

# Cheese

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Cheese is a worldwide produced and consumed commodity. There are many varieties of cheese from soft to hard, white to yellow, and fresh to aged after ripening. Especially, each category has its own producing technology.

Keywords: PDO cheese ; proteomics ; bioactive peptides ; adulterations ; Feta cheese ; Graviera Kritis ; Mozzarella di Bufala Campana ; Parmigiano Reggiano ; Grana Padano

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## 1. Introduction

One of the most widely consumed food is cheese in all forms. Cheese is a dairy product and the result of milk coagulation after its fermentation or acidification. It is used broadly in cooking industry and recently there have been attempts to relate cheese consumption to human health. This product belongs to the most complex, diverse and taste rich foods appreciated today. The characteristics leading to its differentiation from other foods are the various initial substances used, production process and aging. Specifically, these factors include (i) the milk used for the production of cheese, (ii) the method of coagulation used to transform the milk into a gel or coagulum (e.g., acid vs. rennet), (iii) the acidification characteristics (both rate and time), (iv) the additional steps during the cheese-making process controlling moisture levels of the young cheese (e.g., cooking temperature and pressing and salting conditions). In the ripened cheeses, a critical role for the final product is played by the ripening conditions such as temperature, relative humidity, and rates of O<sub>2</sub>, CO<sub>2</sub>, and NH<sub>3</sub>, that affect the character and diversity of cheese microflora. The traits of these microorganisms and activity contribute to the final characteristics of the product, such as flavor and quality <sup>[1][2][3]</sup>. The latter are attributed to the complex dynamics and interactions developed among the microorganisms, growth substrates and proteins in milk and the environment.

Proteolysis is present during cheese production and ripening <sup>[4]</sup>. This phenomenon includes the hydrolysis of milk caseins as a result of chymosin action and other proteases already present in milk, such as plasmin, which originate from somatic cells or naturally present in the milk bacteria <sup>[4]</sup>. The proteolytic enzymes produce large oligopeptides, which are the substrate for proteinases and peptidases coming from starters lactic acid bacteria (S-LAB) and nonstarters LAB (NS-LAB), to exhibit their activity <sup>[5]</sup>. The final outcome of the hydrolysis of milk caseins is the release of 5–30 amino acids length oligopeptides <sup>[6]</sup>.

During cheese making the most uncontrollable step is the one linked with the microflora growth and their interactions <sup>[1][2]</sup>. The traditional method for improving the quality characteristics of cheese is the “vaccination” with bacterial strains isolated from high quality aged cheeses <sup>[8]</sup>. Even though the product was made in the same industry or following the same manufacturing instructions, its final characteristics demonstrate great variability <sup>[9]</sup>.

All these variants make the classification and characterization of cheeses difficult and for that reason many classification models exist. The “European” approach, which is used in Mediterranean countries, is based on the technological processes. The European legislation is very strict regarding the classification of cheese and labeling them as Protected Designation of Origin (PDO). Directives by European Union and National laws require that manufacturers label PDO cheeses only if they provide proof concerning the nature and authenticity of their products. PDO cheese adulteration is a problem that has arisen in the past few years and continues to grow, mostly because of the economic profits for the producers and the availability of milk substitutes such as milk powder and curd <sup>[10]</sup>. To prevent counterfeit products, especially for the PDO ones, the legislation becomes even more strict and as a result the demand for new technologies concerning fraud detection in food grows <sup>[9]</sup>.

The identification of the protein amount of various samples, biological or not, is performed by means of proteomics <sup>[11]</sup>, which is the connecting link among genome, transcriptome and biological function <sup>[12]</sup>. The most frequent proteomic methods applied in cheese analysis are 2D-gel electrophoresis followed by MALDI-TOF-MS and HPLC-MS/MS, which are fundamental tools to perform proteomics on dairy products, whereas mass spectrometry is now the most widely applied technique for accurate quantitative and qualitative protein analysis <sup>[13]</sup>, see also Table 1. Mass spectrometry-based

proteomics relies on appropriate protein fractionation and protein or peptide ionization and MS-fragmentation [14]. The fragmentation pattern of a peptide demonstrates its amino acid sequence and any post-translational modifications (PTMs). By comparing with reference databases (especially SWISS-PROT and PFAM) peptides are assigned to full-length proteins [15]. Recently, proteomics assays have been applied in food analysis regarding microbial contamination, quality and adulterations [16][17][18], mainly in milk [19][20] and meat [21][22][23][24].

This review focuses on how proteomics assays have been used in order to distinguish proteins produced during ripening or after impurity in chosen PDO cheeses. Moreover, with proteomics methods proteins that act as bioactive molecules can be specified, thus raising the economic value of the cheese. The cheeses of interest in this review are related to Mediterranean diet, which has been well studied during the past few decades because of its beneficial properties to human health and well-being. In particular, researchers selected Feta, Graviera Kritis, Mozzarella di Bufala Campana, Parmigiano Reggiano and Grana Padano, which originate from Greece and Italy.

## 2. Feta

Since Ancient Greece, a cheese resembling Feta was produced, and it is believed that this cheese was Feta's ancestor. Especially a Greek historian, Diodorus (Diodorus Siculus; 1st century BCE), wrote that Aristeus, whose ancestors were Apollo and Zeus, father and grandfather, respectively, learnt the art of cheesemaking from the nymphs and was godsent to teach ancient Greeks how to make cheese (Diodorus, Library of History, IV 81.1–81.2) [25].

Feta is a semi-soft, white brined, PDO traditional Greek cheese (EU L277/2002), made from ovine or mixtures of it and caprine milk (up to 30%) accompanied by starter lactic acid bacteria. The cheese is produced all over the country [26]. Feta has a mildly acidic to bitter taste, with no gas holes in the body, but with desirable mechanical openings, (i.e., little undefined holes) resulting from the mechanical pressure applied during manufacturing. Feta has low pH and is made from curds that have not been heat-treated. Dry salting is applied, draining by gravity and ripening is carried out in brine with 7–8% salt for at least 2 months. Its mean gross physicochemical composition (w/w) is the following: moisture 55%, fat-in-dry matter, 51%; protein, 17.5%; salt in moisture, 5.2%; pH, 4.6 [27]. Ney [28] has reported that peptides with a hydrophobicity of >1400 cal per residue and a molecular mass ≤6000 Da are bitter. The beta-casein (b-CN) (f191–205) isolated from Feta cheese has a mean hydrophobicity of 1693 cal per residue and a molecular mass of 1669 Da. Thus, b-CN (f191–205) has likely a bitter taste, which it attributed to the bitterness of Feta [29]. Lactic acid bacteria (LAB), Enterobacteriaceae and coliforms are the major components of the microflora during storage of cheese. The counts of total nonstarter LAB (NSLAB) and *Lactococci* in curd, made during spring, increased initially during ripening for 24h and then declined gradually during storage for 6 months, respectively [25].

Milk caseins during ripening undergo hydrolysis, because of the action of proteolytic enzymes from the starters; therefore, new peptides are created, or the pristine peptides are transformed. The casein hydrolyzed by rennet combined with inorganic milk components, mostly calcium and phosphorus, create paracasein micelles [30]. The latter leads to a three-dimensional paracasein network, consisting of whey, fat and microorganisms; common in almost every cheese. Cheesemaking conditions are responsible for the pH, the concentration of the minerals and the ratio of paracasein to other cheese components. The ripening conditions in Feta are a crucial step, because of the caused changes in the paracasein network that affect all the final traits of the produced cheese. The impact of ripening is mostly seen in the protein content, which is altered by proteolysis affecting the stabilized bonds [31][32]. The contribution of hydrophobic interactions and hydrogen bonds in the structure of Feta paracasein is significant because of the changes in paracasein–calcium interactions induced by low pH and the proteolysis pattern, thus decreasing more extensively the calcium binding sites onto the paracasein elements.

## 3. Graviera Kritis

Graviera Kritis is a hard PDO cheese (EU L148/1996), produced in Crete, made from drained curds, ewe's milk or mixtures with caprine milk up to 20%, scalded up to 50 °C and pH 6.2–6.3, pressed, salted in brine and ripened for at least three months. The mean moisture content, fat-in-dry matter, protein, salt-in moisture, and pH of Graviera is about 33–35%, 53–58%, 25–27%, 4%, and 5.5–5.8, respectively [30][33][34][35].

As a way to keep Graviera authentic, the production process chosen is the traditional one, as described below. After milking, the milk is heated at a range from 33 to 36 °C and a quantity of rennet able to produce a coagulum within 30–40 min is added. Then, it is stirred and cut into small cubes. Afterwards, the curd is heated at 50 °C and then removed from the heat. After one hour of stirring the coagulum, in order to separate the whey from the cheese curds, salt approximately 1% is added and the cheese curd is left to settle and then collected using special cheesecloth. The curd is transferred to a

mold and initially pressed for 24 h with low pressure (1–2 times the weight of the cheese), which is gradually increased (to 12 times the weight of the cheese). For the same reason, the cheese is turned upside down three or four times during its drainage in the mold. The cheesecloth is also changed three times. After the cheese has been sufficiently drained, it is put on shelves in the ripening cellar (14–16 °C) for 14–15 days and every morning is inverted and salted. The cheese ripens for 3–4 months. Enterobacteriaceae and coliforms concentration was reduced in the curd after cooking [25].

In a study conducted by Panteli et al. [30] the Feta and Graviera Kritis' solubilized caseins were compared. In the paracasein matrix of Graviera high insoluble calcium, resulting from cheese's pH and low moisture, was found. The  $\alpha$ s1-casein and para-k-casein contribution in the urea extractants of Feta were significantly higher than those of Graviera. Therefore  $\alpha$ s1-casein interactions with calcium have a key role in Graviera.

## 4. Mozzarella di Bufala Campana

Mozzarella di Bufala Campana is an Italian Protected Designation of Origin (PDO) cheese according to EU product quality legislation [36] and it is a soft cheese made from fresh, raw milk of water buffalo (*Bubalus bubalis*) and natural whey starter cultures, in specific areas of Italy. In order to protect the uniqueness of the product a consortium of dairy producers, the Consorzio di Tutela della Mozzarella di Bufala Campana, acts to guarantee the authenticity of this PDO cheese by monitoring the whole production chain in hundreds of enterprises and marketing of the final product in compliance with the ordinance of the Italian Ministry of Agricultural, Food and Forestry Policies [21][37]. The production of Mozzarella starts by pasteurizing raw milk and then cooling it until the temperature descends to 37 °C. At this stage, thermophilic starters' culture is added to the milk followed by rennet addition and cutting the cheese curd, as the cheese name suggests. Afterwards the curd is cooked for 30–40 min at 35–40 °C and whey-drained. Cheddaring of cheese curd is applied, which leads to acid development up to 0.75% lactic acid (LA) of whey. More specifically, the curd is cut in many small pieces, which are stacked on top of each other, so that the majority of whey and moisture are pressed out. Then, the cheese curd is stretched in hot water 80–85 °C for 3–5 min, shaped in molds and kept in cold brine (20–23% brine strength) for a sufficient period [38].

## 5. Parmigiano Reggiano

Parmigiano Reggiano (PR) is a hard cheese with a protected designation of origin. It is made using a mixture of raw and skimmed cow milk and natural whey starters, mainly thermophilic S-LAB [39]. After cooking and brine salting PR is subjected to at least 12 months of ripening, during which NS-LAB progressively replaces the S-LAB population [40]. The production and maturation procedures of the PR cheese, according to the PDO, are detailed in Tagliazucchi et al. [41][42]. More specifically, the mixture of milk used consists of evening raw milk, partially skimmed by natural creaming, and morning whole milk, with the final fat/casein ratio not exceeding 1.1 + 12%. Milk is then supplemented with natural whey starter arisen from the cheese-making processing of the previous day and mainly composed by homofermentative thermophilic lactic acid bacteria (SLAB) (*Lactobacillus helveticus* and *Lactobacillus del-brueckii* ssp. *bulgaricus*/lactis), followed by *Streptococcus thermophiles* [43][44][45][46]. SLAB convert lactose into lactic acid that lowers the pH, affecting texture, moisture content and the subsequent caseins coagulation, which is carried out with animal rennet in a copper vat with a capacity of about 1000 L of milk. After coagulum formation and cutting, curd grain is cooked at temperature of approximately 55–56 °C, and the rest of the cooked curd remains under the hot whey for about 60 min [47][48][49]. Cheese wheels are salted by immersion in brine for approximately 20 days and, as a consequence, large gradients of salt and moisture develop within the cheese structure [50]. Finally, ripening is mandatory for at least 12 months.

## 6. Grana Padano

Grana Padano is an Italian PDO cheese and for its production 24% of annual cow milk is used, as it is the most PDO produced cheese in Italy (38% of the whole production) [51]. Grana Padano (GP) is an extra-hard cheese ripened from 9 to 20 months, depending on product category, produced in a defined area in Northern Italy [51]. The milk used is partly skimmed raw cow milk, in which calf rennet and natural whey cultures of thermophilic lactic acid bacteria as starter, are added [52][53]. The manufacture process of Grana Padano starts with creaming of raw milk at 10 °C and then the partially skimmed milk is heated to 33–34 °C, at which point rennet is added. After that the curd is cut and cooked again at 53–56 °C followed by whey extraction. Then, the curd is poured into molds and brined for 16–24 days. The final stage is ripening, whose duration lasts for 9–36 months [53].

In order to control the overpopulation of bacteria, such as Clostridia, during ripening and aging (late blowing defect) in Grana Padano, lysozyme treatment was proposed. The results revealed that enzymes, such as acetate kinase, linked to the previous phenomenon, decreased after treatment with lysozyme, which exerts antibacterial activity [54].

## 7. Starters and Proteomics

Cheese, as mentioned above, is a complex biological ecosystem, containing different microbial communities, originating from starter and adjunct cultures, raw milk and adventitious microorganisms from the production environment [55].

Functions and activity of these bacteria can be easily determined through analysis of genes and transcripts. The identity and quantity of proteins produced by the starters can be revealed using proteomics' methods providing data for the post-translational level of the activity and function of microorganisms and the associated enzymes. This information is critical for understanding how microorganisms function and the interactions created between the different strains and the remaining cheese compounds under challenging conditions. That kind of data can be described by means of metaproteomics, since it is the study of the protein amount produced by microbial communities in every environment in a specific time [56]. The challenge with metaproteomics analysis in animal food products is the presence of milk proteins and those produced by different microbiota in cheese.

### 7.1. Lactobacilli

*Lactobacillus* is the largest genus within lactic acid bacteria with over 170 species and subspecies and the most common starter used for fermented food products such as fermented dairy, meat and other foods, such as sourdough and yogurt enriched with probiotics. Aside from fermentation they are used as probiotics and in compounding bioactive and antimicrobial molecules [57][58][59]. The strains of *Lactobacillus* are recognized as safe for human consumption and they are able to metabolize various carbon sources into lactic acid, a pathway with great significance in food technology [58][60][61]. The ability of *Lactobacillus* to rapidly lower pH during lactic fermentation contributes to spoilage prevention and food shelf-life extension, while other metabolic activities improve organoleptic characteristics of food [62]. Since *Lactobacilli* are widely used in food industry and are linked to human health, many experiments have been designed using comparative proteomics, in order to reveal those adaptive and metabolic mechanisms under optimal laboratory and food-like conditions. The latter aims to recognize all those surface-exposed proteins, also known as secretome, that differentiate each strain and make it suitable for starter or probiotic [57].

One of these studies had been conducted by Bove et al. [53] and the authors tried to elucidate the metabolic and proteomic adaptive pathways of *L. rhamnosus* strains isolated at different ripening stages of Parmigiano Reggiano (PR). In order to mimic the ripening environment in PR the strains' cultivation was conducted under optimal conditions (de Man, Rogosa, and Sharpe (MRS) agar) or under environmental conditions (cheese broth, CB). The protein identification was performed with MALDI-TOF-MS and multidimensional liquid chromatography (MDLC) coupled to nano-ESI-MS/MS to obtain comprehensive understanding of the mechanism of environmental adaptation in *L. rhamnosus* strains [53]. A variety of compounds originating from ripening cheese, such as N-acetyl-D-galactosamine, D,L-lactic acid, thymidine, uridine, L-alanyl-L-histidine, glycyl-L-methionine, and FAA, are catabolized by many strains of *L. rhamnosus* [63][64]. The consumption of free amino acid was increased during the late stages of ripening by the predominant bacteria. The amount of proteins responsible for protein biosynthesis, such as Protein translation elongation factor (EF-G), Transcription elongation factor NusA (NusA), 30S ribosomal protein S3 (RpsC) and 30S ribosomal protein S6 (RpsF), was elevated in strains cultivated in laboratory conditions and isolated shortly after the beginning of ripening. CB grown strains (e.g., PR1473) showed increased levels of proteins (RPL1, EF-P, RP 50SL5, Rps10, and Adt) with similar function to the above-mentioned ones. During cheese ripening, the proteolytic system of lactic acid bacteria (LAB) degrades caseins into small peptides and FAA, which fulfill the nutritional and organoleptic requirements [65][66]. The results obtained from Bove et al. (2012) [53] indicated that the amount of protease (PepS16), aminopeptidases (PepA and MAP), endopeptidases (PepS, PepS24, and PepM16) and proline-specific peptidases (PIP and PepQ) was increased during cultivation on CB. The *L. rhamnosus* strains also demonstrated an increase in the amount of proteins responsible for amino acids catabolism (e.g., MetC, SDH), favoring the synthesis of lactic acid, acetic acid, and ATP via the pyruvate pathway.

Cultivation on cheese broth resulted in augmentation of the enzymes responsible for the catabolism of aspartic acid (AspAT and OadA), glutamic acid (GDH), arginine (ADI), aromatic amino acids (ArAT), methionine and cystathionine (MetC), branched-chain amino acids (BcaT) and serine (SDH), as well as proteins responsible for citrate catabolism, acetate production, proteolytic activity, and amino acid catabolism. A decrease was found in the amount of proteins responsible for sugar transport, glycogen biosynthesis, pentose phosphate pathway, exopolysaccharides (EPS) biosynthesis and cell wall biosynthesis [53].

The bacterial exoproteome of *Lactobacilli* is responsible for the hydrolysis of macromolecules, such as polysaccharides, proteins and anti-nutritional compounds; the uptake of nutrients, the communication with other prokaryotic and eukaryotic cells and the colonization of organic and inorganic surfaces [57][67]. For the detection of exoproteome gel-based and, especially, gel-free proteomics are mainly used [57][68]. The S-layer proteins (SlpA, SlpB, and SlpX) of *L.*

*acidophilus* strains have adhesive properties and they are involved in the adhesion to intestinal epithelial Caco-2 cells and induction of inflammatory cytokine IL-12 [57][69][70]. S-layer proteins of *L. acidophilus* and *L. helveticus* strains are also involved in TH-29 cells survival in simulated gastric and intestinal juices and in adhesion capacity of those [57][71]. Recently, LC-MS/MS analyses of the surface-associated proteome (surfaceome), released by trypsin shaving of *L. rhamnosus* strains, identified 102 and 198 proteins from GG and Lc705 strains, respectively [57][72]. Comparative surface-associated proteome (surfaceome) analysis pointed out differences between bacteria strains associated with adaptation to specific niches (response to bile, hydrolysis of casein, host response with immune functions). Most of the identified proteins were moonlighting proteins (e.g., DnaK, GroEL, EFTu, LDH, GAPDH, Eno, PGK). Specific moonlighting antigens were detected in *L. rhamnosus* strains [72]. Many moonlight proteins (e.g., GAPDH, Eno and EF-Tu) were also identified in the secretome of other *Lactobacillus* strains [73][74][75][76][77][78][79]. The mechanisms by which cytosolic proteins change location, regarding moonlighting function, are not completely understood [80]. The adhesion capacity of *Lactobacilli* was affected during growth and changes in the environmental conditions, as the composition of secretome altered [72][73][81]. Labeling of bacterial surface proteins and DIGE separation, followed by MS analysis of *L. rhamnosus*, highlighted that bile stress affected the abundance of surfaceome proteins (including DnaK, LDH, GAPDH, Eno, PG) [82] whereas proteins N-acetyl-glucosamine (GlcNAc) acetylase (NagA) and GlcNAc deaminase/isomerase (NagB) involved in the catabolism of GlcNAc were oversynthesized by *L. rhamnosus* GG and *L. casei* [82][83][84]. NagA and NagB were also oversynthesized by acid stressed *L. casei* cells [57]. Compared to optimal conditions of growth, surface antigen (LGG\_02016) and predicted cell wall hydrolase (NLP/P60 domain) of *L. rhamnosus* cells were upregulated under acidic conditions [85]. *Lactobacilli* cells (e.g., *L. plantarum* DB200) increased the levels of surface proteins like bacteriocins, molecular chaperones, enzymes, lipoproteins, and surface layer under biofilm conditions [74][75][86]. In general, proteins, which are associated with carbohydrate binding and metabolism, protein synthesis, lipid degradation, amino acid binding, and stress tolerance, are found in *Lactobacillus* spp. [57].

## 7.2. Lactococci

*Lactococci* is another family of bacteria used as starters or developed during ripening in cheeses. Studies conducted on Feta cheese have reported the heterogeneity of “wild” *Lactococci* strains used as starters at the production chain in different regions. Identification of the isolates was performed by comparison of their protein patterns to the fingerprints of the reference strains by means of phenotypic criteria and the SDS-PAGE of cellular proteins assay. The non-starter lactic acid bacteria (NSLAB) that are used in household level for the manufacture of Feta in many areas, are usually *Lactococci* strains. During maturation the NSLAB microflora may differ depending from the area of origin and display various activities, with the most interesting being the inhibition of undesirable bacteria through competitiveness, which plays a key role in food and consumers’ safety. Moreover, principal component analysis (PCA) confirmed that acidifying, proteolytic and autolytic activities vary according to the district of origin, leading to different maturation and flavor traits of the produced Feta. Thus, a link seems to be formed between the area of production, the composition of NSLAB and their biochemical properties, which allows the selection of the strain used for starter for cheese production [26].

*Lactococcus* spp. are known to hydrolyze caseins and their micelles [87]. The formation of as1-CN (f40–49) is difficult to explain on the basis of the known specificity of chymosin toward Leu40-Ser41, but not toward Glu39-Leu40 [88]. The nearest bond that has been reported to be hydrolyzed in solution was Val37-Asn38 by a PIII-type proteinase of *Lc. Lactis* ssp. *cremoris* SK11 [89]. Although residue 37 is Ile in the ovine polypeptide chain [87], the Ile37-Asn38 bond would likely be still cleaved by a starter proteinase, and, thereafter, the two residues Asn38 and Glu39 would be released by aminopeptidase or aminopeptidases, several of which are present in *Lactococcus* spp. [29][90][91]. The peptide eluted in peak 5(I) was b-CN (f164–180). The N-terminus suggests that this peptide originated from cleavage of Leu163-Ser164, a probable site for chymosin action in solution and in cheese [29]. This bond is also susceptible to the action of lactococcal cell-wall proteinases [29]. In ovine b-CN, the residues Pro179 and Tyr180 are deleted. Therefore, Gln180-Arg181 in the primary sequence of ovine b-CN corresponds to the Gln182-Arg183 of bovine b-CN, the latter being cleaved by the cell-wall-associated proteinases of *Lactococci* [29]. The isolation of k-CN peptides from cheese has not been reported. In the water soluble fraction of Feta cheese, however, a peptide corresponding to k-CN (f96–105) was found and presumably originated from para-k-CN (k-CN (f1–105)), and its formation could be the result of the action of lactococcal proteinase at Met95-Ala96, which exhibits the characteristics of a susceptible cleavage site for such an enzyme [29][92]. This histidine-rich peptide is of particular physiological significance because His is known to be an essential amino acid for *L. lactis*.

In a metaproteomic analysis with use of isobaric tags for relative and absolute quantitation (iTRAQ) labeling reagents, it was found that proteins that originated from bacteria were present in Swiss-type cheese [11][53]. The concurrent presence of proteins that originated from milk and cheese microbiota complicated the metaproteomic approach. The protein content coming from starter bacteria (*L. helveticus* and *S. thermophilus*) increased during ripening [57][93]. The reference map of

mixed proteins belonging to microbial starter and the cheese proteins 2DEmap comparison showed that some peptidases from *L. helveticus* and *S. thermophilus* were released into the cheese. Thus, metaproteomics approaches were proposed as a means for the determination of the microbial impact on the human health and disease progress [94][95][96].

## 8. Bioactive Peptides

In the past few years there has been a rising need in the industry for reagents that can be received through food and can contribute to improving and maintaining the status of wellness and prevent the appearance of chronic diseases. The need for wellbeing regimes can be obtained by consumption of various meat and vegetable products [97]. Peptides are small molecules with weight less than 10 kDa and can be found naturally in foods or occur by chemical or enzymatic hydrolysis of the parent proteins [98]. Usually peptides are latent, when encrypted into proteins, and become active, when released after proteolysis or digestion in gastrointestinal (GI) tract [99]. They affect many physiological functions in the human body, such as gastrointestinal, cardiovascular, immune, endocrine, and nervous pathways. One of the most important, and accessible to the majority of people, sources of beneficial peptides is milk and dairy products in general [100][101]. As previously discussed, these peptides are well protected in the protein matrix of milk, requiring proteolytic enzymes, originating from various sources, such as natural rennet or starter bacteria, for their release. A growing body of evidence indicates that milk and dairy products have unique metabolic, signaling and antimicrobial effects, apart from their high nutritional content [102][103][104][105]. Such peptides are involved in many physiological activities, including regulation of inflammatory and immune response, signaling and metabolic process, antihypertensive, antioxidant and antimicrobial properties [105].

According to previous studies, pasta filata cheeses (Caciocavallo and Mozzarella) have antibacterial peptides [106]. Rizello et al. [106] conducted in vitro simulated gastrointestinal digestion on six buffalo dairy products (Grana, ice cream, yoghurt, Mozzarella, Ricotta and Scamorza) and the isolated peptide digests were characterized by high resolution mass spectrometry, followed by a database-driven specific bioactivity assessment for each identified sequence. The study revealed a great amount of potential bioactive peptides, with characteristics promoting wellness, including antihypertensive, immunomodulatory, antimicrobial, antidiabetic, anticancer and antioxidant. The different manufacturing process of each product explained the diversity of the released peptides.

Antimicrobial peptides (AMPs) with resistance in proteolysis have a direct impact on the gut microbiome assisting to control dysbiosis, by suppressing opportunistic pathogens, such as *Helicobacter pylori* [107], *Escherichia coli* and *Staphylococcus aureus* [108], so that the GI tract can remain healthy. The milk and dairy products from different animal breeds and species have unique compositions of bioactive peptides offering a broad range of sequences to screen for peptides with functional traits of medical and scientific interest [109]. Tomazou et al. [102] focused on the potential antimicrobial properties of Feta cheese, to probe for AMPs following an assessment of their stability in an intestine-like environment and they characterized the antimicrobial “load” of the proteomes of interest. Protein sequences from Feta cheese were screened using the publicly available tool AMPA [110][111] to find sequence stretches with predicted high antimicrobial potential (i.e., low AMPA propensity). The same protein sequences were digested in silico to identify which peptides, that can actually occur in the GI tract, matched the predicted AMPA stretches. The authors stated that Feta cheese proteome had 63–64 AMPs the same with the milk from the sheep and goat breeds, used to manufacture Feta. Albeit the small proteome size of Feta cheese, the proteome presents to have unique antimicrobial properties and resistance in proteolysis AMPs in quite a large amount. Recent work has suggested that lactic acid microbes have a central role in the release of encrypted bioactive peptides during ripening [112].

The *Lactobacillus helveticus* CP790 extracellular protease hydrolyze both  $\alpha$ s1- and  $\beta$ -CN of sour bovine milk resulting in the release of angiotensin converting enzyme (ACE) inhibitory peptides. These peptides exhibited antihypertensive activity in spontaneously hypertensive rats as monitored by systolic blood pressure as many studies indicated [113][114][115][116][117]. Especially, biological activity analysis revealed great variation among samples of Parmigiano Reggiano with the average ACE-inhibitory and antioxidant activities to be elevated in the LL (low salt–low fat) group compared with the HH (high fat–high salt) group. The differences observed are attributed to factors such as milk quality, somatic cells content and extensive proteolysis by indigenous proteolytic enzymes [118][119][120].

Although there are many ways for the presentation of inhibition of ACE, the results of Solieri et al. [41] were in accordance with that reported by Bütikofer, Meyer, Sieber, and Wechsler [121] for hard and semi-hard cheeses and they were comparable. According to the study [41] the results on DPP-IV-inhibitory activity were lower than those previously reported for the peptide fraction of gouda-type cheese [122], whereas antioxidant activity data were comparable to those already reported in the literature for PR and Cheddar cheeses’ peptide fractions [123][124]. The authors reported for the first time 25 peptides with potential bioactive properties from the total of 40 identified peptides for Parmigiano Reggiano. The



remaining 15 peptides were reported in different ripening times [125]. In fact, 13 peptides were ACE inhibitors and already described for their ability to reduce blood pressure in vivo [6]. In more detail, the  $\beta$ -casein-derived peptide KVLPVPQ, isolated from a commercial functional yogurt, demonstrated strong antihypertensive effects in spontaneously hypertensive rats [114][126]. The peptide LHLPLP, previously found in various cheeses such as Grana Padano, Parmigiano Reggiano, Gorgonzola, and Cheddar, exhibited very low IC50 value against ACE and has been found able to decrease blood pressure in spontaneously hypertensive rats [127][128][129][130]. Additional peptides found in all of the examined PR samples showed potent ACE-inhibitory activity, similar to the peptides NLHLPLLL, YPFPGIPN, and the peptide YQEPVL [131][132][133]. Some other peptides exhibited a high inhibitory effect against ACE and in vivo antihypertensive effect. The S1-casein-derived peptide YKVPQL was detected in the majority of PR samples and has been previously identified as an in vitro and in vivo antihypertensive peptide [129]. Similarly, the  $\beta$ -casein-derived peptide HLPLP has exhibited in vitro and in vivo antihypertensive effect [134]. The lactotripeptides VPP and IPP, antihypertensive molecules in vivo in humans, were also found in all PR samples [135]. For the ACE the same standards and peptides have been identified in Parmigiano and Grana [131]. No information is so far available on cheeses, in which antimicrobial peptides occurred after milk proteins' hydrolysis and interaction with cheese microflora [136]. Knowledge of the sequence plays a fundamental role in the studies of food derived bioactive peptides and may help in the identification of the peptide source. Moreover, it can serve as the starting base for the preparation of synthetic analogues to improve the nutritional characteristics of food products. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was adopted for the identification of bioactive peptides in food matrices using a bioinformatics approach, [137][138][139][140]. An ion trap was used to generate MS/MS and, when required, MS3 spectra, whose potential in solving structural ambiguities in peptide sequences arising from MS/MS measurements/database search has recently been proved to be very successful, leading to the identification of 45 different peptide sequences in fractions of cheese extracts displaying antimicrobial activity [141]. All the peptides were found to be generated by the hydrolysis of milk caseins (of different mammalian species, according to the type of milk used to produce the cheese), which typically occurred in specific regions of the proteins already known for the presence of bioactive amino acid sequences.

Antithrombotic peptides are also present in milk. The mechanisms involved in blood and milk clotting are proportional, leading to the hypothesis that the C-terminal dodecapeptide of human fibrinogen  $\gamma$ -chain (residues 400–411) and the undecapeptide (residues 106–116) from bovine  $\kappa$ -CN are structurally and functionally quite similar. The casoplatelin is produced by hydrolysis of bovine  $\kappa$ -casein with chymosin and shows antithrombotic properties [142]. This casein-derived peptide sequence affected platelet function and inhibited both the aggregation of ADP-activated platelets and the binding of human fibrinogen  $\lambda$ -chain to its receptor region on the platelets' surface [143]. A smaller  $\kappa$ -CN fragment (residues 106–110), casopiasirin, was obtained from trypsin hydrolysates and exhibited antithrombotic activity by inhibiting fibrinogen binding [143][144]. A second segment of the trypsin  $\kappa$ -CN fragment, residues 103–111, inhibited platelet aggregation but did not affect fibrinogen binding to the platelet receptor [143][145][146]. Other studies concluded that bioactive peptides isolated from both casein and lactotransferrin had antithrombotic properties by affecting platelet function [147][148]. Antithrombotic peptides have also been derived from  $\kappa$ -caseinoglycopeptides, that were isolated from several animal species. Bovine  $\kappa$ -caseinoglycopeptide, the C-terminal end of  $\kappa$ -CN (residues 106–169), inhibited von Willebrand factor-dependent platelet aggregation [149]. Two antithrombotic peptides, derived from human and bovine  $\kappa$ -caseinoglycopeptides, have been identified in the plasma of 5d-old neonatals after breast-feeding and ingestion of cow's milk-based formula, respectively [150]. The C-terminal residues (106–171) of sheep  $\kappa$ -casein, or  $\kappa$ -caseinoglycopeptide, decreased thrombin- and collagen-induced platelet aggregation in a dose dependent manner [151]. Finally, thrombin-induced platelet aggregation was inhibited by pepsin digests of sheep and human lactoferrin [152].

Casein phosphopeptides (CPP) are the result of trypsin action in  $\alpha$ 1-,  $\alpha$ 2-, and  $\beta$ -CN [153]. CPP are stable molecules even after gastrointestinal digestion and create a high soluble complex with calcium phosphate [154]. Animals fed with casein diet displayed elevated capacity to absorb calcium through distal small intestines compared to animals fed with soy-based diet [155][156][157]. Calcium physiological is absorbed through passive transport system and this is the way to supplement the human/animal organism with the calcium required for calcification [158]. Caseinophosphopeptides inhibit caries lesions through recalcification of the dental enamel, so their application in the treatment of dental diseases has been proposed [159].

Buffalo (*Bubalus bubalis*) milk contains a lot of bioactive peptides and they have a significant role preventing various disorders [160][161][162][163]. Bioactive peptides with antioxidant properties, regulation of oxidative stress in intestinal epithelial cells and erythrocytes, have been isolated from buffalo milk-derived products [164][165]. Among these, MBCP, a peptide isolated after in vitro digestion of Mozzarella di Bufala Campana DOP, has shown good stability to brush border exopeptidases and a high bioavailability [166]. In a study conducted by Tenore et al. [165], the therapeutic potential of MBCP in inflammatory bowel disease (IBD) was discussed after submitting Caco-2 cell, which resembles colonic enterocytes [167], in in vitro digestion conditions. In mammals, enterocytes are renewed continuously every 4–8 days through an

organized cycle involving proliferation, differentiation and programmed cell death. The proliferation to differentiation transition (PDT) is a critical step in the continual renewal of a normal intestinal epithelium [166]. Indeed, Caco-2 cells express tight junctions, microvilli, enzymes and transporters functionally similar to colonic enterocyte [167]. Moreover, this cell line has the capacity to trigger a pro-inflammatory reaction in response to stimulants like TNF- $\alpha$ , a known mediator of gastrointestinal mucosal barrier injury [168][169]. The results from the latter study revealed that MBCP can modulate the differentiation and permeability in Caco-2 cells stimulated with TNF- $\alpha$  and to attenuate inflammation and hypermotility in murine models of intestinal inflammation. Borelli et al. [170][171] stated that intracolonic administration of DNBS induced intestinal inflammation associated to an increase of epithelial permeability, symptoms that were inverted by oral MBCP administration as demonstrated by the reduction of colon weight–colon length ratio, histological alterations, IkB $\alpha$  phosphorylation and of NF- $\kappa$ B activation associated with DNBS administration. Intestinal permeability is a predisposing factor for the development of IBD, as well as in the IBD ongoing bowel symptoms [168][172]. Moreover, inflammation reduces barrier integrity and affects the normal intestinal permeability [173]. Studies on Caco-2 cells have proved that MBCP restored tight junctions altered by TNF- $\alpha$ . Tight junctions are multiprotein complexes that maintain the intestinal barrier while regulating permeability [174]. Intestinal permeability increased after DNBS administration and, more importantly, MBCP restored the impaired permeability. It is clinically well established that inflammation in the gut causes debilitating symptoms due to motility disturbances [175], which were balanced after treatment with MBPC, without causing other side-effects, like constipation [176][177]. In conclusion, the data obtained in the discussed above study indicated that MBCP, a peptide isolated from Mozzarella di Bufala Campana DOP, exerts anti-inflammatory effects both in vitro and in vivo, which is related to its beneficial activity on adherent junctions mainly during an inflammatory process and it could be used as therapeutic or supportive treatment in intestinal inflammation and possibly reducing colorectal cancer risk.

Apart from the MBCP, antimicrobial peptides are generated by digestion of buffalo Mozzarella. Characteristic examples are two  $\kappa$ -CN derived peptides (YYQQKPVA, f64-69; YYQQKPVA, f64-70) with antimicrobial activity against *E. coli* ATCC 25922 [178], and two other peptides, casecidin 17 and casecidin 15, that were identical to sequences in the C-terminal of bovine  $\beta$ -casein (YQEPVLGPVRGPFPIV,  $\beta$ -CN f208-224; YQEPVLGPVRGPFPI,  $\beta$ -CN f208-222). Conclusively, buffalo Mozzarella is a great source of antimicrobial peptides and it might be used as a supportive treatment to the classic antibacterial approach [164].

Lactic acid bacteria exert the ability to downregulate the expression of virulence genes of enteropathogenic bacteria [179][180][181][182]. In a study conducted by Ali et al. [179], cell free spent medium (CFSM) collected from whey protein (WPI) fermented by *L. helveticus* LH-2 and *L. acidophilus* La-5 reduced the expression of both the *hlyA* and *ssrB* genes of the *S. Typhimurium* DT 104 wild strain. Among the two strains, *L. acidophilus* La-5 had the most significant downregulatory effect on the virulence genes, which acts as an indicator that antivirulence capacity depends on the strain and is affected by the nature and components of CFSM. WPI fermented by La-5 contains nine unique peptides not found in LH-2-fermented or unfermented medium as the results from the same experiment revealed. The downregulatory effect of the synthetic peptide mixture shows the antivirulence effect of specific peptides produced by *L. helveticus* (LH-2) and *L. acidophilus* (La-5 CFSM) on the *S. Typhimurium* virulence genes. In *Salmonella* spp., the antivirulence activity of milk protein derived peptides is related to the presence of the oligopeptide-binding protein (OppA) gene. The undigested and fermented WPI by LH-2 and La-5 strains could be considered as a possible source of natural and functional components, which may be used to increase the biological activity of food products [179]. The endogenous milk proteases can act synergically to LAB, which are plasmin, elastase and cathepsin D, B, and G and are still active, as the presence of peptides in unfermented WPI suggests [183]. Their role is to release bioactive compounds. Plasmin has little or no activity toward  $\kappa$ -casein and whey proteins [184], so the presence of endogenous peptides from these proteins in CFSM of *L. acidophilus* La-5 and *L. helveticus* LH-2 could be attributed to the action of other milk proteases such as cathepsin B, D, and G [185][186]. Dallas et al. [187] concluded that minimal proteolysis by native milk enzymes continued to function during incubation in the heat-treated milk, when compared with that carried out by the proteases of kefir microorganisms, which were mainly *L. acidophilus* and *L. helveticus*.

## **9. Adulteration and Proteomics**

Over the past few years there is a growing consumers' movement regarding products' originality and their distinction from their counterfeit counterparts. Moreover, consumers are interested in the correlation of food products and history. An excellent example regarding history and cheese, is Feta and its ties with ancient Greece through unique traits and characteristics. Anagnostopoulos and Tsangaris [188] tried to gather the full protein content of Feta cheese by employing exhaustive deep-proteome analyses using LC/MS-MS. They collected Feta samples from every Greek area that produces PDO labeled Feta cheese and reported the complete list of Feta cheese proteins. The analysis of Feta contains 489 distinct proteins and eventually this method can be used as an identification tool of the authentic Greek product [188].



Other studies tried to elucidate the caseins behavior through ripening of Feta cheese. Michaelidou et al. [29] stated that in Feta cheese  $\alpha$ s1-CN seemed to be further hydrolyzed; a decrease has been observed during ripening. The formation of  $\alpha$ s1-CN is important because it indicates that the N-terminus of  $\alpha$ s1-CN has been hydrolyzed during the ripening of Feta cheese. Para- $\kappa$ -casein is the hydrophobic part of  $\kappa$ -casein and is a component of the paracasein matrix in cheese curd. Chymosin or rennet are added to form the coagulum and the residuals of the enzymes have proteolytic action to the paracasein matrix, with  $\alpha$ s1- and  $\beta$ -caseins being the most susceptible whereas  $\alpha$ s2- and para- $\kappa$ -casein rather resistant to their action during cheese ripening. Alexandraki and Moatsou [189] stated that the greatest part of para- $\kappa$ -casein remained intact during ripening and storage of Feta with high residual chymosin activity, although other studies indicated the opposite [190][191]. The positive aspect in their findings is that since para- $\kappa$ -casein remains intact, it can be used for the quantification of the composition of mixtures of sheep and goat cheese milk and other adulterations, even though the para- $\kappa$ -casein is similar but not identical in the two species. The latter characteristic was proved by cation-exchange HPLC. In particular, sheep and goat para- $\kappa$ -casein were efficiently separated, and the changes of their chromatographic areas indicated that hydrolysis happened during the early stages of ripening. Thereafter, and in accordance to the evolution of free amino groups, para- $\kappa$ -caseins remained stable [189].

One of the main consumers' concerns is the milk origin of the PDO cheeses they are buying, which also is applied to Mozzarella di Bufala Campana. One of the cheesemaking rules is that the milk must come from Mediterranean water buffalo raised in Italy. Caseins are fully incorporated in cheese and they can create a "fingerprint" useful for the identification of milk samples origin [10]. Mediterranean Italian buffalo is reared in Italy for centuries and is a pure breed without any crosses with animals from other countries, as being indicated by their casein polymorphism absence. The  $\beta$ -CN locus was found to be monomorphic [192] and  $\alpha$ s1-CN showed only two silent variants [193] in Mediterranean Italian buffalo while milk samples from Romanian, Canadian, Polish and Venezuelan WB displayed additional CN variants, such as  $\beta$ -CN A and  $\alpha$ s1-CN with eight internally deleted amino acid residues in positions 35–42 [192]. The new caseins variants have been detected in all the foreign countries, but not in the Mediterranean area, proving that WB herds in the Mediterranean area are pure without any crossbreeding, thus proving that the region of milk origin is significant for the PDO labeling in Mozzarella di Bufala Campana [10].

The above-mentioned traits of WB caseins allowed Caira et al. [10] to develop a new laboratory approach through an analytical detection method using the MALDI-TOF-MS data of signature peptides from wild and variant WB and bovine CN, thus allowing the identification of milk adulterations with non-Italian WB or bovine milk in commercial Mozzarella di Bufala Campana cheese samples [10].

A great increase in Mozzarella di Bufala Campana production demands has been observed in the past few years, which has not been followed by an increase in Mediterranean WB milk availability [21]. Furthermore, the price of buffalo milk varies within each year, creating an unstable economic environment for Mozzarella's producers. In order to deal with these challenges, dairy owners turn to non-compatible with PDO rules and techniques. In more detail, they use frozen curd as a substitute for fresh milk, something highly prohibited by European Union laws, resulting in different flavor and other traits compared to the original product. To restrict this fraudulent procedure new laboratory methods were applied [194][195], which identified the marker  $\beta$ -casein fragment (69–209) [194][195][196], resulting from protein proteolysis as result of the activity of endogenous plasmin [197][198][199], in curd. Dedicated controls in Italy from the Anti-Sophistication Nucleus for foods revealed recently this technological approach to become progressively adopted for Mozzarella DOP production [21]. In fact, this adulteration practice allows for several stable proteolytic and lipolytic enzymes from psychrotrophic bacteria to induce non-desired coagulation of milk proteins and production of unwanted organic compounds [200][201][202][203], affecting final Mozzarella organoleptic characteristics. Nineteen discriminant marker signals in frozen buffalo milk with respect to the fresh counterpart were linked to specific polypeptides/proteins based on literature data [19][194][196][204][205], mass value calculations, and additional mass spectrometric investigations. Among protein/polypeptide marker signals identified by MALDI-TOF-MS profiling and worth mentioning are those related to  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin and  $\beta$ -casein coupled with the ones associated with GLYC-AM1- and  $\beta$ -casein-derived fragments which possibly originated as a result of the activity of proteolytic enzymes in buffalo milk.

In view of this fact and in order to find a parameter to assess the presence of cow or ewe milk in samples of water buffalo Mozzarella cheese, attention was focused on the mass region of the whey proteins. As the molecular masses of cow, ewe and water buffalo whey proteins are very different and due to the MALDI-TOF-MS resolution in this mass region (14–19 kDa) being high enough to resolve them, lactalbumins and lactoglobulins have been used as biological markers for the evaluation of possible fraudulence in water buffalo Mozzarella cheese production. The mass difference between buffalo and ewe whey proteins is  $\frac{3}{4}$ 78 Da for lactalbumins and  $\frac{3}{4}$ 116 Da for lactoglobulins, therefore making it possible to use both proteins for the evaluation of ovine milk sophistication of fresh water buffalo Mozzarella cheese [206]. The situation is very similar for the MALDI-TOF-MS analysis of Mozzarella prepared with a 90:10 mixture of water buffalo and ewe milk.

Even in the case of two mass ranges presented (14–18 kDa), the mass signals of water buffalo and ovine milk proteins are well separated, and the existence of ewe milk can be readily evaluated. The study of other Mozzarella cheese samples obtained from different percentages of ewe milk added to water buffalo milk demonstrate that the response of the instrument is linear up to a limit of 2%.

The EU reference methodology to detect bovine proteins in dairy products is based on gel isoelectric focusing of g-caseins after plasminolysis (EC Regulation No. EC 273/2008) with a detection limit for bovine milk in buffalo cheese products of about 1%. However, the overlapping of species-specific bands generates complex protein profiles, thus impairing the interpretation of results. To overcome the detection of false positive responses, an additional laborious immunoblotting step may be performed [9][207]. The phosphorylated b-casein f33-48 tryptic peptide was identified as a novel species-specific proteotypic marker whose limit of detection was three orders of magnitude lower than that declared by the methodology officially recognized by the European Commission (Reg. CE n. 273/2008). The high sensitivity of MRM-based mass spectrometry and the wide dynamic range of triple quadrupole spectrometers provide a valuable tool for the analysis of complex matrices such as dairy products.

## 10. Conclusions

This entry is an attempt to summarize the use of proteomics methods in cheese manufacturing process, as well as in cheese per se, offering useful methods for cheese analysis. The knowledge of protein content in each PDO cheese is a useful tool in order to avoid misleading of consumers and to valorize the products. Moreover, it provides information about their bioactive peptides and antimicrobial properties ensuring the consumer's health. The overall conclusion is that cheese is a very useful component of Mediterranean diet offering unique elements and nutrients.

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