidobacteria (46). It has also been demonstrated that GMP is able to bind the toxins produced by *Vibrio cholerae* and by *Escherichia coli.*

Worthy of mention is also the fact that glycomacropeptide seems to be able to induce a shift of the microbial flora of dental plaque from *Streptococci* to *Actinomyces* which are less cariogenic. For this reason, in order to prevent plaque formation and therefore, the development of cavities, glycomacropeptide has been incorporated in toothpastes and chewing gum, where in synergy with xylitol, it enhances the remineralisation of teeth (44).

Finally, CMP as such but especially some of its specific tryptic hydrolysates, has an inhibitory effect on the enzyme that converts angiotensin I to angiotensin II and this makes them potentially useful in the control of arterial hypertension (46).

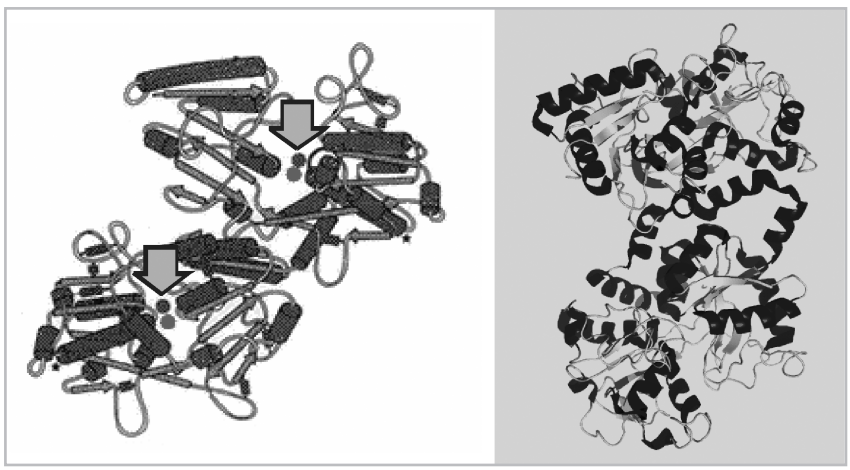
Serum albumin

Bovine serum albumin is a protein of haematic origin (29) with a high molecular weight (9). It is a good source of essential aminoacids, and is the whey protein that contains the highest number of cystine residues (a good 17!), whose biological importance has already been mentioned, and one cysteine residue. Even though its physiological role is still to be completely understood, it has long been known that it has a striking ability to adsorb not only lipid substances but also chemical species with a radical nature, in a similar way to that observed for serum albumins in general; this property seems to enable serum albumin to play an important role respectively in the absorption of liposoluble micro and macronutrients and in the control of oxidative stress (see below).

Lactoferrin

Lactoferrin is a high molecular weight (80 kDa) conjugated protein composed of a long single chain of aminoacids (708) covalently linked to a highly expressed glucidic component (approximately 7%) that makes it highly hydrosoluble (glycoprotein). Its sequence is very similar to that of two human proteins correlated functionally with it, namely the lactoferrin (68%) and the transferrin (60%) (46) whilst its tertiary structure allows as many ferric state iron atoms (Fe^{3+}) to localise in two specific "pockets" (47) (Fig. 3). The term apolactoferrin is normally used to indicate iron-depleted lactoferrin. It is present both in colostrum and in whey representing around 1-2% and 7%, respectively but it may also be found in

Figure 3 - Structural models of lactoferrin. The arrows on the left indicate the iron binding site.



other biological fluids, such as tears (48).

Over the years numerous biological properties have been attributed to lactoferrin, even though only a few of them are supported by sufficient scientific evidence. However, there is the general consensus among academics that it may play a role in the modulation of the immune system, especially at intestinal level and in the control of oxidative stress.

Firstly, by binding iron with an extremely high affinity (even higher than that for circulating transferrin), lactoferrin is particularly resistant to enzymatic proteolysis and more in general also to denaturing treatments (heat and acid treatments) (32). Due to these properties, it favours the gastrointestinal absorption of iron, therefore playing a fundamental role in the nutrition of babies. Furthermore, being active at a neutral-basal pH, it may also function within the intestinal lumen starting from the duodenal area.

However, lactoferrin is well-known especially for its bacteriostatic effects at intestinal level on pathogenic bacteria (especially *Escherichia coli* but also *Salmonella typhimurium*) with favourable repercussions on the development of probiotic microorganisms (49). This effect can be obtained through two mechanisms: a direct mechanism of interaction with the bacterial surface components (lipopolysaccharide A and/or porins) leading to an increase in permeability and cell lysis, and an indirect mechanism of iron chelation, where growth processes and bacterial replication (iron depleted bacteriostasis) are deprived of iron (50). It is not surprising in fact, that lactoferrin concentration in ruminants' milk can markedly increase during an intramammary infection, probably in an attempt to contrast the development of pathogenic bacteria, similarly to what is seen in human secretions. The bacteriostatic effect above described seems to be enhanced by an immunostimulant action that is yet to be properly defined and in particular by the inhibition, both *in vivo* and *in vitro*, of the release of histamine from mast cells (51). In any case, once lactoferrin is subjected to proteolysis through pepsin, it generates peptides with antimicrobial activity, such as lactoferricin that are toxic for *Candida albicans* (52), *Giardia lamblia* and for a series of viruses (herpes zoster, cytomegalovirus and HIV-1). Some synthetic peptides (HTL1 and HTL2) have a greater effect on *Escherichia coli*. Human lactoferricin is composed of residues 1-46 and has marked antibacterial activity attributable especially to sequences 20-35, 24-35 and 31-35 (53).

Finally, lactoferrin, due to its ability to keep iron bound, is today rightly considered a preventive antioxidant (54, 55). In fact, when this transition metal is found in its "free" state, in other words not chelated, in a biological system, it acts as a generator of free radicals

in the so-called Fenton reaction (56). More precisely, iron – due to its characteristic of reversibly going from an oxidised state (Fe^{3+}) to a reduced one (Fe^{2+})– catalyses the breakdown of hydroperoxides (products that originate from an oxidative injury of a wide class of biological molecules, such as lipids, aminoacids, peptides, etc.) into alkoxyl radicals ($RO^{\cdot}$) and/or hydroperoxyl radicals ($ROO^{\cdot}$) (56). Therefore, once the iron has been "sequestered", lactoferrin blocks this undesired chain of events that would lead to oxidative damage to the whole cellular system.

The effects described above are enhanced by the high number of cystine residues (a good 16!), that are considered precursors of glutathione biosynthesis, the most powerful intracellular antioxidant.

Lactoferrin is today available on the market in different formulations with concentrations varying between 0.3% and 2.0% (48).

Lactoperoxidase

Lactoperoxidase constitutes approximately 0.25-0.50% of whey proteins and at the same time it is one of the main enzymes (proteases, lipases, hydrolases, liases, transferases etc.) of this fraction (9). In biological systems its main catalytic activity is the breakdown of potentially harmful hydroperoxides to "innocuous" alcohols (57). From this point of view, it creates a potent antioxidant system that acts in synergy with lactoferrin. In fact, whilst lactoferrin reduces the availability of iron necessary for the transformation of hydroperoxides into free radicals, lactoperoxidase completely neutralizes these reactive oxygen metabolites. However, additional biological activities potentially responsible for anti-bacterial effects cannot be excluded (58). It is interesting to underline that lactoperoxidase activity is not destroyed by pasteurization processes, thus suggesting that it could act as a natural milk preservative (9).

Whey proteins. Technological aspects

Overview

Milk is consumed in its 'natural' or non-fermented form only in the Western world and this is a relatively new practice. Before the Industrial Revolution, Europeans used milk to make yoghurt, cheese, cream, curd and whey. In general, fermentation is the basis of many traditional procedures that have been used over the centuries to preserve and at the same time improve the nutritional value and enhance the organoleptic properties of food and therefore its taste.

In particular, dairy products to which cultures have been added or that have been fermented have been part of the food culture of many populations for millenniums. It is believed that an angel revealed to Abraham a form of yoghurt; the particles found in 'kefir', a substance similar to yoghurt but with a more fluid consistency, are called "Prophet Mohamed's grains', because it is believed that Prophet Mohamed introduced their use (59). Yoghurt and kefir both represent paradigmatic examples of 'functional' foods, in other words, foods with peculiar biological effects (see above). In this context, whey has always been considered, in folk medicine, a panacea to alleviate a series of common ailments such as articular or gastrointestinal problems (9).

Icelandic people collect and store fermented whey in barrels to obtain the so-called syra (60). When necessary, this typical Icelandic product is drunk as such (or after having been diluted in water) or used to marinate or preserve meat and other food (9, 60). For centuries syra was the most popular Icelandic drink and it is believed that it replaced beer following a scarcity of cereals in this region (9, 60).

For a long time, however, whey was considered as a waste product of dairy processes and for this reason was at the most used as a raw material in the feeding of food-producing animals such as pigs.

Over the last decades, however, due to numerous scientific discoveries and to the development of food and pharmaceutical technologies, it has been possible to isolate and characterise a series of proteins from whey that all together or only in part form today the "active ingredient" of numerous proposed formulations – thanks to its proved functional properties described at least in part in the previous paragraph – in several "applications", from sport food supplementation to supportive care in patients with chronic-degenerative diseases (9).

In order for these formulations to be put on the market it is necessary to create systems capable of maintaining and then enhancing the biological properties attributed to whey proteins so they can be transferred as integrally as possible to consumers. Hence the crucial role played by the industrial production processes that should consider not only the quality and the quantity of raw materials but also the most common changes that milk goes through following various treatments before being transformed into the final formulation.

Impact of physical, chemical and enzymatic treatments on the biological properties of the protein fractions of milk, and in particular of the serum protein fraction

In addition to sterilization of the raw material, heat treatments (pasteurisation, thermization, UHT) can also lead to a series of structural changes of the milk's protein fraction, the entity of which varies depending on a series of parameters that are able to influence the interactions between proteins, and between proteins and other macromolecules (Tab. 2).

Therefore, a strong treatment can induce an irreversible transformation of the whey proteins from their native to the denatured state, improving the final product's nutritional profile. For example, it has been seen that the digestion of denatured whey proteins at duodenal level enhances the proteolytic activity of pancreatic enzymes and therefore, improves the absorption of their relative aminoacids or peptides. Furthermore, heat treatment allows the neutralisation of certain anti-tryptic agents present in milk. Among all the whey proteins, immunoglobulins are the most sensitive to heat treatment. In the case

Table 2 - Effects of the heating process on certain milk constituents

Modified substances	Possible modifications	Main consequences
Whey proteins	Structural changes, exposure to the -SH group with β–lactoglobulin, insolubilization	Formation of protein polymers, flocculation, inhibition of the formation of cream, cooked taste
Whey proteins/casein	Modifications of the air/liquid interface, formation of macromolecular complexes, reduction of solubilization	Formation of β–lactoglobulin/ κ–casein complexes, polymerization, reduction of nutritional value
Whey proteins/lactose	Maillard reaction between the aldehyde and amino groups, generation of coloured condensation products	Formation of reducing complexes, oxidation of fats, browning, reduction of nutritional value
Enzymes	Inactivation at temperatures > 60°C	Inhibition of catalytic activity
Lipids	Hydrolysis, oxidation, lactone formation	Release of fatty acids and peroxides with a nasty taste
Vitamins	Destruction (B_1, C and B_{12})	Decrease of nutritional value
Mineral salts	Shift of the Ca/P equilibrium towards the formation of insoluble complexes, modifications of the superficial status of micelles	Decrease of calcium bioavailability

of β–lactoglobulin, as already mentioned, the final effect is represented by a series of structural changes which also translate into the exposure of the free thiol to the solvent, which normally is not accessible as inside the protein structure; this favours the creation of disulphide bridges between the different chains, in other words between these and κ–casein, with a subsequent change in the micellar stability of the caseins with calcium. Unfortunately, an intense heat treatment may also favour the formation, within the protein, of covalent adducts that can cause a reduction of its digestibility and of the bioavailability of its relative hydrolysate. Examples are represented by glutamine and asparagine deamination (at temperatures higher than 120°C), cysteine desulfurization and serine dephosphorylation with formation of the lysine-alanine complex having minor nutritional value, and the protease-resistant isopeptide bonds between the lysine lateral amino group and the glutamine or asparagine amino groups (at neutral pH and at temperatures higher than 120°C). Finally, heat treatment increases the probability of reactions between the lactose aldehyde group and the whey protein amino groups with the generation of anomalous glycosylated complexes or even of oxidised derivatives (Maillard reaction), responsible for the end product's brown colour (61).

Chemical treatments that lead to the breakdown of the peptide bonds of the whey proteins through hydrolysis of the raw material under drastic conditions (HCl 6N at 110°C for 4-12 hours), are not applicable in a technological transformation.

Finally, enzymatic treatments are practically due to the presamic coagulation of milk by curd or rennet found in the abomasum of a young calf and that is specific for caseins and is at the basis of any cheese production process. In this process, rennet causes the hydrolysis of a specific peptide bond (Met_{105}-Phe_{106}) of κ-casein, whose removal leads to a destabilisation of the casein micelles that in turn cause the formation of a coagulus that is at the basis of cheese production (see also above glycomacropeptide).

The role of technologies in guaranteeing the quality of the end product

Whey proteins, as has often been mentioned, are produced starting from whey that in turn represents a by-product of the cheese making process. Normally whey is composed on average of 65 g/Kg of dry matter, of which 50 g is represented by lactose, 6 g by protein, 6 g by ash, 2 g by non-protein nitrogen compounds and by 0.5 g of fat. The industrial production process normally allows to obtain from whey some protein concentrates that, when necessary, can be further modified, for example depleted of their fat and/or lactose, due to additional passages through the column of ion exchange chromatography or when subjected to selective hydrolysis, through incubation with specific enzymes with the objective of obtaining partial or total hydrolysates (9). In this process all changes in temperature and pH should be reduced to a minimum in order to preserve as much as possible the 'native' protein conformation and therefore, their original biological activity (9). The end product is diversified in several preparations, such as whey protein concentrates (protein content 80-95%), whey proteins with a low content in lactose/delactosed and/or lipids/delipidised, demineralised whey proteins, proteins isolated from whey, whey protein hydrolysates, etc. (9). The "quality" of the end product depends not only on the raw material (milk or whey) but also and above all on the production process. Currently, excellent results can be achieved due to specific ultra-filtration and spray-dry techniques, that allow to obtain elevated protein performance/protein concentrations (up to 95%), with a transformation of the raw material in dehydrates or concentrates, finally obtaining a crystalline-like powder, which can easily be used in dif-

ferent food stuffs and/or pharmaceutical formulations, from sachets to tablets (9, 46).

It is just the use of ultra-filtration membranes that seems to best guarantee the nutritional quality of the whey proteins destined to human nutrition. In this process the whey proteins are withheld and therefore concentrated, whilst lactose and mineral salts pass through the filter. With the appropriate adjustments one can obtain whey protein solutions that are pure enough and extremely concentrated, up to 65% of dry weight. Performance can be further increased (up to 80%) through additional diafiltration that guarantees the elimination of almost all lactose and mineral salt residues. Clearly, the ultrafiltration procedure is not free of influence on the other micronutrients contained in the raw material. Therefore, whilst the vitamins that are strongly bound to the proteins (cobalamine and folic acid) are almost completely withheld, those belonging to the group B complex remain at a percentage of approximately 60%, versus a 15% of vitamin C (mainly lost because of its low thermal stability), compared to initial concentrations. For these reasons, sometimes it is necessary to add to the end product of whey protein formulations vitamins and/or mineral salts (2). Finally, the quality of the end product depends on the type of drying process. Pulverisation or spray-drying processes should be preferred compared to the traditional heated drum drying technique: unlike drum-drying, which is associated to a median loss of lysine of up to 40%, spray-drying markedly reduces nutritional losses to less than 10% or even less. Instead, aromatic aminoacids, such a tryptophan and tyrosine, are not influenced by dehydration treatments (61) (Fig. 4).

Clearly, the preparation process leads to a marked concentration of proteins and therefore, of their respective aminoacids. Hence, the levels of aminoacids known to be critical for GSH synthesis, for example, can show dozen-fold in rise on equal weight, compared to the starting raw materials (in this case cow's milk) (Fig. 5).

When cow's milk undergoes adequate ultracentrifugation and ultrafiltration processes, these prevent the denaturation of the whey proteins allowing to obtain various titres of concentrates, as for example, 57% of β–lactoglobulin, 24% of α–lactalbumin, 10% of serum albumin and 10% of immunoglobulins.

Potential clinical utility of whey protein formulations. Biochemical, physiological and pharmacological aspects

Overview

Whey proteins have a series of biological properties that make

Figure 4 - Schematic representation of the preparation process of whey proteins

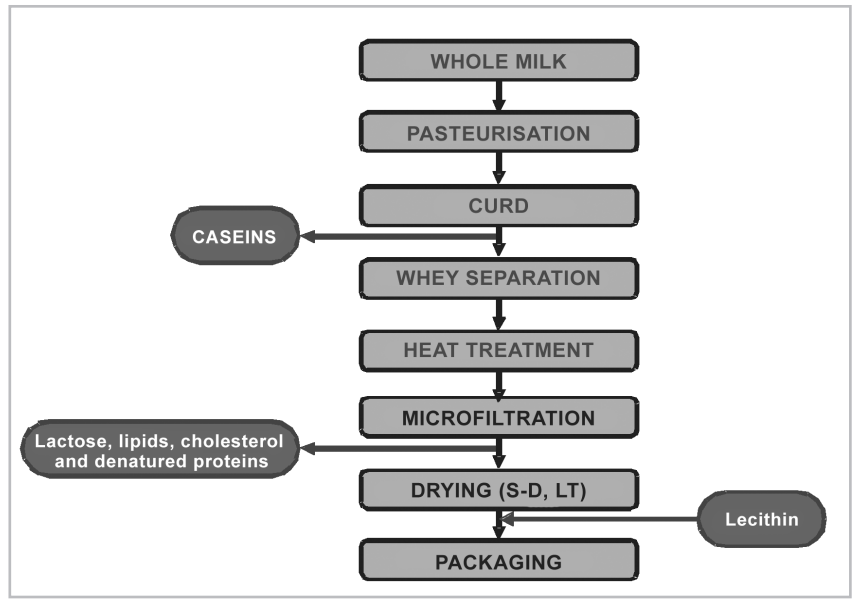
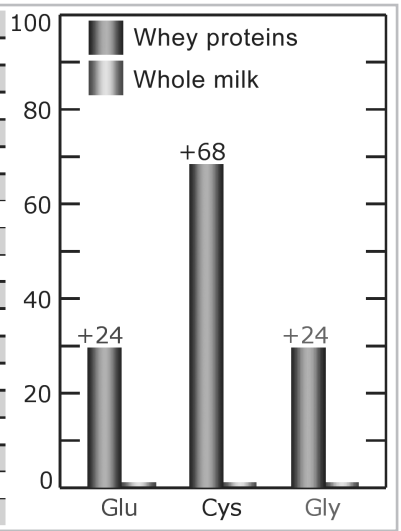


Figure 5 - Comparison between the aminoacid composition of a typical whey protein formulation (Prother™) and of the whole milk it originated from. Results are expressed as grams of aminoacids per grams of product. As shown in the histogram on the right, compared to whole milk, on equal weight (10 grams), the formula taken into account contains glutathione precursors, i.e. glutamic acid, cysteine and glycine, in quantities that are respectively 24-, 68- and 24-fold higher than in the milk they originate from.

Aminoacid	Whey proteins	Cow's whole milk
Glutamic acid	1.89	0.078
Cysteine	0.27	0.004
Glycine	0.17	0.007
Aspartic acid	1.19	0.027
Threonine	0.80	0.016
Serine	0.53	0.020
Alanine	0.53	0.012
Valine	0.63	0.023
Isoleucine	0.72	0.019
Leucine	1.12	0.035
Tyrosine	0.32	0.014
Phenylalanine	0.32	0.018
Histidine	0.21	0.009
Lysine	0.88	0.027
Arginine	0.22	0.011
Proline	0.78	0.037
Methionine	0.22	0.008
Tryptophan	0.19	0.005



them potentially useful in the nutrition of Humans and more in general in different clinical, physiological and pathological situations. In fact, they have been proposed with variable success as a support therapy or integration of conventional therapies in several clinical conditions (6), with particularly interesting results recorded in sports medicine, cardiovascular and metabolic disease, in oncology, in paediatrics, in gastroenterology etc. (9) (see Section II).

However, a transfer of know-how from research to the clinical practice is not as easy as may seem.

In fact the properties above described in paragraph 1 of this article are not 'automatically' transferable to Humans through all the formulations currently available on the market. In fact, these formulations, when obtained through technologies complying the highest qualitative standards possible and keeping whey proteins in a condition very similar to the "native" one, beyond the possible undesired contaminations (i.e. lactose), once ingested, in any case then have to deal with physiological absorption, digestion and broadly speaking, assimilation processes. These processes, as is well-known, are all more or less dramatically influenced by the raw material's and in this case the proteins' susceptibility to conformational changes (i.e. denaturation) and/or covalent changes (hydrolysis, reactions at the level of the aminoacid lateral chains etc.) able to influence *in vivo*, sometimes markedly, those effects that initially were attributed to an *in vitro* situation.

A striking example in this respect is represented by certain glycomacropeptide tryptic hydrolysates that have the ability to inhibit the enzyme (ACE) converting angiotensin I to angiotensin II (33). At the current state of knowledge it would be premature to propose today this whey protein or any of its specific sequences as a coadjuvant treatment of arterial hypertension. Firstly because proteins and peptides are cleaved at gastric and duodenal level into single aminoacids that lack the effect in question and then because even imagining the generation of small peptides through a partial proteolysis still capable of preserving their initial biological property (in this particular case the inhibition of an enzyme), it would still be necessary to demonstrate that these peptides are absorbed at intestinal level and reach their molecular target in pharmacokinetic and pharmacodynamic conditions

sufficient to guarantee a significant decrease of the arterial blood pressure in hypertensive subjects.
In other words, the simple application of clinical and pharmacological principles to physiology suggests that as a general rule, whey proteins can be validly used in human nutrition only when their effects can be recorded *in vivo* despite their submission as proteins to denaturation and hydrolysis at gastrointestinal level.
Therefore, by strictly applying this principle, whey proteins can be used as food supplements as, despite being hydrolysed, they are in any case capable of providing aminoacids variably indispensable for those functions that are normally ascribed to the proteins of the human body, perhaps with some advantages, that have yet to be documented, compared to other similar sources of food protein (for example, caseins), because of its particular composition (for example, a higher level of aminoacids compared to others).
Corollary of this statement is the fact that in order to obtain the desired effect, the above mentioned formulations should be administered in doses that are proportional to the subject's needs and that depend on various factors, physiological (i.e. age, gender, body weight, growth, pregnancy, breastfeeding) and/or pathological factors (i.e. cystic fibrosis, neoplasias) and that in any case may be expressed, at least at the beginning, in terms of grams of product per kilo of body weight.
In fact, numerous studies carried out over the last decades both in animals and in Humans have widely documented that in actual fact when whey proteins are used following the above mentioned principles and in generous dosages (tens of grams a day), these can be useful as nutritional supplementation.
However, today it seems more and more evident that despite the whey proteins' limitation due to their intrinsic susceptibility to denaturation and hydrolysis processes at gastrointestinal level, they can induce *in vivo* a series of biological effects that do not depend on a "ponderal" type of effect, in other words when they are used as a noble food source of aminoacids, but rather on their ability to increase in the body, this time at markedly lower doses than those necessary exclusively for nutritional purposes, the bioavailability of chemical mediators involved in the control of important cell functions. In this respect, it seems paradigmatic that whey proteins are used as precursors of glutathione biosynthesis that thus justifies their use as antioxidants.
On the basis of these observations, a description of the bases of potential clinical applications of whey proteins in the human nutrition will be dealt in this article discussing separately the available evidence relative to the use of whey proteins as a source of aminoacids in nutrition and evidence of their ability to modulate important physiological phenomena. The borderline between these two different types of application - not always clearly distinguishable - is represented above all by their dosage, higher in the first case, lower in the second one.

Whey proteins as "conventional" nutritional supplementation

Whey proteins have a 3-fold higher level of branched aminoacids in comparison to those of caseins and therefore, are among those proteins with the highest nutritional value used in human nutrition, as demonstrated by their high biological value (104) and their effective protein dose (3.2) (62).
Whey protein intake is accompanied by a globally "anabolizing" effect that can be ascribed not only to a stimulation of the biosynthesis processes but also to an inhibition or slowing down of the degradation processes (63).
The proteo-anabolic effect is firstly due to the high concentrations of branched aminoacids, and in particular to leucine, that is metabolised mainly in peripheral

muscles rather than in the liver as an energetic substrate through a mechanism attributable to the m-TOR kinase-independent ancestral pathway (64-66).
Furthermore, when administered to adults, whey proteins generate a high, rapid and transitory peak of plasmatic aminoacids that significantly stimulate (+68%) the protein synthesis without modifying the protein catabolism. In contrast, the administration of caseins induces a rise of plasma aminoacid levels that is less intense and more prolonged over time with a subsequently minor effect on protein synthesis (-31%).
Therefore, the different behaviour of these two classes of proteins may depend on their different digestion and absorption rate as confirmed by studies carried out by adding other macronutrients, including carbohydrates and lipids, to whey proteins. For this reason, whey proteins are considered "fast proteins" in contrast with caseins, considered "slow proteins" (63).
Moreover cysteine and glutamine that are sufficiently represented in all whey proteins, could contribute to the anabolizing effect analysed in this article. In fact, in the liver cysteine can be transformed into sulphur hydrogen ions and H^+ ions that tend to bind to the carbonate-hydrogen ion (HCO_3^-). Therefore, HCO_3^- becomes less available and enters the urea cycle; consequently, the excess of ammonia is eliminated through glutamine, that is considered by the cell as an anabolic stimulus. This phenomenon seems to help to explain the effect of certain whey proteins on the increase in body weight observed in weakened patients (*Chiarla and Giovannini, unpublished data*).
Clearly, hormonal mechanisms cannot be excluded. In fact, even though the percentage of aminoacids capable of stimulating an insulin response is similar in both proteins (63), whey proteins exert a less marked effect on glucagon; therefore, biosynthesis mechanisms prevail over degradation also through this pathway (67).
Also compared to single chain aminoacid solutions, whey proteins that similarly to lactoferrin are not denatured at acidic pH and resist the action of gastric chyme, guarantee a better absorption and a greater bioavailability of their end products of hydrolysis (68).
The ability to stimulate biosynthesis to the detriment of the catabolism - that can also contribute to explaining the more favourable effect of whey proteins on the sense of satiety than compared to caseins (69) – makes whey proteins themselves potentially useful in several clinical conditions, for example, in sarcopenia in the elderly, in whom the leucine equilibrium seems better than that following intake of slow proteins, even when compared to younger subjects (62-67, 69, 70). Other promising fields of application, in this respect, are sepsis, skeletal trauma, HIV infection etc. (67, 69) which will be mentioned in section II.

Whey proteins as "physiological modulators"

Whey proteins represent the prototype of a recently identified class of chemical agents, the so-called "physiological modulators" (71). This definition includes all the agents potentially able, through a fine metabolic regulation, to prevent or slow down the appearance of or influence in a favourable way the evolution of a series of pathologies and in general (but not only) degenerative or chronic disorders, often highly invalidating for the affected subject and onerous for the community in which the subject lives (71, 72).
In other words, with physiological modulation we mean when we use natural agents able to regulate in a harmonious way specific biochemical events responsible for vital cell functions (i.e. maintenance of membrane potential, signal transduction, metabolic cycles etc.), nearly always "derailed" – even though not exclu-

sively – due to the excessive level of chemical species with an oxidant action (73). In fact, numerous biological activities have often been attributed to whey protein formulations - antioxidant, detoxicating, immunomodulatory, anti-inflammatory, anti-hypertensive, lipid lowering activities etc. – that in turn depend on the properties of the single fractions that compose them (β–lactoglobulin, α–lactalbumin, immunoglobulins, glycomacropeptide, serum albumin, lactoferrin, lactoperoxidase) (see pages 9-14).

Antioxidant action

Whey protein based formulations have a potent antioxidant action that can efficiently limit the undesired effects of the so-called oxidative stress.

Oxidative stress is a 'transversal' pathological condition, common to many diseases, characterized by an impairment of the physiological equilibrium between production and elimination, by the antioxidant defence systems, of a series of chemical species with oxidant action such as, for example, oxygen free radicals (74).

The increased production of oxidising chemical species and/or the reduced efficiency of antioxidant systems leads to the generation in cells of a series of oxidation by-products such as hydroperoxides (ROOH) (see also page 15) that are released into the extracellular matrix and therefore, into the microcirculation (75). Here, under conditions of cell suffering (hypoxia→acidosis), the hydroperoxides can be transformed into free radicals that are extremely harmful due to the catalytic action exerted by iron (which in the meantime has been released by transferrin due to a conformational change induced by microacidosis) (75, 76). Usually, an excess of free radicals is buffered both by the plasma antioxidant barrier (composed of albumin, uric acid, bilirubin, ascorbic acid, tocopherols, reduced thiols etc.) and the intracellular ubiquitous enzyme glutathione peroxidase (GPx) (77).

However, since oxidative stress does not lead to a characteristic clinical picture but is often masked by symptoms and related diseases (more than 100 known to date), when there is a real suspect of disease, this must always be confirmed by specific laboratory investigations (76, 77). Among these, the following are emerging as being particularly useful in clinical practice as based on the simple principle of photometry: the determination of the total oxidizing capacity of serum/plasma on N,N-diethylparaphenylendiamine (d-ROMs test, with normal values ranging between 250 and 300 CARR U), the evaluation of the total serum/plasma antioxidant capacity in terms of iron reducing activity (BAP test, optimal value > 2200 micromol/L), the dosage of glutathione (GSH) and the dosage of GPx activity, these last two elements on formed elements of the blood (in general lymphocytes and erythrocytes, respectively) (77, 78).

We believe that no supplement with antioxidant action should be administered to a subject whose oxidative balance has not been evaluated (77). In fact, some formulations on the market can be inefficient or even harmful when taken without a real need and this need can be identified exclusively using objective measures, in other words laboratory tests (77).

On the basis of this, the antioxidant action of whey proteins could be imputed to four main mechanisms: the shock adsorber action, the chelation of transition metals, the direct inactivation of peroxides and, above all, the stimulation of endogenous GSH synthesis.

The shock-adsorber action is a scavenger type antioxidant action that all the proteins, and in particular albumins, are endowed with (79). By analogy with hematic serum albumin, also milk serum albumin, due to the lateral chains of its numerous aminoacids, can contrast, already in the intestinal lumen, the potentially harmful action of an excess of free radicals and this is due to its ability to donate reducing equivalents to the oxidizing species.

More in general, all whey proteins, being quite rich in cysteine residues, once assimilated can make their own thiol groups available at plasma level and these, in their reduced state (-SH), form an essential part of the antioxidant barrier against free radicals (77). Today plasma levels of thiols can be determined photometrically through a specific test (-SHp test, with normal values between 450 and 650 micromoles/L) (80). We preliminarily checked the validity of this analytical approach by monitoring the level of thiols before and after administration of a whey protein based formulation (*unpublished data*).

The chelating action on transition metals is mainly due to lactoferrin that by blocking iron prevents it from catalysing the conversion of hydroperoxides into extremely reactive free radicals, such as alkoxyl and peroxyl radicals (see also pag. 15) (54, 55).

The inactivating action on peroxides is due to lactoperoxidase that, as already mentioned, transforms agents that are still potentially oxidising (and precisely, peroxides) into relatively innocuous products (see also pag. 15.) (35).

Finally, the stimulation of endogenous GSH synthesis is practically linked to all the whey proteins (6).

As is well-known, GSH is a tripeptide composed of glutamic acid, cysteine and glycine (γ-L-glutamyl-L-cysteinyl-glycine) (Fig. 6); compared to common polypeptides, its peculiarity consists in the fact that the first of its two carbamidic bonds (glu-cys) uses the carboxylic group of the lateral chain and not the one bound to the α-carbon of glutamic acid (35).

Glutathione performs a series of functions mainly due to its nucleophilic properties (Tab. 3).

The role of glutathione transferase (see below, detoxification properties of whey proteins) and of GPx, an ubiquitous intracellular enzymatic selenoprotein, stand out from all of GSH's known and studied biological roles (81). Glutathione peroxi-

Figure 6 - Glutathione structure in its reduced (GSH) and oxidised (GSSG) form

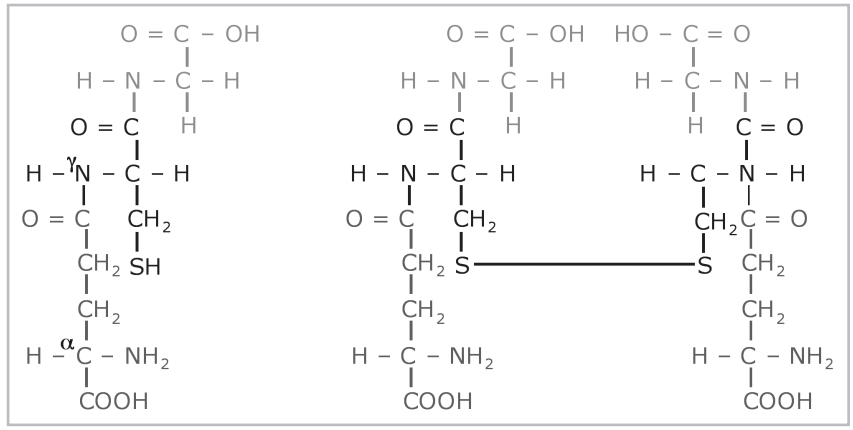


Table 3 - Main glutathione functions.

1. Directly dependent on its oxidant-reducing properties:
   - it is indispensable for glutathione-peroxidase (GPx) activity
   - it can act as a direct scavenger of free radicals
   - it maintains vitamins C and E in their reduced and active form
   - it can modulate the function of proteins (e.g. enzymes) having thiol groups
   - it can take part in phase I detoxification reactions
   - it can reduce transition metals
   - it can prevent the formation of methemoglobin.

2. Dependent on its "conjugating" ability:
   - it takes part in phase II detoxification reactions
   - it can form undesired "adducts"

3. Miscellaneous
   - it maintains and transports cysteine in a non-toxic form
   - it intervenes indirectly in the phospholipid and homocysteine metabolism
   - it intervenes in the arachidonic acid metabolism enabling leukotriene T synthesis
   - it can act as an NO carrier and more in general as a modulator.

dase catalyses the breakdown of peroxides to alcohols by subtracting a couple of reducing equivalents (hydrogen atoms) to two molecules of reduced glutathione (GSH) that is released in its oxidised form (GS–SG) (81). Glutathione peroxidase acts both on lipoperoxides and hydroperoxides and therefore, also on hydrogen peroxide (81). Therefore, it plays a key role in the antioxidant defence system.

Glutathione is synthesised right from the beginning especially, but not only, in hepatic cells through 2 ATP-dependent reactions starting from 3 constituent aminoacids (82). In the first reaction catalysed by glutamate-cysteine ligase [E.C. 6.3.2.2], once called γ-glutamylcysteine synthethase (GCS), a cysteine residue is added to a glutamic acid residue leading to the generation of the dipeptide γ-L-glutamyl-L-cysteine (82, 83). In the second reaction, catalysed by glutathione synthethase [E.C. 6.3.2.3], a glycine residue is added to the dipeptide that is formed leading to the formation of γ-L-glutamyl-L-cysteinyl-glycine, in other words GSH (82, 83).

The intracellular levels of GSH are normally elevated, in the order of mmol per liter, according to the various districts and metabolic needs, thus justifying its important biological role (Tab. 4).

One of the peculiarities of GSH is represented by the fact that it must be synthesised inside cells in which it is needed. It is possible to bypass this obstacle by administering intravenously the previously formed tripeptide, but in this case it is not homogeneously distributed within organs and tissues but is more or less rapidly sequestered by the liver and kidneys. Therefore, in cases of documented GSH deficiency and therefore, in cases where a specific supplementation is need, it will be necessary to deal with the singular and still not well-known pharmacokinetics of this molecule.

In fact, at least in humans, the oral administration of GSH does not seem to cause an increase in the intracellular levels of this substance: on the contrary, a study carried out on volunteers clearly demonstrated that for oral boluses of up to 3 g, an increase in the systemic bioavailability of the tripeptide was not observed and this was due to the hydrolytic phenomena that it faces both at gastrointestinal (protease) and hepatic level (γ–glutamyltransferase) (85).

Awaiting further and more enlightening studies, one thing seems certain: endogenous GSH synthesis is a process that is markedly limited by cysteine availability (83, 86, 87).

Therefore, a valid alternative to the oral administration of GSH to stimulate the endogenous synthesis of the tripeptide is to increase the cysteine bioavailability within tissues through the administration of "donors" of this aminoacid.

There are two possibilities.

The first one is to administer N-acetylcysteine or oxothiazolidine carboxylate: it has been observed that with this approach one can favour endogenous GSH synthesis and protect against cell death induced by oxidative insult (88–92).

The second one is to administer protein-rich foods normally rich in cysteine, in other words whey proteins. In fact, GSH biosynthesis strictly depends on the concentration of its 3 aminoacid precursors (glutamic acid, cysteine and glycine) and competes with albumin synthesis for available cysteine (93, 94). On the basis of the Michaelis and Menten constants (K_M) – 0.003 *vs.* 0.35 mmol/L – it has been deduced that albumin biosynthesis is activated at cysteine concentrations 166-fold lower than those required for GSH synthesis. Therefore, GSH synthesis, under conditions of reduced cysteine availability, will be

Table 4 - Levels of glutathione in certain body districts (84)

Organ/cell/districts	Concentration
Crystalline	~10 mM
Liver	5–7 mM
Lung, kidneys and heart	2–3 mM
Erythrocytes	~2 mM
Plasma	<0.05 mM

more compromised in comparison to albumin synthesis (94). Since an important source of cysteine is in fact represented by whey proteins, that have up to 6 times higher levels of cysteine than compared to those recorded in casein (Tab. 5) with an even more striking difference when the content of aminoacids is expressed in mmol/kg (Tab. 6), it is acceptable that these enhance the hepatic synthesis of GSH under conditions of oxidative stress due to their high concentrations of cysteine, exceeding the limiting threshold for the hepatic protein synthesis (94-97).

In fact, the administration of whey proteins has proved to be effectively able to increase tissue GSH levels (98, 99). On the other hand, the cystine (cys-cys) that is reduced to two molecules of cysteine, that can be used in GSH synthesis (36), is present in significant quantities in whey proteins (100) (Tab. 1).

Therefore, it has been observed that the consumption of these proteins is associated with an increase of GSH plasma concentrations in Humans (101, 102) and can also reduce the incidence of breast and colon cancer in rats (98, 103–106).

In one experience our group evaluated comparatively, compared to casein the effect of whey protein administration on glutathione levels, and therefore on oxidative stress in

Table 5 - Percentage of some aminoacids in whey proteins and casein

Aminoacids	Whey proteins	Casein
Glutamic acid	10.9	23.7
Cysteine	2.9	0.5
Glycine	2.0	1.8
Alanine	4.6	3.0
Arginine	2.8	3.6
Asparagine	6.8	7.1
Histidine	1.9	2.9
Isoleucine	5.0	5.1
Leucine	12.3	9.2
Lysine	9.7	8.0
Methionine	2.0	2.9

Table 6 - Aminoacid content in whey proteins and casein (mmol/kg).

Aminoacids	Whey proteins	Casein
Glutamic acid	1.15	1.41
Cysteine	0.50	0.05
Glycine	0.22	0.20
Aspartic acid	1.04	0.56
Threonine	0.40	0.32
Serine	0.39	0.50
Alanine	0.55	0.32
Valine	0.46	0.49
Isoleucine	0.46	0.37
Leucine	1.02	0.66
Tyrosine	0.24	0.25
Phenylalanine	0.23	0.28
Histidine	0.11	0.15
Lysine	0.74	0.49
Arginine	0.18	0.26
Proline	0.35	0.88
Methionine	0.14	0.18
Tryptophan	0.08	0.03

rats subjected experimentally to carbon tetrachloride (CCl_4) (a powerful oxidant agent) intoxication (107). Compared to treatment with casein, whey protein supplementation was accompanied by a significant increase in hepatic levels of glutathione (total and oxidized) and also by a significant reduction of peroxidation levels (evaluated using the TBARs test) (Tab. 7).

In conclusion, the evidence here reported suggests that whey proteins exert a potent antioxidant effect through diversified mechanisms and among them the GSH precursors seems to play an important role.

Detoxifying action

Whey protein based formulations are well-known for their detoxicating property. In fact by favouring GSH synthesis, they activate glutathione-transferase that catalyses phase II detoxification reactions (generation of hydrophilic xenobiotic conjugates that can be eliminated more easily with bile and/or urine) (6, 82, 83, 108, 109). Furthermore, it seems that α–lactalbumin is able to chelate transition metals, thus contributing to the prevention of tissue damage due to mercury and lead (6).

Immunomodulatory action

Whey proteins can exert an immunomodulatory action through different mechanisms. *In vitro*, for example, it has been observed that whey proteins can boost the immune defences inducing an increase in GSH synthesis inside lymphocytes (108, 110). Similar results have been observed *in vivo*, in several animal models. In the guinea pig, the administration of whey proteins as a source of protein was associated at 2 weeks with a marked increase in the number of lymphocytes in the spleen that was 5-fold higher than in the control group treated with casein (95). In the same way, the administration of buthionine sulforximine, an inhibitor of glutathione biosynthesis, annulled the immunostimulant effect of whey proteins observed in Rodents (95, 111, 112). The action of whey proteins on the immune system was even more evident in clonal expansion experiments carried out on lymphocytes stimulated by antigens. It was observed that their proliferation requires adequate intracellular levels of GSH (62), that in turn, at least in part, depend on the presence of cysteine (113). Since whey proteins contain approximately 8-fold more cysteine than casein, it has been hypothesised that the immunostimulant action observed

Table 7 - Effects of carbon tetrachloride intoxication on the production of plasma and hepatic reduced and oxidised glutathione.

	Casein group		Whey protein group	
Treatment	C-CTR control arm	C-CCl_4	P-CTR	P-CCl_4
Hepatic GSH nmol/mg	2043 ± 258.0	2196 ± 323.2	2431 ± 456.7	4994 ± 652.6 (a)
Hepatic GSSG nmol/mg	30.73 ± 5.399	77.57 ± 10.22 (e)	27.95 ± 5.250	231.1 ± 90.65 (b)
Plasmatic GSH nmol/mL	1528 ± 86.36 (f)	1088 ± 48.35	1188 ± 40.04	1368 ± 69.56 (c)
Plasmatic GSH nmol/mL	6.388 ± 1.039	9.227 ± 1.629	7.565 ± 0.8313	13.18± 3.006 (d)

GSH: reduced glutathione; GSSG: oxidised glutathione; C-CTR: rats treated with casein and not intoxicated with carbon tetrachloride; C-CCl_4: rats treated with casein and intoxicated with carbon tetrachloride; P-CTR: rats treated with whey proteins and not intoxicated with carbon tetrachloride; P-CCl_4: rats treated with whey proteins and intoxicated with carbon tetrachloride; a) $p<0.001$ vs. C-CTR; $p<0.01$ vs. C-CCl_4; $p<0.001$ vs. P-CTR; b) $p<0.05$ vs. C-CTR e vs. C-CCl_4; c) $p<0.001$ vs. C-CTR; $p<0.01$ vs. C-CCl_4; $p<0.001$ vs. P-CTR; d) $p<0.05$ vs. C-CTR; $p<0.01$ e vs. P-CTR; e) $p<0.01$ vs. C-CTR; f) $p<0.01$ vs. C-CCl_4 e vs. P-CTR.

depends on an increase of splenic GSH levels (107). Together with this indirect effect, whey proteins, can in any case exert a direct immunomodulatory action (6, 9), probably through lactoferrin. Lactoferrin, according to a series of studies, is capable of activating Natural Killer lymphocytes, stimulating neutrophils, favouring CSF (colony stimulating factor) activity and potentiating the cytotoxic effect of macrophages (114-117). Moreover, by subtracting the iron from growing microorganisms, it can also exert an antiviral, antibacterial and antimycotic effect (118). Finally, through a direct interaction with lipopolysaccharide, it can show antibiotic properties on gram-negative bacteria (119).

Anti-inflammatory action

An anti-inflammatory action, probably due to lactoferrin, has also been attributed to whey proteins. In fact, in a study carried out on rats this protein proved to have the ability to regulate Tumor Necrosis Factor (TNF) and interleukin-6 levels, thus reducing inflammation and ultimately mortality in animal models (120).

Anti-hypertensive action

Anti-hypertensive peptides have been isolated from bovine β-lactoglobulin and this suggests that whey proteins may be useful in reducing blood pressure values. These peptides are associated with a significant inhibitory activity on ACE enzyme, subsequently blocking the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor agent (121, 122).

Lipid lowering action

It seems that β-lactoglobulin has a potent cholesterol lowering action (6). This effect, as emerges from studies carried out on animals, seems to originate from the ability of the above mentioned protein to interfere with the micellation phase of edible fatty acids in the intestine with a subsequent slowing down of cholesterol absorption (123).

Antineoplastic action

In preliminary studies (93, 124, 125) carried out on mice treated with Dimethylhydrazine-dihydrochloride (DMH) to induce colon tumours after 28 weeks of administration of feed containing casein or whey protein or purine (20 g/100g) a marked decrease was observed in the incidence and area of tumours in the group that was fed with whey proteins and casein in comparison to the group fed with purine, particularly marked in the whey protein group. These positive results were confirmed in subsequent studies on breast cancer both *in vitro* (cell cultures) (63, 126-128) and *in vivo* (again in the rat) (62). Studies carried out on cell cultures show that when whey proteins are used as a medium for immortalised cells of the MCF-7 line and reactive to the oestrogen and for the prostate cancer cell line, they significantly reduce cellular growth (126). Some whey protein fractions and in particular β-lactoglobulin, and serum albumin, inhibit neoplastic growth in the MCF-7 cell line (63) and show a potent antimutagenic effect against 4-nitroquinoline-L-oxide in the epithelial cell line of Chinese hamsters (128).

Conclusive considerations

Milk is the oldest functional food that has been available to mammalians. Since birth it provides essential nourishment and is absolutely unique for its numerous functional merits, as described above. On this solid basis, over the past decades numerous formulations have been developed containing one or more whey proteins, each of them with its specific characteristics, and these formulations have been used in various clinical applications as supportive care to conventional therapies.

Whey proteins that in the past represented a waste product of the treatment process of milk, due to the evolution of technological

processes have become part of the possible treatments of malnutrition and sarcopenia. In particular, due to their elevated content in branched aminoacids and like leucine, they act as an important driver of protidosynthesis. At the same time its 4-fold higher content in cysteine and glutamine favours the production of glutathione enhancing an antioxidant response in situations in which oxidative stress may play an important role in physiopathologic dynamics, as during chemo and radiotherapy. Great attention should be paid when choosing supplementation according to the type of technological processes used that lead to synthesis favouring the use of sources of ultracentrifuged and ultrafiltered whey proteins rather than denatured and coagulated ones.

Adjustments, such as the elimination of lipids, have extended their use in clinical nutrition also to hypocaloric diets, whilst other adjustments, such as the elimination of lactose, have made their administration possible also to subjects intolerant to this disaccharide (i.e. Prother™).

Moreover whey proteins, thanks to their chemical-physical properties, have proved to be a useful 'vehicle' for other biologically active substances, they are already on the market in docosoesanoic acid enriched protein bars (Prother™ Metabolic 250) or granules enriched with super oxide dismutase (Prother™ SOD) or arginine (Prother™ FC), and further research continues on new possible clinical applications, such as malnutrition in cancer patients, neurodegenerative diseases, cystic fibrosis etc., conditions that are all characterised by a profound alteration in the oxidative balance.

By using low doses (10-20 g/day) these new formulations are in line with the modern concept that antioxidants are like 'physiological modulators', i.e. substances capable of contrasting even in small doses (precisely, physiological doses) the undesired effects of free radicals, due to their ability to interact catalytically with the key substrates of the oxidative metabolism (129).

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