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Valorisation of Cheese Whey, a By-Product from the Dairy Industry

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1. Introduction

Whey is the by-product of cheese or casein production, it is of relative importance in the dairy industry due to the large volumes produced and the nutritional composition. Worldwide whey production is estimated at around 180 to 190×10⁶ ton/year; of this amount only 50% is processed [1]. Approximately 50% of worldwide cheese-whey (CW) production is treated and transformed into various food and feed products. About half of this amount is used directly in liquid form, 30% as powdered cheese-whey, 15% as lactose and its byproducts and the rest as cheese whey- protein concentrates [2].

A total of 40×10⁶ tons/year of whey is produced in the European Union [3]; the annual surplus of whey is 13×10⁶ tons, containing about 619,250 tons of lactose. Nowadays this surplus is not utilized for further production of lactose; consequently, whey disposal represents a serious problem from both an economical and an environmental point of view. On the contrary, recovery of whey components and/or use of whey as fermentation medium may be advantageous not only for the environment but also for a sustainable economy [4,5].

Whey contains more than half of the solids present in the original whole milk, including whey proteins (20% of the total protein) and most of the lactose, water-soluble vitamins and minerals. Consequently, whey can be considered a valuable by-product with several applications in the food and pharmaceutical industries.

From a valorization point of view, two different options in CW management can be considered: the first one is based on the application of technologies to recover valuable compounds such as proteins and lactose. Currently, valorization processes applied to CW constitute the preferential option to treat this by-product, only exceeded by the production of powdered CW. The second option relies on the application of fermentation processes to obtain value

added products [6] such as: organic acids (e.g. lactic, succinic and propionic), single cell proteins and oils, biopolymers (enzymes, polyhydroxyalkanoates, exopolysaccharides) and bacteriocins. Sometimes whey permeate, obtained from ultrafiltration step, has been used as fermentation medium; in this case, both the management options are applied.

The ultrafiltration process produces a whey permeate rich in lactose (about 80% of the original lactose in milk) new technologies have been developed (using nanofiltration or reverse osmosis) for concentration of the lactose which can be applied in the sweet industry or in pharmaceutical fermentation procedures [7]. In addition to lactose, whey permeate containing other nutrients essential for microbial growth; so the possibility to use it as a fermentation medium to obtain high value products represents an interesting opportunity [4] which must not be neglected. Moreover, whey permeate is an attractive source of oligosaccharides for potential application in human nutrition [8].

Among the different possibility of whey valorization, reported in Figure 1, individual whey protein purification and application of fermentation technology on whey permeate will be discussed.

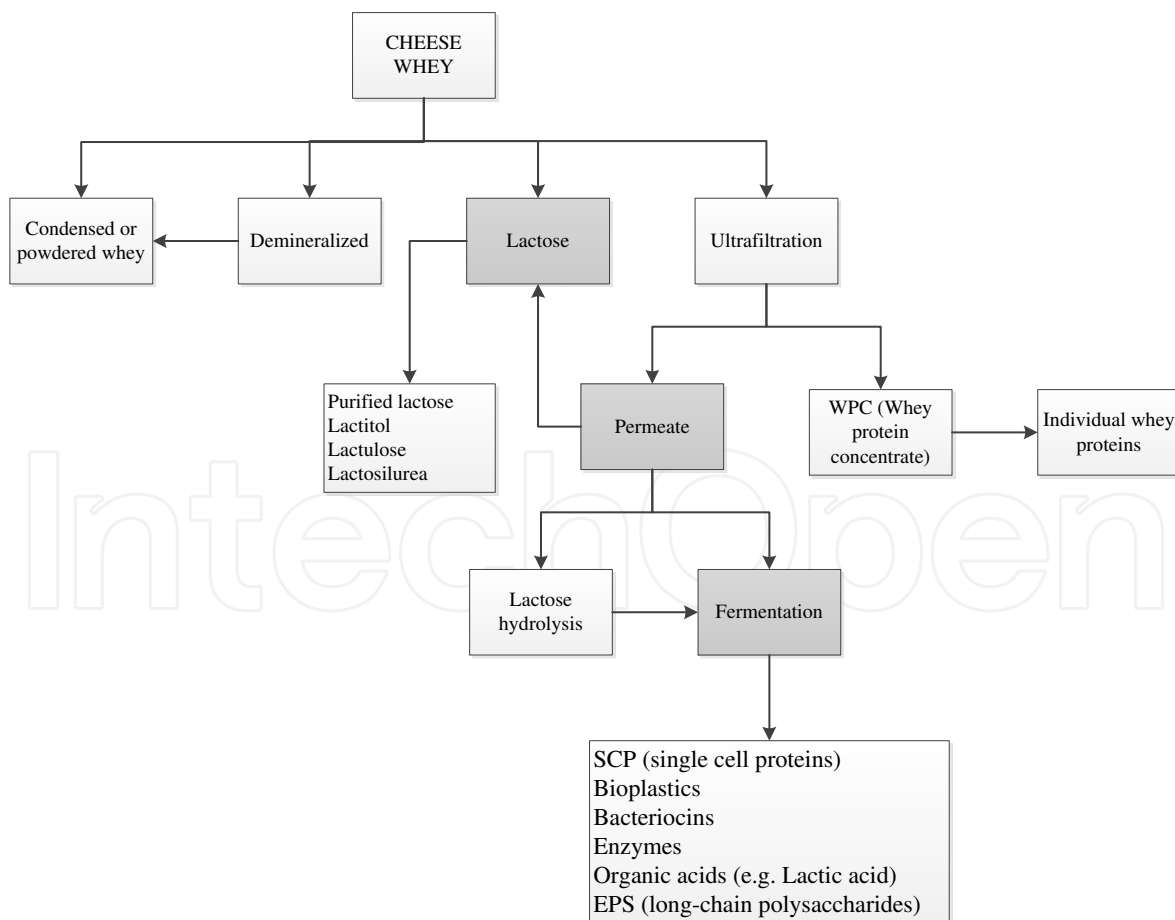


Figure 1. Scheme of current possibility of whey valorization.

2. Whey proteins

Today, whey is a popular dietary protein supplement purported to provide antimicrobial activity, immune modulation, and to prevent cardiovascular disease and osteoporosis. In addition, whey has the ability to act as an antioxidant, antihypertensive, antitumoral, hypolipidemic, antiviral, antibacterial, and chelating agent [9].

Advances in processing technology, including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange, have resulted in development of several different finished whey products: whey protein concentrates (WPC) containing between 50 - 85% protein on a dry basis, whey protein isolate (WPI) containing between 90-98% protein and very small amounts of lactose and fat, reduced lactose whey, demineralized whey and hydrolyzed whey [10]. Each whey product varies in the amount of protein, carbohydrates, immunoglobulins, lactose, minerals, and fat in the finished product [9].

Nowadays whey ultrafiltration (UF) and diafiltration (DF) are standard operations in the dairy industry that allow protein recovery without significant loss of their functional properties and with a low salt content, making it suitable for human consumption [11,12].

The recovery of proteins by UF and DF represents the first step in whey valorisation. UF has been used in the dairy industry to produce WPC, because this technology allows the selective concentration of the proteins in relation to the retention of protein and selective permeation of lactose, minerals, water and compounds of low molecular weight [10]. DF is used for the production of WPC with a high protein content and purification grade. WPC, which are obtained by whey UF and DF are available in great variety according to protein content and functional properties [7,9].

Whey proteins have a high nutritional value, due to the high content of essential amino acids, especially sulfur-containing ones [13]. They are high quality proteins with a protein efficiency ratio (PER) of 3.4, higher than casein (2.8) and similar to egg albumin [14].

Moreover whey proteins have functional properties (e.g. high solubility, water absorption, gelatinization and emulsifying capacities) essential in food application [15].

Thanks to the excellent nutritional and functional properties, commercial value of WPC is from 3 to 40 times greater than that of whey powder [1].

Moreover, the possibility of a different use of whey proteins is taken into account to obtain the so-called "functional foods". Individual whey proteins have their own unique nutritional, functional and biological characteristics that are unrealised in whey protein concentrates. WPC micro-, submicro- and nanocapsules have been applied in the encapsulation of bioactives of interest in the development of novel functional foods (e.g. the antioxidant β -carotene) [16].

The major components among whey proteins are β -lactoglobulin (β -LG), α -lactalbumin (α -LA), bovine serum albumin (BSA) and immunoglobulin (IG), representing 50%, 20%, 10% and 10% of the whey fraction, respectively. All of these major proteins, except for BSA and

IG, are synthesized by epithelial cells in the mammary gland. Besides these, whey contains also numerous minor proteins, called low abundance proteins, such as lactoferrin (LF), lactoperoxidase (LP), proteose peptone (PP), osteopontin (OPN), lysozyme (LZ), among others; LF and LP are the most abundant minor proteins [17,18].

The concentration of whey proteins depends on the type of whey (acid or sweet), the source of milk (bovine, caprine or ovine), the time of the year, the type of feed, the stage of lactation, and the quality of processing. Whey proteins are globular molecules with a substantial content of α -helix motifs, in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a fairly balanced way along their polypeptide chains [19]. Major characteristics of whey proteins are summarized in Table 1.

Protein	Molecular mass (Kg/mol)	Isoelectric point	Concentration (g/l)	Number of amino acids
β -LG	18	5.4	3.2	162
α -LA	14	4.4	1.2	123
Immunoglobulin G	150	5 ÷ 8	0.7	*
BSA	66	5.1	0.4	582
LF	77	7.9	0.1	700
LP	78	9.6	0.03	612

*variable values

Table 1. Major characteristics of whey proteins [20,18,21].

The three major forms in which whey protein products are available, such as concentrates (WPC), isolates (WPI), and hydrolysates (WPH), have limited acceptance by the food processing industry because of the lack of consistency in the gross composition and functionality. Whereas each whey protein has unique attributes for nutritional, biological and food ingredient applications; otherwise individual milk proteins exhibit better functionality than in their native protein mixtures [22,9].

As a matter of fact whey represents a rich mixture of proteins with wide-ranging chemical, physical and functional properties. These proteins play an important role in nutrition and, in a number of instances, also appear to have specific physiological actions, such as: ability to bind metals, functions related to the immune or digestive systems, source of essential amino acids also branched chain amino acids (leucine, isoleucine, and valine) which are thought to play a role as metabolic regulators in protein and glucose homeostasis, involvement in lipid metabolism, etc. [23,24].

By this way these bioactive compounds are able to reduce disease risk and/or to prevent disease development and have been reported to have utility in many different applications ranging from effects on bone, muscle, blood, brain, pancreas, immune, cancer, infection, me-

tabolism, wound healing, learning, and aging [25]. Moreover, these proteins, once partially digested, serve as a source of bioactive peptides with further physiological activities.

All of these biological and physiological activities offer to the food industry several opportunities; in particular they provide the basis for development of valuable whey protein-based food ingredients targeted to the functional food sector [26].

The term functional food was first introduced in Japan in 1980's: it refers to healthy food similar in appearance to conventional food, consumed as part of a usual diet, and claimed to have physiological benefits like health-promoting or disease-preventing properties beyond the basic function of supplying nutrients. Various definitions of functional food, proposed by authorities, academic bodies and industries, exist worldwide; the difficulty to give a unique definition depends on the fact that foods consumed perform some functions in one way or the other, in particular depending on the state of health of the consumer. Moreover various different terms, listed in Table 2, are sometimes linked or interchanged with the term functional foods [13,27,28]. Considering on one hand the ambiguity among these definitions and on the other the wide set of functions of whey proteins and related peptides, it is quite difficult to establish whether they fall within a definition or into another.

Terms	Definition
Bioactive compounds:	they are chemical compounds derived from a plant, animal, or marine source, that exert the desired health/wellness benefit.
Dietary supplements:	they act as a supplement to the diet in which the active ingredient is added to the food or it can be consumed in the form of pills, powders, or in liquid forms; they do not replace the complete food or meal.
Functional ingredients:	they are preparations, fractions or extracts containing bioactive compounds of varying purity, that are used as ingredients by manufacturers in the food.
Medical foods:	they are formulated to be administered under the supervision of a physician, for the specific dietary management of a disease or condition for which distinctive nutritional requirements are established.
Natural health products (NHP):	they include homeopathic preparations, substances used in traditional medicines, minerals or trace elements, vitamins, amino acid, essential fatty acids, or other botanical, animal, or microorganism derived substances. They are generally sold in medicinal to diagnose, treat, or prevent disease, restore or correct function, or to maintain or promote health. NHP also include nutraceuticals.
Nutraceuticals:	they are substances, either a food or part of a food, that provide medical or health benefits, including the prevention and treatment of disease. They are derived from foods and can be used in the form of pills, capsules, potions, and liquids.

Table 2. Different terms linked or interchanged with functional foods [29,28].

2.1. Whey proteins separation

Protein functions are related to their native structure, which depends on pH, temperature, pressure and solvent effects. Changes in native structure affect functional properties, by this way in the last years there has been a great interest in developing efficient separation and purification processes that prevent denaturation and loss of biological activity. On the other hand fractionation can emphasize the functional and nutritional properties of the individual proteins [30,31].

Separation processes tend to exploit to the maximum extent different molecular masses, concentrations, and isoelectric points of whey proteins. The main available processes for commercial-scale production of whey protein fractions belong to four categories: selective precipitation, membrane filtration, selective adsorption, and selective elution [20].

A brief description and some examples for each separation category are after-presented.

- Selective precipitation involves adjusting the solution physical properties to promote insolubility. Proteins are typically least soluble at a pH near the isoelectric point (pI) and in low ionic strength solutions, and most likely to aggregate under these conditions. While β -LG precipitates rapidly and selectively at high temperature (70-120 °C) and pH near neutral (pH 8), α -LA precipitates and aggregates better at acidic pH (3.5-5.5) and moderate temperature (50-65 °C) with long reaction times, usually accompanied by the precipitation of bovine serum albumin, immunoglobulins and lactoferrin, while β -LG remain soluble [32,33].
- Membrane filtration is commonly used to make whey protein concentrate, a mixture of all the proteins in whey, with the aid of membranes with a 5,000 to 10,000 g/mol molecular mass rating. As regard fractionation, traditionally it has been based solely on differences in molecular mass; by this way in the past fractionation was possible only for proteins with great differences in molecular mass (e.g. α -lactalbumin vs. IGG) or for proteins with a great combined difference in charge and size. Nowadays it is possible to achieve separation also when proteins have little or no difference in molecular mass together thanks to a careful adjustment of the solution pH and ionic strength [34,35]. The electrostatic rejection by the membrane, due to their slight residual charge can be enhanced or reduced by adjusting the solution pH. Furthermore separation is enhanced by operating near the pI of the smaller protein and far from the pI of the larger protein to maximize the difference in effective hydrodynamic size: this because the effective diameter of a protein increases with decreasing ionic strength. Low salt concentrations (1–20 mmol/L) increase electrostatic and steric rejection by the membrane: by this way a multi-step adjustment of the pH and ionic strength of the whey may allow fractionation of proteins using a sequence of membrane separation processes [20]. As an example reference [36] is reported; in this experimental study, the effect of working pH was evaluated, employing a 300 kDa tubular ceramic membrane in a continuous diafiltration mode, by measuring the flux-time profiles and the retentate and permeate yields of α -LA, β -LG, BSA, IgG and LF. It was found that a 300 kDa membrane could be employed to fractionate the original array of whey proteins in two parts: α -LA and β -LG in the permeate and BSA, IGG and LF in the reten-

tate. As a matter of fact important protein yields for α -LA and β -LG were obtained in the permeate (except for pH 4 and 5), while for the rest of the proteins studied, there was no significant diffusion through the membrane.

- In selective adsorption, a single purified protein is produced in conjunction with a treated whey solution depleted in that protein; the cost of manufacture must be borne by the income generated from that single purified protein product and the depleted whey solution [20,36]. There are many examples of selective adsorption processes for whey proteins: the immobilized hexapeptide ligand affinity resin or immobilized phenyl groups were used for α -LA, while for β -LG immobilized retinal or ceramic hydroxyapatite chromatography with sodium fluoride as a displacer were applied [37-40].
- In selective elution all the proteins in a mixture are trapped simultaneously onto the adsorbent, rinsed free of contaminants, and then eluted one by-one to obtain different purified proteins. The process uses an adsorbent and buffers that are inexpensive and food-grade, and it is operated at a high flow rate. Further on the cost of manufacture is spread among many purified protein products with the possibility to manufacture different products simply by using different elution buffers; by this way it is considered an attractive alternative to selective adsorption [41]. Differently from precipitation and membrane separation processes, which are volume-dependent separation methods, selective adsorption and selective elution processes are less volume dependent because adsorbent capacity depends mostly on the mass of protein recovered, not the volume of liquid processed [42]. Various studies about the development of ion exchange or affinity chromatography processes to separate whey proteins exist; referring as a case in point [43] various superparamagnetic anion-exchangers and their use together with cation-exchangers in the fractionation of bovine whey proteins (LF, LP, IG, and β -LG) were studied. While in reference [41] all the positively charged proteins in whey were bound simultaneously to a cation exchange column, rinsed free of contaminants and then eluted selectively to produce different fractions: with a single piece of equipment they were able to manufacture WPI, or α -LA and WPI depleted in α -LA or LF and LP only.

2.2. Biological properties of individual whey proteins

2.2.1. β -lactoglobulin (β -LG)

β -lactoglobulin, a member of the lipocalins family, is the major whey protein of ruminant species, 58% (w/w). It is present in many mammalian species but absent in human milk. Genetic variants of a single gene, of which β -LG A is the most common, have been widely reported particularly in the cow [44,21].

The lipocalins family presents various kind of features, most of which involve some ligand-binding functions; these last must be the physiological reason for the significant quantities of β -LG found in milk (the domestic cow produces 2 to 3g L⁻¹) [45].

In isolated form, despite its globular nature, it exhibits a low solubility and a low ionic strength. It contains normally 162 aminoacidic residues and has a molecular weight of

18,362 g/mol. The secondary structure of this protein comprises nine strands of β structure, a short α helix segment and three helicoidal turns [46].

Its quaternary structure depends on the medium pH: at pH values between 7 and 5.2 it is a stable dimer (molecular weight of 36,700 kDa); at pH values between 5.2 and 3.5, an octamer (molecular weight of ca. 140,000 kDa); and below pH 3.0 and above 8.0, a monomer, with two-cysteine residues per monomer [47].

2.2.1.1. β -LG biological functions

The endogenous function of β -LG is not clear, but it is known that it is a source of amino acids. Indeed β -LG, over-expressed in the lactating mammary gland of many species, is primarily an important source of amino acids for the offspring of those animals that produce it [45]. The amino acid content fuels muscle growth and it is a source rich in cysteine, which is important for the synthesis of glutathione [13].

It participates in the digestion of milk lipids in the neonate: β -LG binds to free fatty acids as they are released by pregastric lipases, by this way the digestion of milk fat is facilitated [48].

β -LG is the major allergen in cow's milk, responsible for causing milk allergy [25]. It was evidenced that milk's major allergen can be rendered non-allergenic and it can also be modified or administered inhibiting rather than stimulating the allergic process. β -LG has been conjugated with acidic oligosaccharides to reduce its antigenicity. Immunization of mice with the conjugates leads to a reduced T cell response, predominantly Th1-mediated, suggesting that the conjugates may have utility in preventing Th2-mediated allergy [49]. Oral administration of recombinant *Lactococcus lactis* expressing bovine β -LG induces a specific Th1 response, suggesting that probiotics expressing β -LG could be useful in the management of food allergy [50]. Intranasal co-administration of live lactococci expressing IL-12 and β -LG produces a protective Th1 response that inhibites allergic airway disease in mice [51]. Acidic β -LG-derived peptides hydrolyzed with *Lactobacillus paracasei* peptidases repress lymphocyte stimulation, upregulate IL-10 production, and downregulate IFN- γ and IL-4 secretion [52].

β -LG structure comprise a ligand-binding site. The ligands that bind to β -LG tend to be hydrophobic and include long-chain fatty acids, triglyceride, retinoids, cholesterol and, more weakly, hydrocarbon molecules [53].

β -LG can also be a source of peptides with different functions:

- Lactokinins, Tyr-Leu (f102–103) and Ala-Leu-Pro-Met-His-Ile-Arg (f142–148), are inhibitors of angiotensin-I-converting enzyme (ACE) and represent potential nutraceuticals/functional food ingredients for the prevention and/or treatment of high blood pressure [54].
- β -lactorphin (f102–105) has ACE-inhibitory activity, improved vascular relaxation in spontaneously hypertensive rats, and it is an opioid receptor agonist suggesting that it can modulate absorption processes in the intestinal tract [55].
- Beta-lactotensin, His-Ile-Arg-Leu (f146 –149), is an ileum-contracting peptide, which exhibits hypertensive activity. It is a natural ligand for neurotensin NT2 receptors, has an

anti-stress effect, promotes the abolition of fear memory, reduces sensitivity to painful stimuli, and consolidates memory. It is able to reduce blood cholesterol levels, but only when given parenterally, and not orally [56-58].

2.2.2. α -lactalbumin (α -LA)

α -lactalbumin is one of the most studied proteins: it is an important component of milk and it contributes significantly to its physical, biological and nutritional characteristics [59]. It is biosynthesized in the mammary gland during lactogenesis, and participates actively in the synthesis of lactose [60].

In human whey, α -LA is a major protein (1.7 mg/mL); however, in bovine whey it is ranked second with respect to protein content after β -LG. The bovine protein is characterized by its relatively low molecular mass (14.2 kDa) and its acidity (isoelectric point of 4.8); α -LA possesses a high mineral content and a balanced amino acid composition indeed its sequence is composed of 123 amino acids, that includes four residues of tryptophan and a high proportion of essential amino acids, Cys, Ile, Leu, Lys [61,62].

The structure of bovine α -LA is highly stabilized by four disulphide bonds and by the association of Ca^{2+} at the binding loop that joins together two domains of the protein: a large α -helical and a small β -sheet domains [63,64].

In addition to Ca^{2+} ion, α -LA is able to bind several other cations such as Mg^{2+} , Mn^{2+} , Na^+ and K^+ and also contains various Zn^{2+} binding sites [65]. Nevertheless, only the binding of Ca^{2+} ion is fundamental for the maintenance of the native conformation of the protein [66,67].

2.2.2.1. α -LA biological functions

α -LA and its biological and functional peptides are characterized by potential health benefits; as regard of that there is a growing scientific interest in the context of health-promoting functional foods. In particular, α -LA and α -LA-peptides can be used as supplements of essential amino acids in food to improve/maintain the immune system, to reduce the stress, for opioid activity, antihypertensive action, regulation of cell growth, immunomodulation etc. [68].

α -LA is cytotoxic and this property can be exploited for therapeutic uses. α -LA has protective properties against mucosal injury [69,70].

α -LA is particularly rich in essential amino acids. It has a high content of lysine and cysteine and a particularly high content of tryptophan (5.9% of the total amino acid content) [61].

The high content in tryptophane makes α -LA a nutraceutical; in particular it may help improve mood, sleep, and cognitive performance [71,72]. On the other hand the content of cysteine is valuable in boosting the immune system and promoting wound healing. As a general consideration, thanks to the high content in essential amino acids, α -LA is an invaluable supplement for infant formulas [68].

The protein may possess bactericidal or antitumor activity. The active form of the protein is called "human α -LA made lethal to tumor cells" (HAMLET), a complex formed by which

induces apoptosis in tumor cells but spares mature cells and has received much attention due to its potential use as a new therapeutic agent against tumor cells [73].

The digestion of α -LA with trypsin and chymotrypsin creates some polypeptides with bactericidal properties, mostly against Gram-positive bacteria and a weak bactericidal activity against Gram-negative strains. The peptide α -lactorphin behaves like opioid receptor agonists [74,75].

α -LA has a marked suppressive effect against the increased release of proinflammatory cytokines and tumor necrosis factor- α , from the D-galactosamine induced liver injury rat model or ischemia/reperfusion induced intestinal injury rat model [76]. Two synthetic peptides corresponding to the sequences f50-51 (Tyr-Gly) and f18-20 (Tyr-Gly-Gly) of α -LA also enhance both the in vitro proliferation and protein synthesis of concanavalin A-stimulated human peripheral blood lymphocytes [77].

2.2.3. Immunoglobulins (IG)

Immunoglobulins constitute a complex group of elements produced by B-lymphocytes and their concentration in whey is 0.7 g/l. IG are divided into three basic classes: IGG, IGA and IGM. IGG is often sub-divided into two subclasses, IGG1 and IGG2. IGG represents up to 80% (w/w) of all IG in milk or whey. Qualitatively the family of IG found in bovine whey and colostrum include IGA, secretory IGA, IGG1, IGG2, IGG fragments, IGM, IGE, J-chain or component, and free secretor component [78,21].

IG make a significant contribution to the whey protein content and they exert an important immunological function (especially in colostrum). IG are subject to postnatal transfer via colostrum because the placenta does not permit passage of macromolecules. These proteins are present in the serum and physiological fluids of all mammals; some of them attach to surfaces, where they behave as receptors, whereas others function as antibodies, which are released in the blood and lymph [79].

In terms of quaternary structure, IG are either monomers or polymers of a four-chain molecule, consisting of two light polypeptide chains (with a molecular weight in the range 25,000 kDa) and two heavy chains (with molecular weight of 50,000–70,000 kDa) [80].

2.2.3.1. IG biological functions

Milk immunoglobulins normally provide passive immunity for the neonate, but they are also potentially powerful agents that could be incorporated into diets to remove toxic, or undesirable dietary factors. As an example, naturally occurring antibody in milk can be extracted which binds to cholesterol in the human digestive tract and prevents its absorption into the bloodstream. This anti-cholesterol antibody can be a useful food supplement for the functional food market [25].

Concerning IG antimicrobial and antiviral properties it is known that concentration of colostrum whey antibodies against a particular pathogen can be raised by immunising cows with the pathogen or its antigens. Antibody concentrates derived from immune milk collected

from cows immunised with inactivated human rotavirus possess preventive/treatment features in enteric disease caused by said viruses in therapeutics of child infections [81]. The hyperimmune whey that results can potentially provide prophylactic protection against various infectious gut microbes including rotavirus and *Helicobacter pylori* [82]. Infant gastritis originated by *H. pylori* is well fought via a diet including immune milk containing specific anti-*H. pylori* antibodies [83]. There is also evidence of protection via bovine antibodies against dental caries caused by cariogenic streptococci [84].

IG can also act in the immune system modulation; they are recognised to provide protection against diseases in the newborn through passive immunity. Systemic immunisation of pregnant cows increases the levels of antibodies against immunising bacteria, and also reduces susceptibility to disease [85]. Vaccination of pregnant cows originates colostrum characterized by high concentrations of specific antibodies against the antigens of the vaccine used [86].

Other metabolic features of IG are also known. Immune milk is suggested to lower blood pressure. In reference [87] a clinical-trial study regard the effects on reduction of cholesterol and blood pressure of immune milk, produced by dairy cows previously hyper-immunised with a multivalent bacterial vaccine, was described. The involved human hypercholesterolemic subjects consumed 90 g of immune milk daily: this was a useful adjunct in the dietary management of hypercholesterolemia.

2.2.4. Bovine Serum Albumin (BSA)

Bovine serum albumin represents the 5% of the whey proteins and its concentration in whey is about 0.4 g/l. It appears in milk following passive leakage from the blood stream indeed it is not synthesized in the mammary gland [21,25]. The most outstanding property of BSA is its ability to bind reversibly various ligands; in particular it is the principal carrier of fatty acids [88].

It contains 582 amino acid residues, which lead to a molecular weight of 66,267 kDa; it also possesses 17 intermolecular disulphide bridges and one thiol group at residue 34 [89]. BSA molecule is heart-shaped; it consists of three homologous α -helical domains and each domain contains two sub-domains that share common structural motifs [88].

Thanks to its size and higher levels of structure, BSA can bind to free fatty acids and other lipids, as well as flavor compounds, this feature is severely injured upon denaturation [90].

Its heat-induced gelation at pH 6.5 is initiated by an intermolecular thiol-disulphide interchange, similar to what happens with β -LG [78].

2.2.4.1. BSA biological functions

BSA has the ability to inhibit tumor growth thanks to the modulation of activities of the autocrine growth regulatory factors; this was evidenced by *in vitro* incubation with human breast cancer cell line MCF-7 [91]. In relation to this activity [92] the delivery system MTO-BSA-NS (mitoxantrone nanoparticles loaded with bovine serum albumin) was studied; human MCF-7 breast cancer in nude mice and animal model of P388 lymphnode metastases in

Kunming mice were applied to investigate the therapeutic efficiency. The inhibition rate of the nanospheres against breast cancer was high and lymphnode metastases were efficiently inhibited.

BSA is able to bind fatty acids stored in the human body as fat; this allows BSA to participate in synthesis of lipids [93]. BSA has also antioxidant activities; *in vitro* it is able to protect lipids against phenolic-induced oxidation [94]. BSA is also a source of essential amino acids, whose therapeutic potential is largely unexplored.

Biological functions of some BSA-derived peptides have also been examined. The peptide serorphin (Tyr-Gly-Phe-Gln-Asn-Ala) (f399–404) has opioid agonist activity, while the peptide albutensin A (Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg) (f208 –216) is an ACE inhibitor and is reported to have ileum contracting and relaxing activities [54].

2.2.5. Lactoferrin (LF)

Lactoferrin together with lactoperoxidase is one of the minor whey proteins. It belongs to the transferrin family which is composed by proteins capable of binding and transferring Fe^{3+} ions. LF is a glycoprotein characterized by a molecular weight of 80,000 kDa. It is synthesized by glandular epithelial cells and mature neutrophils, and can be found in milk, saliva, tears, nasal and intestinal secretions, pancreatic juice, seminal fluid, and in secondary granules of neutrophils. Bovine milk contains between 0.02 and 0.35 mg/ml of LF, depending on the period of lactation [25,95].

LF is a bilobal protein that contains two homologous metal-binding sites with high affinities for ferric iron. The iron-binding sites are situated in the inter-domain clefts. The requirement for an anion, bound synergistically with the Fe ion is a unique feature of LF. Iron release can occur at low pH and is associated with a large-scale conformational change in which the two domains that enclose each iron-binding site move wide apart [96].

LF possess a wide range of biological functions, many of which are not connected with its iron binding ability; it is the most valuable biomedical protein present in whey due to the various therapeutic properties it exhibits [97,25].

2.2.5.1. LF biological functions

LF has quite important role in iron metabolism. It may contribute to local iron accumulation at sites of inflammation and it has been known to be responsible for hypoferraemia through binding free iron and shuttling it back to macrophages [98,97]. LF might also have a control function in situations when increased amounts of iron are released from its depots [99]. Further on LF seems to affect intestinal iron absorption in infants depending on the organisms need for iron [100].

LF is a part of the innate immune system and it also takes part in specific immune reactions; it represents one of the first defense systems against microbial agents [101,95].

LF can have a bacteriostatic effect thanks to its ability to bind free iron, essential for the growth of bacteria (e.g. *E. coli*). LF, as an iron donor, supports the growth of *Lactobacillus* sp.

or *Bifidobacterium* sp., characterized by a lower iron demands and generally considered as beneficial [102,103]. LF owns also a bactericidal effect, which is not iron-dependent, against Gram-negative and Gram-positive bacteria. In the first case there is the disruption in the cell wall because the microorganisms have the receptors for the N-terminal region of LF; while for Gram-positives, changes in the permeability of the membrane occurs mediated by electrostatic interactions between the lipid layer and LF surface [104,105]. LF may contribute to defense against the invasion of facultative intracellular bacteria such as: *E. coli* HB101, *Yersinia enterocolica*, *Y. pseudotuberculosis*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. LF binds both target cell membrane glycoaminoglycans and bacterial invasins. The proteolytic activity of LF inhibits the growth of *Shigella flexneri* and *E. coli* through degrading proteins necessary for colonization [105,106].

The main contribution to antiviral defense of LF consists in its binding to glycosaminoglycans of cell membrane: viruses (Herpes simplex virus, cytomegaloviruses, human immunodeficiency virus) cannot enter cells and infection is stopped at an early stage [106]. LF is also capable of binding certain DNA and RNA viruses [107].

LF may support the proliferation, differentiation, and activation of immune system cells and strengthen the immune response; LF also acts as an anti-inflammatory factor. Thanks to its antimicrobial activity and capability of binding components of bacterial cell walls or their receptors, LF may prevent the development of inflammation and subsequent tissue damage caused by the release of pro-inflammatory cytokines and reactive oxygen species [101].

As regard the influence of LF on tumor growth, it is known that it is able to halt the growth of human mammary gland carcinoma cells between the G1 and S stage; this effect may be ascribed to the altered expression or activity of regulatory proteins [108]. The lactoferrin-dependent, cytokine-mediated stimulation of activity of NK cells and lymphocytes CD4+ and CD8+, represents an important factor in defense against tumor growth: after the oral administration of lactoferrin the number of these cells increases [109]. Even if the exact mechanism has to be discovered completely, it seems that LF-mediated inhibition of tumor growth might be related to apoptosis of these cells induced by the activation of the Fas signaling pathway [110].

LF is also able to act as a growth factor activator; it can have effect on small intestine epithelial cells and endometrium stroma cells [111]. It has also been identified as a transcription factor [112].

LF contributes to the stabilization of the osseous tissue; it may affect bone cells through the inhibition of osteolytic cytokines whose levels rise during inflammation. LF is a potent anabolic factor affecting osteocytes; it stimulates osteoblast proliferation, enhances thymidine incorporation into osteocytes, and reduces apoptosis of osteoblasts. By this way LF might be potentially useful in the treatment of diseases such as osteoporosis [113].

2.2.6. Lactoperoxidase (LP)

Lactoperoxidase is member of the family of mammalian peroxidases. It is present in a variety of animal secretions (e.g. tears, saliva and milk), it is one of the most abundant enzymes

in plain milk and represents 1% (w/w) of the total protein pool in whey; its concentration in whey corresponds to 0.03 g/l [114].

LP consists of a single polypeptide chain containing 612 amino acid residues and its molecular mass is about 80 kDa. It contains 15 half-cystine residues and carbohydrate moieties comprise about 10% of the weight of the molecule [115].

The complete LP system (i.e. enzyme plus substrate) was originally characterized in milk [116]; its activity depends on many factors (e.g. animal species, breed and lactation cycle). Other members of that group of oxidoreductases include myeloperoxidase (present in neutrophils and monocytes), eosinophil peroxidase and thyroid peroxidase; they are characterized by a close evolutionary relationship [21].

2.2.6.1. LP biological functions

LP is characterized by the antimicrobial activity related to the LP system, formed by LP, thiocyanate anion, and hydrogen peroxide, which is active only in the presence of all these three components: LP catalyses the oxidation of thiocyanate by H_2O_2 and generates intermediate products with a broad spectrum of antimicrobial effects against bacteria, fungi and viruses. The LP system is naturally occurring in milk and saliva and has been used in foods, cosmetics and in clinical applications because of its safety and broad spectrum of action; in particular it is fundamental in the dairy industry for the preservation of raw and pasteurized cheese, and yogurt [117,118].

The thiocyanate anion is significantly present in saliva, milk and airway secretions. The amount of the anion in cow's milk ranges from 0.1 to 15 mg/kg and its concentration varies according to animal species, breed, lactation cycle, season and composition of feed [114,21].

Hydrogen peroxide is not normally detected in raw milk. It may be generated endogenously by polymorphonuclear leucocytes or under aerobic conditions by many lactobacilli, lactococci, and streptococci. H_2O_2 is normally present to very small levels because its content is rapidly reduced by catalases and peroxidases that are adventitious in milk [119-121].

The major intermediary oxidation product, at physiological pH, is hypothiocyanate; it mediates bacterial killing, as it is cell-permeable, and can inhibit glycolysis, as well as (NADH)/(NADPH)-dependent reactions in bacteria. It is bactericidal for enteric pathogens including multiple antibiotic resistant strains of *E. coli*. Other reaction products of the LP system, such as cyanosulphurous acid and cyanosulphuric acid, are able to oxidise sulphidril groups of bacterial proteins [114,21].

The LP system inhibit Gram-negative, catalase positive organisms, such as pseudomonas, coliforms, salmonellae and shigellae; these microorganisms can also be killed if H_2O_2 is supplied exogenously. Gram-positive, catalase negative bacteria, such as streptococci and lactobacilli are generally inhibited but not killed by the LP system [122,121].

The LP system exerts both bacteriostatic and bactericidal activities against strains of *Salmonella typhimurium*, *S. aureus*, and *L. monocytogenes*; the system is bactericidal against *Campylobacter jejuni*, *Brucella melitensis*. As regard *Bacillus cereus*, *Streptococcus mutans*, *Streptococcus*

sanguis, *Streptococcus mitis*, and *Streptococcus salivarius* the LP system has an inhibitory effect [123-127,121].

The antifungal activity of the LP system with glucose oxidase as H_2O_2 source has been reported against *Rhodotorula rubra*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Aspergillus niger*, and *Byssoschlamys fulva* in salt solution and in apple juice. The LP-thiocyanate- H_2O_2 system was found to inhibit the growth and proliferation of many fungal species (e.g. *Aspergillus flavus*, *Trichoderma* spp.); the same system showed also antifungal activity against *Alternaria* spp., *Penicillium chrysogenum* and *Claviceps* spp. [128,121].

Finally, as regard the antiviral properties of the LP system, the ability to kill both poliovirus and vaccina virus with halides (I^- , Br^-) as electron donors has been reported [129]. In referen-ces [130,131] the LP- H_2O_2 -halide system virucidal activity against HIV-1 was reported.

3. Whey fermentation products

During last 50 years, cheese whey was used in different bioconversions; for examples the microbial biomass production for animal feed supplement [132], biogas production using anaerobic methanogenic bacteria [14], bioethanol production by *Kluyveromyces marxianus* [133,134] or recombinant *Saccharomyces* cells [135,136], hydrolized lactose solution in sweeteners and dietary supplements production [14].

Therefore, at present, it can be very interesting and promising to consider again the possibility to use cheese whey and, particularly, glycidic component because of new demands, as bioplastic synthesis (poly-hydroxyalkanoates -PHA- and polylactate acid -PLA-), antimicrobial peptides (bacteriocins), enzymes and esopolisaccharides (EPS).

Just below, there are the information regarding the fermentative production of the molecules and macromolecules previously mentioned.

3.1. Polyhydroxyalcanoate

Polyhydroxyalkanoates (PHA) are aerobic bacteria synthesized macromolecules (polyesters); they are carbon and energy reserve, accumulated as intracellular granules. These microbial compounds play the same role as glycogen and starch in animal and plant cells, respectively [137].

Among biodegradable plastics, PHA are very interesting polymers because of their chemical, physical and mechanical properties comparable to petroleum-derived plastics (polyethylene and polypropylene); otherwise, PHA are different for their complete biodegradability, UV-resistance, oxygen-impermeability (fundamental property for food packaging) and biocompatibility (essential property for medical and surgery applications) [138,139].

Under unbalanced growth conditions (limitation of essential nutrients, e.g., nitrogen, phosphate, or oxygen), several microorganisms redirect the acetyl-CoA flux from biomass formation towards accumulation of PHA [140,141].

At present, these biopolymers are synthesized in pure cultures, using synthetic pure substrates (as monosaccharides and organic acids), in macronutrient limiting conditions (N, O, P). Because of substrate weights on whole process cost at 40%, it becomes necessary to search alternative cheaply available source materials for PHA production, as food by-products (e.g. cheese whey).

In the last two decades, a broad number of studies were related to the production of PHA from milk whey permeate using pure cultures of wild type microorganisms or recombinant ones. In several of these works, whey lactose was hydrolyzed by lactase: poly-(3HB-3HV) was produced with *Ralstonia eutropha* DSM545 on whey permeate and inverted sugars [142], poly-3-(hydroxybutyrate-co-hydroxyvalerate) was produced by *P. hydrogenovora* with hydrolyzed whey permeate and sodium valerate [143].

In [141] *Hydrogenophaga pseudoflava* DSM1034 was reported as unique example of wild type microorganism able to synthesize PHA directly from lactose, three possible routes from whey lactose to PHA have been suggested: direct conversion of lactose to PHA, hydrolysis of lactose (chemically or enzymatically) and conversion of glucose and galactose to PHA, lactose fermentation to lactic acid and then conversion of lactic acid to PHA. In reference [144] a recombinant *E. coli* strain GCSC 6576 and whey powder was used in a pH-stat fed-batch fermentation. After 47 h of fermentation, it was obtained a dry cell weight and P(3HB) concentration of 109 g L⁻¹ and 50 g L⁻¹, respectively. Further, with the same organism and using pH-stat fed-batch culture and concentrated whey solution containing 210 g L⁻¹ lactose as nutrient feed, a dry cell weight of 87 g L⁻¹ and P(3HB) concentration of 69 g L⁻¹ containing 87% dcw P(3HB) was achieved. Using the recombinant *E. coli* strain (K24K), the authors successfully produced 70.1 g L⁻¹ biomass containing 51.1 g L⁻¹ P(3HB) in a pH controlled fed-batch fermentation at pH 7.20 with whey and corn steep liquor as carbon and nitrogen sources, respectively [145]. Previously, PHB production by *Methylobacterium* sp. ZP24 on lactose and whey with similar values of biomass polymer content was described [146].

Poly-β-hydroxybutyrate production was also described in lactic acid bacteria belonging to *Lactococcus*, *Lactobacillus*, *Pediococcus* and *Streptococcus* genera [147].

Lactic acid producing bacteria such as *Lactobacillus lactis* [148], *Propionibacterium* [149], *L. delbrueckii* [150-152] and *C. necator* have been also used in a co-culture fermentation system: LAB converted sugars into lactic acid which was later taken up by *C. necator* to produce PHAs. Generally, it has been demonstrated that co-culture fermentations resulted in increased yield, improved control of product qualities. A further advantage in the application of co-cultures is the possibility of utilizing secondary products (e.g. whey) cheaper than glucose as substrates for production of PHAs [153]. Bacteria that have a “generally recognised as safe” (GRAS) status for PHA-production such as lactic acid bacteria and bacilli belonging to probiotic species [147,154] might constitute an added value to these biotechnological processes [155].

3.2. Lactic acid

Acetic, propionic, lactic, lactobionic, citric, gluconic and itaconic acids can be obtained from lactose/whey fermentation. Among organic acids that find applications in specialty chemicals, lactic acid is the most important from an economical point of view [156].

Lactic acid is used in food and chemical industries (pharmaceutical products, textiles, leather), primarily as a preservative and as acidulant [157-159]. Also it has applications as a biodegradable plastic component (polylactide, polymers, polyhydroxybutyrate) [159]. Notably, the industrial demand for lactic acid (LA) has been increasing considerably in recent years, owing to the promising applications of its polymer, the polylactic acid (PLA), as an environment-friendly alternative to plastics derived from petrochemicals [160].

Polylactic acid (PLA) is a biodegradable polyester made by condensation of lactic acid (LA) monomers [161]. It can be worked with conventional facilities and techniques to produce implant devices and internal sutures [162]. Due to its low toxicity, it is classified as GRAS and can be also used in food packaging [161,163], which is one of the widest fields of plastics market. PLA is completely biodegradable under compostable conditions: however, if disposed in landfills, it will last in the environment for years, likewise oil-based plastics [164].

Cheese whey effluents have been used in fermentation processes to produce lactic acid [165-168,158,169,159]. Microorganisms used in lactic acid production are *Lactobacillus casei* [166,157,158,169], *Lactobacillus helveticus* [170-172,168,156,159]; *Lactobacillus acidophilus* [167]; *Lactobacillus delbrueckii* [165,167,168]; *Lactobacillus salivarius* [169]; *Lactococcus lactis* [158]. *Streptococcus thermophilus* [167]; *K. marxianus* [168]; *Leuconostoc* and *Pediococcus* [4].

Among different lactobacilli species employed in lactic acid production, *L. helveticus* is the generally preferred organism, it is a homolactic LAB that produces LA in racemic mixture (DL) [173]. *L. helveticus* showed enhanced lactose utilisation and lactic acid production at 42°C and pH 5.8 [159].

Currently, a high fraction of generated CW is managed by membrane processes, mainly, ultrafiltration. The obtained permeate has a low protein content and an elevated lactose and mineral salts concentration, both aspects are advantageous in lactic acid fermentation. As a consequence, several works have been carried out aimed at obtaining lactic acid after ultrafiltration of CW [171,157,172,169]. Highest lactic acid production rate was obtained with *L. helveticus* cultivated in whey permeate, with corn steep liquor (CSL) as the nitrogen source [174]. Lactic acid productivity of 9.7 g/L/h using *L. helveticus* strain milano has been obtained in continuous fermentation of whey-yeast extract permeate medium [173,175]. In a work with *L. salivarum* YE supplementation was replaced by *in situ* treatment of fermentation medium with proteolytic microorganisms [169].

However, lactic acid production from cheese whey or its permeate obtained without nutrients supplementation [165,168,159,169] is of limited application to industrial scale because of the low productivity, nutrients supplementation is a key factor limiting the process efficiency.

Some studies report the use of mixed cultures in lactic acid production with synergistic effects [168,158]. Other research groups tried to improve LA production using *rE.coli* harbouring an inducible expression plasmid containing D-lactate dehydrogenase encoding gene of *Lactobacillus plantarum* [176] or using metabolic engineering of LAB, fungal or yeast systems [177,178,179], but all of these strategies, if compared with the use of mixed cultures, involve higher costs due to genetic engineering studies and the need of sterilization.

LAB have been immobilised by several methods on different supports (calcium alginate, k-carragenane, agar and polyacrylamide gels) [4] and the immobilised systems have been investigated for lactic acid production from whey. A two-stage process was used for continuous fermentation of whey permeate medium with *L. helveticus* immobilised in k-carrageenan/locust bean gum, which resulted in high lactic acid productivity ($19\text{--}22\text{ g L}^{-1}\text{ h}^{-1}$) [172].

In [4] *L. casei* was immobilized in Ca pectate gel. A high lactose conversion (94.37%) to lactic acid (32.95 g L^{-1}) was achieved and the cell system was found highly stable, no decrease in lactose conversion to lactic acid was observed up to 16 batches.

3.3. Exopolysaccharides

Exopolysaccharides (EPS) are long-chain polysaccharides ($4\cdot 10^4\text{--}6\cdot 10^6\text{ Da}$). They are produced by bacteria and microalgae and can be divided in homopolysaccharides (Homo-EPS) and heteropolysaccharides (Hetero-EPS) [180]. Homo-EPS consist of either D-glucose (glucans) or D-fructose (fructans) residues, with different types of linkage and branching degree. Hetero-EPS are constructed from multiple copies of an oligosaccharide and show little structural similarity to one another: glucose, galactose, xylose, mannose, arabinose and rhamnose are the most represented sugars but also amino-sugars and polyols can be occasionally present as well as glucuronic acid. They are often highly branched with different binding types [181].

The physiological role of EPS is related to microbial cell protection from toxic compounds, phagocytosis, antibiotics effect and osmotic stress [182]. In the last years, their prebiotic role was identified and described [183,184]. In addition to role as food additives and prebiotics, LAB synthesized EPS have been implicated as anti-tumor agent, immuno-stimulator and blood cholesterol lowering agent [185,184].

On the basis of their rheological properties, EPS are applied as stabilizers and emulsifiers in food industry, particularly for yogurt, fermented milk and mozzarella production [186,187]. EPS used in food industry must be considered additives and, consequently, must ensure safety qualification. EPS synthesized by LAB, generally used in food production, possess this safety characteristics [184]. The GRAS and probiotic status of some lactobacilli give to them more preference for consumable EPS production. Main drawbacks limiting their industrial expansion are their low yields of production [188]. Approximately 30 species of lactobacilli are described as EPS producers, among them, the best known are *L. casei*, *L. acidophilus*, *L. brevis*, *L. curvatus*, *L. delbrueckii bulgaricus*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*. They are principally cultivated between 30 and 37 °C on rich media as Man Rogosa Sharp (MRS), milk or milk derivatives [189,188]. But *Lactobacillus* sp. are not the best polysaccharide pro-

ducers compared to some soil bacteria, as *Xanthomonas campestris* [190,188]. It is known that different strains of *X. campestris* can produce xanthan gums of different composition, viscosity and yield. In reference [191], cheese whey was used as substrate in the production of xanthan gum with optimised high gum production in a bioreactor and at a wide range of viscosity values. With the strain *X. campestris* C7L, [192] a production level of 14.7 g kg⁻¹ was obtained, in a 40 g L⁻¹ whole milk whey-based medium (powdered cheese whey) with the addition of 0.1 g L⁻¹ magnesium sulphate and 5 g L⁻¹ potassium phosphate. In [193], the feasibility of using cheese whey as carbon source for xanthan gum production was investigated using two different strains: *Xanthomonas campestris* pv *mangiferaeindicae* 1230 and *X. campestris* pv *manihotis* 1182. At 72 h of fermentation, using cheese whey as sole carbon source, in presence of 0.1% (w/v) MgSO₄*7H₂O and 2.0% (w/v) of K₂HPO₄, maximum xanthan gum productions were observed. Although the xanthan gum concentration was similar for the two strains, approximately 25 g L⁻¹, chemical composition and ionic strength presented several differences.

A modelling approach was used to describe the influence of temperature and pH on the kinetics of both growth and EPS production of *Streptococcus thermophilus* ST 111 in milk-based medium; addition of whey protein hydrolysate to milk medium resulted in an increased growth and EPS production [194].

Growth and EPS production during free and immobilized cell chemostat culture of *Lactobacillus rhamnosus* RW-9595M D was described in [195]. Whey permeate powder was used, both very high EPS production (1800 mg L⁻¹) and volumetric productivity (542 mg L⁻¹ h⁻¹) were obtained during chemostat culture with free cells for a D of 0.3 h⁻¹.

3.4. Bacteriocins

Bacteriocins are antimicrobial peptides synthesized, at ribosomal level, by various Gram positive and Gram negative bacteria, acting towards strictly correlated bacteria (growth inhibition). Generally, bacteriocins are produced at the end of the exponential growth-phase and their spectrum of action can vary, depending on the producing specie [196].

Positive effect of bacteriocins was spotlights in different types of food: dairy and bakery products, meat and fishing products, fruit and vegetables, beverage [197]. Moreover, further application are known, in medical, pharmaceutical and veterinary fields [198].

LAB are particularly prolific in bacteriocins production and can biosynthesize different types of antagonistic molecules. Many LAB are able to synthesize different classes of bacteriocins, currently, nisin is the only bacteriocin industrially produced and which use in food is allowed and authorized [199,200]. Over the last two decades, there has been an explosion of basic and applied research on lactic acid bacteria (LAB) bacteriocins, primarily due to their potential application as biopreservatives in food and food products to inhibit the growth of food-borne bacterial pathogens [196]. Different experimental models demonstrated efficiency on pathogens and spoilage microorganisms growth control, both adding bacteriocins in food [201,202] and using bacteriocins *in situ* synthesized [203].

Although bacteriocins can be produced in the food matrix during food fermentation (*in situ*), bacteriocins by LAB can be produced in much higher amounts during fermentations under optimal physical and chemical conditions [204-207].

A recent review [208] summarized information on nisin production by *L. lactis* in batch cultures utilizing skimmed milk or whey as inexpensive medium. Nisin biosynthesis occurs during the exponential growth phase, several cultural factors influence nisin production: producer strain, media composition, pH and temperature values, agitation and aeration. Nisin production with *Lactococcus lactis* ATCC 11454 was carried out in two different media with pasteurized milk whey, filtered or not. In filtered milk whey nisin titer was 11120.13 mgL⁻¹ up to 1628-fold higher than the filtered milk whey. The higher nisin concentration was probably related to insoluble proteins released into the media [204]. In [209] the utilization of milk whey was studied in batch cultures, a higher nisin production was observed in diluted whey (mixed with wash waters), 22.9 BU mL⁻¹ (BU – bacteriocin units) in relation to concentrated whey (liquid remaining after the first cheese pressing), 8.3 BU mL⁻¹.

In reference [205], the production of nisin by *L. lactis* UQ2 in a bioreactor using supplemented sweet whey (SW) was optimized by a statistical design of experiments and response surface methodology (RSM). A 2nd-order model built from a CCD experiment predicted a maximum nisin production of 178 IU/mL at 6 h of incubation in the bioreactor, leading to a productivity of 0.74 mg nisin/([L][h]), which increased 1.62 times when using controlled pH (6.5) fermentation.

Continuous production of nisin in whey permeate, supplemented with casein hydrolysate, was investigated using a packed-bed bioreactor. *Lactococcus lactis* subsp. *lactis* ATCC 11454 was immobilized by natural attachment to fiber surfaces and entrapment in the void volume within spiral wound fibrous matrix. Optimal conditions for continuous nisin production were pH5.5, 31°C, 10–20 g/l casein hydrolysate, and D 0.2 h⁻¹. A maximum nisin titer of 5.1×10^4 AU/ml was observed. The bioreactor was operated continuously for 6 months without encountering any clogging, degeneration, or contamination problems [206].

Previously, nisin-Z production was studied during repeated-cycle pH-controlled batch (RCB) cultures using *Lactococcus lactis* subsp. *Lactis* biovar. *diacetylactis* UL719 immobilized in k-carrageenan/locust bean gum gel beads in supplemented whey permeate [210,211].

Bacillus bacteriocins are increasingly becoming more important due to their sometimes broader spectra of inhibition (as compared with most LAB bacteriocins), which may include Gram-negative bacteria, yeasts or fungi, in addition to Gram-positive species, some of which are known to be pathogenic to humans and/ or animals. Bacteriocins from Bacillus species offer a much broader spectrum of potential applications compared with LAB bacteriocins. The use of Bacillus bacteriocins in food preservation is just starting to be investigated [212]. A BLIS with a broad spectrum of activity against pathogenic and spoilage bacteria (*L. monocytogenes*, *B. cereus* and clinical isolates of *Streptococcus* spp.) was produced by *B. licheniformis* P40 when cheese whey concentration in the growth medium was about 70 g L⁻¹ [213].

Pediocin production on whey by *Pediococcus acidilactici* NRRL B-5627 was described in [214], pediocin was obtained both in batch and re-alkalized fed-batch fermentations on diluted whey supplemented with 2% (w/v) yeast extract.

3.5. Single cell protein and single cell oil

The term SCP refers to dried cells of microorganisms such as algae, actinomycetes, bacteria, yeast, molds, and higher fungi grown in large-scale culture systems for use as protein source in human food or animal feed. Among suitable microorganisms to be grown on whey lactose as a substrate for SCP production, more research work has been carried out on yeasts, in particular *K. marxianus* [215-217]. The use of lactose or whey as a carbon source for the production of yeast biomass is a simple treatment process for increasing the value of food industry co-products [14,218].

Biomass produced from both batch and continuous processes [219-221] is mostly used as animal feed supplement [215] but also in production of baker's yeast [217].

In reference [221] deproteinized sweet and sour cheese whey concentrates were investigated as substrates for the production of SCP with *Kluyveromyces marxianus* CBS6556 up to a 100-l scale. Biomass concentrations up to 50 g l⁻¹ ($Y_{x/s}$ 0.52) for sweet whey and 65 g l⁻¹ ($Y_{x/s}$ 0.48) for sour whey concentrates were obtained.

Use of whey or buttermilk supplemented with YE for the growth of thermophilic LAB was reported in [222]; cell yields and kinetic parameters obtained were comparable or better than those obtained from control media, which appear to be too expensive for growing LAB on an industrial scale.

High added value probiotic biomass from deproteinized and non-supplemented milk whey was reported in [223]. Growth kinetics of *Lactobacillus casei* in deproteinized goat milk whey was analyzed in batch, continuous and fed-batch conditions.

In [224] a technology for kefir SCP production using whey was developed, a three-step process to scale-up kefir biomass production at a semi industrial scale pilot plant (100- and 3,000-L bioreactors) has been described.

The biotechnological production of SCO has been focused on the ability of various oleaginous microorganisms to convert agro-industrial wastes or raw materials into specialty lipids [225], or equivalents of plant oils that contain poly-unsaturated fatty acids (PUFAs) [226]. Three Zygomycetes, *Mortierella isabellina*, *Thamnidium elegans* and *Mucor* sp., were tested for their ability of producing biomass and lipid-containing g-linolenic acid (GLA) during their cultivation on cheese whey. All the tested microorganisms presented appreciable microbial growth; GLA concentration presented differences related with the strains and the fermentation time. *M. isabellina* produced noticeable quantities of SCO resulted in a maximum GLA production of 301 mg/L [227].

3.6. Enzymes

β -D-Galactosidase most commonly known as lactase, is one of the most important enzymes used in food processing, which catalyses the hydrolysis of lactose to glucose and galactose. The enzyme has been isolated and purified from a wide range of microorganisms but most commonly used β -D-galactosidases are derived from yeasts and fungal sources [228,229].

Yeast strains have been grown successfully on whey based medium among different strains of *Kluyveromyces* spp., *K. marxianus* and *K. fragilis* strains showed the maximum enzyme yield [230,14]. *Streptococcus thermophiles* and *Bacillus stearothermophilus* can be considered as potential bacterial sources of lactase. *S. thermophilus* was grown in deproteinized cheese whey [231], supplementation of whey and whey permeate basal media resulted in enhancement of specific growth rate and enzyme activity in bacterial cultures [232].

A simple feeding strategies to obtain high-cell-density cultures of *K. marxianus* (35 g L⁻¹) maximizing β -galactosidase productivity using cheese whey as basic medium was reported in reference [233].

A fermentation process for the production of penicillin acylase by a recombinant *Escherichia coli* and using whey as unique carbon was developed in [234].

Serratia marcescens ATCC 25419 was used for production of secreted proteases on reconstituted whey. A major metallo-protease and a minor serine protease were produced during growth [235]. In reference [236], a mixed culture *Serratia marcescens*–*Kluyveromyces fragilis* was tested on whey, microorganisms showed a synergistic effect in protease production.

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