SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE E TECNOLOGIE DELLE PRODUZIONI AGRO-ALIMENTARI

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DIPARTIMENTO DI SCIENZA DEGLI ALIMENTI

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"Production and evolution of peptides naturally derived from milk protein hydrolysis or generated by technological processes: proteomic and bio-functional characterization"

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Alla mia famiglia,

RIASSUNTO

Il progetto di dottorato è stato condotto presso il Dipartimento di Scienza degli Alimenti della Facoltà di Agraria della Federico II. Per la realizzazione di tale progetto sono state instaurate delle collaborazioni con alcuni laboratori: in particolare si è collaborato con l'Istituto di Scienza dell'Alimentazione ISA-CNR di Avellino per la parte riguardante la caratterizzazione peptidomica dei sieri di lavorazione della mozzarella di bufala e con il Dipartimento di Biochimica e Biofisica della Seconda Università di Napoli per lo studio bio-funzionale delle componenti peptidiche estratte dai sieri di lavorazione su cellule umane di adenocarcinoma del colon CaCo2.

L'obiettivo di questo lavoro di Dottorato è stato quello di sviluppare una strategia analitica che, mediante l'applicazione di procedure di tipo proteomico/peptidomico, consentisse di valutare la presenza, l'evoluzione e la bioattività dei peptidi generati sia per effetto proteolitico indotto dal processo tecnologico che per quello dovuto alle proteasi endogene al latte.

Per questo motivo, il lavoro di tesi si articola in due capitoli, ciascuno per le rispettive tematiche sopra menzionate:

I. studio dell'evoluzione dei pattern peptidici prodotti durante il processo tecnologico di produzione della Mozzarella di Bufala Campana DOP nei sieri di caseificazione a partire dal latte di bufala.

II. studio dei profili peptidici in campioni individuali di latte di asina.

Per quanto riguarda il primo capitolo di questa tesi di Dottorato, è stato sviluppato un approccio integrato di tipo proteomico e bio-funzionale al fine di:

1) delineare mediante approccio proteomico il pathway di formazione dei peptidi a potenziale attività biologica lungo l'intera filiera di lavorazione della Mozzarella, seguendone la produzione a partire dal latte di bufala e passando per il siero dolce, per il siero acido fino ad arrivare alla scotta.

2) studiare l'effetto di modulazione delle componenti peptidiche estratte sulle cellule CaCo2 in termini di attività antiproliferativa preliminarmente e, successivamente, di ulteriore approfondimento del biochimismo coinvolto nella risposta cellulare per le frazioni risultate più attive.

Per quanto riguarda il primo punto in cui è articolato il I capitolo, è stato messo a punto un metodo di estrazione delle componenti peptidiche a basso peso molecolare, basato su un processo di ultrafiltrazione su membrane seguito da estrazione in fase solida, pertanto senza l'utilizzo di precipitanti chimici. Il latte di bufala (BM) della mungitura mattutina, il siero dolce (BSW) ottenuto appena dopo la coagulazione del latte, il siero acido (BWW) residuo alla maturazione della cagliata e la scotta (BS), nella fase di campionamento in caseificio, sono stati immediatamente aggiunti di inibitori di proteasi per ovviare al progredire di fenomeni proteolitici indesiderati durante la successiva fase di estrazione. La caratterizzazione proteomica dei profili peptidici così estratti ci ha consentito di constatare che sebbene BM disponga di un profilo peptidico intrinseco prodotto dall'azione delle proteasi endogene al latte, la proteolisi durante la produzione della Mozzarella di Bufala Campana DOP, contribuisce ad aumentare ulteriormente la complessità e l'eterogeneità di ciascun estratto peptidico. La caratterizzazione strutturale ci ha permesso di concludere che la proteolisi del latte è il risultato di una complessa serie di eventi dovuti all'azione combinata di proteasi native o endogene al latte, di enzimi della coagulazione del latte e microflora contaminante. Dalla comparazione dei profili peptidici di BM, BSW, BWW e BS emerge che quello di BWW è alquanto eterogeneo, con un gran numero di oligopeptidi diversi tra cui alcuni di essi con sequenze bioattive criptate.

Il secondo punto del I capitolo, è stato delineato partendo dalla considerazione che lo studio *in vitro* dell'interazione tra modelli cellulari e bio-molecole rappresenta attualmente una metodologia analitica innovativa per le scienze dell'alimentazione. Le cellule epiteliali del tratto gastrointestinale, a differenza delle cellule di altri tessuti, sono direttamente esposte al contatto con micro- e macronutienti assunti con l'alimentazione; l'intestino si trova all'interfaccia tra l'organismo e il suo ambiente lumenale, rappresentando una barriera difensiva fondamentale contro gli agenti tossici. Le cellule epiteliali di adenocarcinoma umano del colon (CaCO2) sono state utilizzate come modello per studiare *in vitro* la potenziale attività citomodulante dei peptidi estratti da BM, BSW, BWW e BS. Preliminarmente all'incubazione con gli estratti peptidici, le CaCo2 sono state indotte allo stress ossidativo mediante trattamento con H_2O_2 (H-CaCo2) al fine di simulare la risposta delle cellule intestinali, costantemente esposte ad agenti ossidanti durante la digestione degli alimenti.

Solo l'esposizione delle H-CaCo2 a BM (1 mg/ml) e BWW (1 mg/ml) comporta una riduzione della proliferazione cellulare quantitativamente più significativa in quest'ultimo caso: BWW, infatti, riduce del 43% la proliferazione cellulare. Tuttavia, la constatazione che BWW è un

"rifiuto organico speciale" ed un inquinante ambientale con valore commerciale trascurabile rispetto al latte di bufala, ci ha spinto ad approfondire esclusivamente l'attività di BWW sia in termini di riduzione degli anioni superossido mitocondriali (6,2% per il controllo e 3,7% con BWW), che di modulazione del ciclo cellulare. Questi risultati suggeriscono che BWW è in grado di inibire la proliferazione cellulare interferendo con il ciclo cellulare ed esercitando una possibile attività pro-apoptotica *in vitro*. La reiterazione dei test biologici con sub-frazioni semplificate ottenute da RP-HPLC di BWW, ci ha permesso di dimostrare che solo una sub-frazione peptidica (f3) esplica un effetto antiproliferativo superiore a BWW riducendo gli anioni superossido mitocondriali con conseguente diminuzione dell'espressione delle proteine Hsp (Heat Shock)70 e Hsp90 nelle cellule sopravvissute. E' comunemente noto che le cellule CaCo2 sono difficili da trattare e proliferano abbastanza velocemente anche in condizioni avverse per le cellule normali. Inoltre esse esprimono costitutivamente alti livelli di Hsp70 e Hsp90 anche in condizioni di non-stress: questo è alla base della loro elevata resistenza ai trattamenti immunologici e chemio terapeutici. Dunque la riduzione di Hsps indotta dal trattamento con f3 rende le CaCo2 significativamente più sensibile agli effetti dannosi degli agenti ossidanti. Inoltre, la diminuzione dell'espressione di ciclina A e l'arresto del ciclo cellulare nella fase G1/G0 indotte da f3, sono state associate con un aumento dell'attività della fosfatasi alcalina e di beta-galattosidasi A, marcatori di differenziazione e di senescenza, rispettivamente. In questa fase, l'analisi di spettrometria di massa della frazione solubile in cloroformio dei mezzi di coltura cellulari, ci ha consentito di individuare sfingolipidi tra cui ceramidi e cerebrosidi che esplicano normalmente la funzione di secondo messaggero cellulari: l'aumento della loro concentrazione intracellulare in risposta a segnali extracellulari induce l'arresto delle cellule e l'inibizione della crescita delle cellule tumorali. f3 aumentando la secrezione di ceramidi, provoca l'arresto del ciclo cellulare, la differenziazione, e una successiva morte cellulare per "senescenza accelerata".
In conclusione, l'effetto biologico di f3 ne lascia ipotizzare un suo possibile utilizzo come nutraceutico una volta che un opportuno processo industriale di purificazione è stato messo a punto a partire da una materia prima a costo zero o nullo, quale BWW. Quest'ultimo,infatti è attualmente considerato un rifiuto organico speciale che pone non pochi problemi per il suo smaltimento.

Nel secondo capitolo di questa tesi di Dottorato, invece, la strategia di tipo proteomico è stata applicata a 18 campioni di latte di asina individuali forniti nell'ambito del progetto SELMOL.

L'approccio peptidomico mediante spettrometria di massa MALDI-TOF-MS, ha permesso di caratterizzare la componente peptidica in ciascun latte di asina individuale e di individuare, in funzione dei cluster peptidici generati, gruppi omogenei di campioni tra loro simili; negli stessi campioni è stata valutata l'attività antiossidante in modo da determinare il ruolo dei peptidi del latte d'asina nel prevenire le reazioni di perossidazione lipidica.

Dalla valutazione complessiva dei pattern peptidici delineati, è emerso che l'attività delle proteasi endogene al latte di asina si esplica con una specificità di azione intrinseca se comparata a quella studiata per il latte di bufala. In particolare è risultato che:

- in nessun campione sono stati identificati peptidi derivanti dall'α_{s1}-CN ad eccezione del campione P32. Questo dato lascia emergere da un lato una maggiore resistenza alla proteolisi di questa proteina e dall'altro dimostra la presenza di una zona di suscettibilità proteolitica interna alla sequenza proteica diversa rispetto a quella nota per l'omologa proteina bovina, normalmente localizzata nella zona N-terminale della proteina.
- alcuni peptidi vengono prodotti dalla porzione N-terminale dell'α_{s2}-CN, notoriamente non soggetta all'azione proteolitica primaria nel latte dei ruminanti. In particolare, tali peptidi, tra cui anche caseinfosfopeptidi, sono stati identificati solo in alcuni dei 18 campioni oggetto di studio, ossia P28, P29, P30, P31, P32 e P33.
- diversi peptidi identificati provengono dalla β-CN ed in particolare non solo dalla porzione prossima al C-terminale, notoriamente sensibile all'azione di proteasi di parete di tipo I e III di *Lactococcus sp*, ma anche da una zona interna alla stessa proteina compresa tra i residui 40-55. Ad eccezione dei campioni P28, P29, P30, P31, P33 e P39 accomunati da un elevato numero di frammenti generati dal peptide 40-55, tutti gli altri presentano pochi frammenti derivati da questa zona di sequenza. Relativamente alla porzione C-terminale della β-CN, invece, il campione di latte risultato più ricco di peptidi è il P42.

L'attività antiossidante, è stata valutata con il metodo della perossidazione lipidica mediante TBARS-test incubando le componente peptidiche estratte da ciascun campione di latte in presenza di acido linolenico. Essa è stata studiata in presenza di perossido di idrogeno al fine di ricreare condizioni di forte stress ossidativo quali sono quelle cui normalmente è esposto l'organismo anche con la normale respirazione cellulare. E' noto, infatti, che la perossidazione dei lipidi induce un processo radicalico a catena che comporta la formazione di un ampio range di effetti dannosi per l'organismo come mutagenesi, citotossicità ed alterazione dell'espressione genica. I risultati ottenuti permettono di constatare che ad eccezione di P38 e P45 che non esprimono nessuna variazione quantitativa rispetto al controllo, tutti gli altri campioni determinano un effetto di diminuzione del contenuto di

TBARS, particolarmente accentuato in due campioni -P28 e P43- che riducono di circa l'80% la perossidazione dell'acido linolenico. Comparando i profili peptidici di questi ultimi due, è ipotizzabile che l'effetto antiossidante riscontrato, sia ascrivibile ad una azione sinergica di due classi di peptidi a potenziale bioattività antiossidante: i caseinfosfopeptidi provenienti dall'α_{s2}-CN e quelli generati dalla porzione C-terminale della β-CN. Questi ultimi, infatti, contengono criptata una sequenza a nota attività antiossidante quale quella 177-183 della vacca corrispondente a 183-189 dell'asina. Tuttavia, il peptide β-CN f183-189 di asina presenta, rispetto all'omologa sequenza vaccina una sostituzione all'N-terminale $Val^{183} \rightarrow Ala^{177}$: poiché gli amminoacidi coinvolti nella sostituzione sono entrambi idrofobici, ed è noto che la presenza in sequenza di questi ultimi rappresenta un requisito strutturale fondamentale per l'attività antiossidante, è possibile ipotizzare che la sostituzione amminoacidica non né alteri la bioattività.

Tuttavia, l'identificazione dei componenti peptidici può essere effettuata solo se è nota la sequenza della proteina madre da cui essi sono generati. Per questo motivo, preliminarmente alla caratterizzazione dei profili peptidici di ciascun campione di latte di asina, è stata condotta una caratterizzazione della componente caseinica, mediante uno studio combinato dei profili ottenuti da separazione elettroforetica 1-DE (PAGE, UTLIEF) e 2-DE (PAGE → UTLIEF) ottenuti grazie a colorazione con Comassie Brilliant Blue ed anticorpi policlonali, con l'analisi strutturale mediante spettrometria di massa.

L'applicazione combinata di queste metodologie, ci hanno consentito di identificare contemporaneamente l'α_{s1}-CN, α_{s2}-CN, β-CN e k-CN con le relative eterogeneità dovute a fosforilazione (α_{s1}-CN, α_{s2}-CN, β-CN), glicosilazione (k-CN) e ad un incorretto splicing dell'RNA in m-RNA (forme delete di α_{s1}-CN e β-CN).

Inoltre sono state individuate 11 diverse componenti diversamente glicosilate per la k-CN, 6 forme fosforilate per la β-CN e l'α_{s1}-CN, 3 isoforme diversamente fosforilate per l'α_{s2}-CN ciascuna con 11, 12 e 13 gruppi fosfato. Relativamente all'α_{s2}-CN, per la prima volta è stata definita la sequenza primaria di questa proteina a partire dalla sequenza dedotta dal cDNA noto in letteratura.

Inoltre, la β-CN è stata identificata sia nello stato omozigote che eterozigote per la presenza di una variante genetica con MW di 28 unità di massa in più rispetto al più comune fenotipo di β-CN.

PREFACE

Proteins are a very diverse family of large organic compounds involved in many important biological processes. The process involving protein degradation both in a partial way, giving peptides, oligopeptides and/or in a complete one, giving free amino acids, is commonly known as “proteolysis” and is due to proteolytic enzymes.

Although the proteolysis is a conceptually simple phenomenon, it is quite complex in its development and evolution dynamics: the proteolytic activity of enzymes is responsible for structural, functional and nutritional changes in food proteins. Thus, proteolysis is an event of overriding importance in the technology of dairy products processing: in vegetables, bread, and other food products, the protein metabolism by proteolytic enzymes is somewhat less important compared to proteolysis in dairy products.

Milk proteolysis is due to enzymatic hydrolysis on casein induced by a series of enzymes.

The degradation of caseins involves a series of very important effects: it contributes to the softening of cheese texture, and because of a subsequent formation of peptides by lower molecular weight, it leads to a significant increase in the number of carboxylic and amine-free functions, with a resulting decrease of water (aw). The proteolytic process plays an important role in the development of cheese sensorial characteristics, in fact many low molecular weight peptides (di-and tripeptides) and many amino acids, representing the ultimate product of proteolysis, have a particular taste (often bitter, especially in the case of compounds with strong hydrophobicity) and therefore their concentration in the final product will be critical for the taste.

Finally, the same amino acids released during proteolysis are themselves substrates of catabolic reactions leading to the formation of volatile aromatic compounds responsible for the development of cheese flavour.

During the cheese making process, the action of exogenous proteases simultaneously with native ones is responsible for a kind of metamorphosis of the first in processed milk gelled of curd until then to get the cheese passing from a rubbery mass of protein and fat into a final flavourful, textured fusion of complex substances found in the cheese.

The capabilities of the metamorphosis/proteolysis is similarly explicit during milk gastrointestinal digestion: a complex of digestive reaction steps produces simpler peptide fragments easily absorbed and assimilated.

So, following enzymatic hydrolysis during food processing or digestion, proteins may release fragments from their primary amino acid sequence. These fragments are called peptides, and

many of them are known to be physiologically active. The possible beneficial effects of bioactive peptides have attracted increasing interest in recent years.

Dietary proteins are traditionally known to provide a source of energy and amino acids essential for growth and maintenance of various body functions. In addition, they contribute to the physicochemical and sensory properties of protein-rich foods.

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LIST of ABBREVIATIONS and SYMBOLS

BM	buffalo milk
BSW	buffalo sweet whey
BWW	buffalo waste whey
BS	buffalo scotta
PDO	protected denomination origin
PA	plasmin activator
PrtP	cell envelope associated serine proteinases
Opp	oligopeptide transport system
PP	proteose peptone
NPN	non nitrogen protein
LAB	lactic acid bacteria
CN	casein
CMP	casein macro peptide
p-Glu	pyroglutamic acid
ACE	angiotensin converting enzyme
CPP	caseinphophopeptide
gCMP	glycocaseinmacropeptide
MS	mass spectrometry
MALDI TOF	matrix assisted laser desorbtion/ionization time-of-flight
PSD	post source decay
RP-HPLC	reversed phase- high performance liquid chromatography
ESI	electrospray ionization
nanoESI-MSMS	Nano-electrospray ionization tandem mass spectrometry
f3	BWW purified peptide sub-fraction
CaCo2	human epithelial colorectal adenocarcinoma cells
H-CaCo2	human epithelial colorectal adenocarcinoma cells treated with H_2O_2
Hsp	heat shock protein
ROS	reactive oxygen species
SA-β-gal	senescence associated β-galactosidase activity
HE	hydroethydine
PI	propidium iodide
AKT	serine/threonine kinase
PI3-K	phosphoinositide 3-kinases
p-GSK-3	p-Glycogen synthase kinase

CMPA	cow's milk protein allergy
DMP	donkey milk peptide
MDA	malondialdehyde
GRAVY	grand average of hydropathicity

GENERAL INTRODUCTION

1. Milk casein proteolysis by endogenous, exogenous and gastrointestinal proteases

Among constituents of secreted milk there are several endogenous enzymes including proteases. The principal constituents of milk (lactose, lipids and proteins) can be modified by exogenous enzymes, added to induce specific changes. In addition, milk and most dairy products contain viable micro-organisms which secrete extracellular enzymes or release intracellular enzymes after the cells have died and lysed. Some of these enzymes may cause undesirable changes, e.g. hydrolytic rancidity of milk and dairy products, bitterness and/or age gelation of UHT milks, bitterness in cream, malty flavours or bitterness in fluid milk, or they may cause desirable flavours, e.g. in ripened cheese.
The following paragraphs are devoted mainly to the significance of endogenous milk proteases and exogenous enzymes with a proteolytic activity on caseins during gastrointestinal digestion and cheese-making.

1.1 Endogenous milk proteolytic enzymes

Milk contains essentially two proteinase systems, both derived from blood: one is involved in dissolving blood clots (plasmin) and the other in defence against invasive micro-organisms (lysosomal proteinases of somatic cells). Both systems which are involved in milk caseins hydrolysis, are complex in their regulation and sensitivity to external factors, and vary in activity due to factors such as stage of lactation and mastitis. While plasmin is the principal proteinase in good-quality milk, other proteinases, including cathepsins and elastase, are probably also active, particularly as the somatic cell count of milk increases. In terms of activity and technological significance, plasmin is the most important of the endogenous proteinases and has been the most controversial subject.
Plasmin (EC 3.4.21.7) is a serine proteinase, similar to trypsin in its activity and characteristics. Plasmin is a heat-stable alkaline serine proteinase with a relatively broad specificity on the caseins; it exists in milk as a component of a complex system, including its zymogen, plasminogen, plasminogen activators (PAs) and inhibitors of both plasmin and PAs [1]. Plasmin cleaves proteins on the carboxyl side of L-Arg and L-Lys residues, with a

preference for the latter. Plasmin certainly hydrolyzes α_{s1}-, α_{s2}- and β-CN, while although κ-CN contains several Lys and Arg residues, it appears to be quite resistant to plasmin, presumably due to a relatively high level of secondary and tertiary structure. Plasmin has little or no activity on the whey proteins β-lactoglobulin and α-lactalbumin. Degradation of α_{s2}-and β-CN occurs at the same rate while α_{s1}-CN is hydrolyzed at a slower rate [2]. β-CN is the most susceptible milk protein to plasmin action: it is hydrolysed rapidly at Lys^{28}-Lys^{29}, Lys^{105}-His^{106} and Lys^{107}-Glu^{108}, to yield γ^1 (β-CN f29-209), γ^2 (β-CN f106-209) and γ^3 (β-CN f108-209) caseins and proteose-peptone (PP)5 (β-CN fl-105/7), PP8 slow (β-CN f29-105/7) and PP8 fast (β-CN fl-29). From a quantitative point of view, γ-caseins normally represent about 3% of total N in milk while the concentration of proteose peptones is about half that of the γ -caseins. Although α_{s2}-CN in solution is hydrolysed very rapidly by plasmin at bonds on Lys^{21}, Lys^{24}, Lys^{149}, Lys^{150}, Lys^{181}, Lys^{187}, Lys^{188} and on Arg^{114}, it is not known if it is hydrolysed in milk. Similarly, α_{s1}-CN in solution is also readily hydrolysed by plasmin but it does not appear to be hydrolysed to a significant extent [3].

The second proteinase identified in milk is the more heat-labile lysosomal (i.e., somatic cell-derived) aspartyl proteinase, cathepsin D even if other lysosomal proteinases (e.g., elastase, cathepsin B as well cathepsins G, S, K, H and L) are almost certainly present in milk [4].

Undoubtedly the casein breakdown product is a function of the milk endogenous enzyme activity, but it also depends on the time for which the enzyme(s) acts on the caseins, as for example during dairy products storage. A number of peptides have been identified in freshly drawn bovine milk as a consequence of a simultaneously action of plasmin and cathepsins D and B on specific cleavage sites of α_{s1}- and β-CN [4].

1.2 Microbial fermentation

The function of lactic acid bacteria (LAB) in the manufacture of cultured dairy products is to ferment lactose to lactic acid so that milk pH decreases and the isoelectric point 4.6 of casein is reached giving the formation of a coagulum. Anyway, like many bacteria, LAB have a limited capacity to synthesize amino acids and are therefore dependent on the use of exogenous nitrogen sources for optimal growth [5].

Since most foods contain only a small pool of free amino acids, this means that LAB must first be able to degrade proteins and large peptides and then also be able to transport the free amino acids and small peptides released during proteolysis. The proteolytic system in LAB,

especially those species involved in dairy fermentations, has been well studied. Certain LAB, mainly the strains belonging to the genera *Lactobacillus*, are increasingly marketed as health-promoting, that is, probiotic bacteria [6], while certain *Lactobacillus* strains can release bioactive health-beneficial peptides from milk proteins [7]. The caseins utilization system in LAB, involves three main steps: a) caseins are hydrolyzed by proteinases to form peptides; b) the peptides are transported into cells via peptide transport systems; c) the peptides are hydrolyzed by intracellular peptidases to form free amino acids.
Each of this step is described below.

a. The proteinase system

Caseins hydrolysis by LAB occurs via a cell envelope-associated serine proteinase called PrtP which is actually synthesized as a large inactive pre-pro-proteinase (>200 kDa). The "pre" portion contains a leader sequence with the function to direct the protein across the cytoplasmic membrane while the "pro" sequence presumably stabilizes the protein during its synthesis. After both "pre-pro" regions are removed, the mature proteinase remains linked to the cell envelope. More than 100 products are formed by PrtP on the casein substrate. The majority are large oligopeptides (up to thirty amino acid residues), but most are between four and ten residues.

b. Peptide transport systems

Lactococci and other LABs transport peptides directly into the cell without further extracellular hydrolysis. In addition, although there is a myriad of peptides formed by PrtP, relatively few transporters can deliver these peptides into the cell, and even fewer are actually essential for growth in milk. An oligopeptide transport system (Opp) is used by Lactococci and LABs to satisfy their amino acid requirements. The Opp system transports about ten to fourteen different peptides of varying size (between four and eleven amino acid residues).

c. Peptidases

In the final step of protein metabolism, the peptides accumulated in the cytoplasm by the Opp system are hydrolyzed by intracellular peptidases. There are more than twenty different peptidases produced by Lactococci and LABs that generate the pool of amino acids necessary for biosynthesis and cell growth: included are endopeptidases (that cleave at internal peptide bonds) and exopeptidases (that cleave at terminal peptide bonds). The latter group consists entirely of aminopeptidases (hydrolyse the peptide bond located between the amino terminal

amino acid residue and the penultimate residue), and carboxypeptidases (acting at the peptide carboxy end). To fully use peptides accumulated by the Opp system, LAB requires the combined action of endopeptidases, aminopeptidases, dipeptidases and tripeptidases. Anyway, some peptidases may have specificity with regard to the amino acids and their position within a given peptide bond: aminopeptidaseP (PepP) from *L. lactis* subsp. *lactis* hydrolyzes peptides containing proline at the penultimate residues.

However, the specific casein substrates and hydrolysis products of the different PrtP enzymes can vary considerably. For example, some proteinases hydrolyze α_{S1}-, β-, and k-CN, whereas others have preference for β-CN and relatively little activity for α_{S1}- and k-CN [8].

In milk fermentation processes, in addition to its vital role for bacterial growth in milk, certain PrtP can release bioactive health-beneficial peptides during milk fermentation [9].

Along the way, LAB's culture may also produce other small organic molecules, including acetaldehyde, diacetyl, acetic acid, and ethanol. Although these latter compounds are produced in relatively low concentrations, they may still give important contributions to the overall flavour profile of the finished product. The culture may also produce other compounds which contribute to the viscosity, body, and mouth feel of the product.

1.3 Milk clotting enzymes

Rennet is a natural complex of enzymes produced in any mammalian stomach to digest the mother's milk. Rennet contains many enzymes, that are able to clot milk, causing it to separate into solids (curds) and liquid (whey). The active enzyme in rennet is called chymosin or rennin but there are also other important enzymes in it, e.g., pepsin or lipase. Chymosin and pepsin exhert a proteolytic activity on proteins while lipase acts on lipids. Limited to our interest, we are concerned about the first two.

The chymosin exerts its proteolytic activity on casein: the cleavage sites are well known in literature[10].

Experiments in solution showed the presence of seven cleavage sites on β-CN [10], many of which are located near the C-terminal hydrophobic sequence; in particular, the Leu^{192} and Tyr^{193} may lead to the formation of a series of hydrophobic peptides with a bitter taste.

The α_{S1}-CN also has several cutting sites and among them the best known is Phe^{23}-Phe^{24} giving the peptide α_{S1}-CN f1-23 with a high content of basic residues. The α_{S1}-CN f1-23 is rapidly degraded by peptidases of starter microorganisms [11]. The α_{S2}-CN is much more resistant to chymosin. Similarly also the para-κ-casein (κ-CN f1-105) does not appear to be

hydrolyzed, although still contains a number of potential cutting sites [12] probably because of its secondary structure unaccessible to chymosin action [13]. Another very important bovine rennet protease is pepsin, which has a chymosin like proteolytic specificity but a higher proteolytic activity compared to the chymosin one.
However because of pepsin optimum pH is about 2, its action is severely limited by the pH of the medium which could be milk or cheese.

1.4 Milk proteolysis during gastrointestinal digestion

Dietary proteins and peptides are subjected to hydrolysis during the various stages namely ingestion, digestion and absorption. Ingested proteins are first hydrolyzed in the acid environment of the stomach, where low pH initiates protein hydrolysis and gastric pepsin cleaves proteins into peptides of various sizes. The next stage of luminal protein digestion, known as pancreatic digestion, comprises the small intestinal digestion by the major enzymatic secretions from the pancreas trypsin, chymotrypsin, elastase, and carboxi-peptidases. The gastro-pancreatic digestion results in free amino acids and 3 to 6 amino acid peptides, which could have biological activity. Subsequent brush-border peptidases at the surface of the epithelial cells digest peptides into dipeptides and tripeptides and free amino acids while some peptides remain intact.
The intestinal brush-border metabolism is due to the action of aminopeptidases, carboxypeptidases and also enterokinases and dipeptidases. For example, dipeptidyl carboxypeptidase preferentially cleaves di-peptides from the C-terminus of oligopeptides with Pro, Phe or Leu in the ultimate position. Dipeptidyl aminopeptidase IV releases di-peptides from the N-terminus of oligopeptides with Pro or Ala in the second position. Hence, the di-peptides released by these enzymes are generally of the X-Pro type while tri-peptides containing C-terminal Pro-Pro are reported to be resistant to Pro-specific peptidases. Anyway, Pro- and hydroxyl Pro-containing peptides are generally resistant to degradation by digestive enzymes. However, certain peptides are resistant to the action of proteolytic enzymes and remain intact in the intestinal tract. For example, in a recent work of Picariello et al., 2009 on peptides surviving the simulated gastrointestinal digestion of caseins and whey proteins, the β-Lg f125-135 showed an exceptional stability to gastro-pancreatic and brush-border membrane proteolysis [14].

Different transport systems for the absorption of peptides have been described: oligopeptides seem to be transported by endocytosis, although it has been reported that more than 90% of the transported peptides are hydrolyzed in the absorptive cells; oligopeptides could be passively transported via paracellular pathways, which is known to be the main mechanism for transport of intact peptides; oligopeptides could be transported by transcytosis (vescicle-mediated transcellular transport); di-peptides and tri-peptides are actively transported via PepT1, a specific transport system that exists in the brush-border membrane [15]. Much research has been carried about the transport of bioactive peptides on CaCo2 cells: opioid peptides [16], ACE inhibitors [17] and anti-hypertensive peptides [18] have been shown to cross the epithelial cell monolayer.

These studies found that the method of transport is not only dependent on the size of the peptide but also on charge, molecular weight, hydrogen bonding and hydrophobicity. Whichever mechanism was used, a variety of peptides would reach the portal circulation, showing different physiological functions. Therefore, following digestion, bioactive peptides can either produce local effects in the gastrointestinal tract or be absorbed through the intestine to enter intact into the blood circulation and exert systemic effects.

1.5 Proteolysis in cheese

Although it was long argued that milk fat was the primary constituent responsible for cheese flavour, it is now generally accepted that, for most cheeses it is the protein fraction that is the most important overall contribution. So cheese made under controlled conditions in which proteolysis does not occur because of absence of starter and non-starter LAB, develops neither the flavour nor the texture of normal cheeses: these cheeses do not produce cell wall proteinase and casein degradation occurs to a very limited extent. Moreover, it should be noted that residual chymosin and milk proteinases (mainly plasmin) may also contribute to the peptide pool, especially during the early stages of cheese ripening. However, the starter and non-starter LABs are responsible for most of the subsequent casein hydrolysis. During the ripening process, because of the starter bacteria lyse (due to salt, low water activity, lack of fermentable substrates, and other factors), the entire array of intracellular enzymes is released into the curd matrix. Thus, the various peptidases important for peptide degradation are free to hydrolyze available substrates. Likewise, enzymes that act directly on amino acids, triglycerides, and other substrates are also released.

The evolution of milk casein proteolysis varies greatly among different cheese varieties because of different technological productions involved, which may be cooking temperature, pH during whey drainage, time of ripening, moisture conditions, residual coagulant activity, possible development of an highly proteolytic microflora: these differences make the pattern proteolysis essentially unique and characteristic for each type of cheese.

Without going into details of thecnological parameters, we can identify the proteolysis steps involved during the milk transformation on cheeses [19].

The α_{S1}-CN Phe^{23}-Phe^{24} is the first peptide bond to be early hydrolyzed by chymosin action. The high temperatures involved during curd cooking as in Swiss-cheeses, rapidly inactivate chymosin, so the plasmin, which is more resistant to high temperature, is the first proteolitic enzyme in this kind of cheeses. The α_{S1}-CN f1-23 and the α_{S1}-CN f24-199 peptides, themselves become a substrate for the action of other proteolytic enzymes. A more prolonged cheese aging corresponds to an enrichment of peptides and amino acids while little seasoned cheeses have a low rate of nitrogen soluble at pH 4,6; on the other end, the proteolysis degree in fresh cheeses like mozzarella is very small compared to those aged. In mould-ripened cheeses, the *P. Roqueforti* induced proteolysis, degrades these fragments along with β-CN. Consequently at the end of the ripening process the caseins are completely hydrolyzed [20].

In Swiss-cheese types, the β-CN is degraded faster than α_{S1}-CN: the increasing levels of γ-casein, indicating that, as previously reported, the proteolytic activity of plasmin is particularly strong in this kind of cheeses.

A trend common to the Swiss-cheeses can also be seen in particularly fresh cheese such as mozzarella, whose cooking temperatures results in a partial chimosin inactivation [21].

However, the mozzarella is not a ripened cheese, so its index of proteolysis is very low; on the contrary, cheeses like Parmigiano-Reggiano, also cooked at high temperatures, are widely hydrolyzed by plasmin and LAB proteases because of the long periods of ripening (from the 12 months) [22].

2. Milk biologically active peptides

In recent years, food proteins have gained increasing value due to the rapidly expanding knowledge about physiologically active peptides. Although bioactive peptides from different animal or plant origins as soybean have been described, those derived from milk have been in

the last two decades the most studied. Milk proteins provide a rich source of peptides which are latent until released and activated, e.g. during gastrointestinal digestion or food processing [23]. Once liberated, these peptides are potential modulators of many regulatory processes in living systems as shown in Figure 1 and may act even as regulatory compounds with hormone-like activity [24].

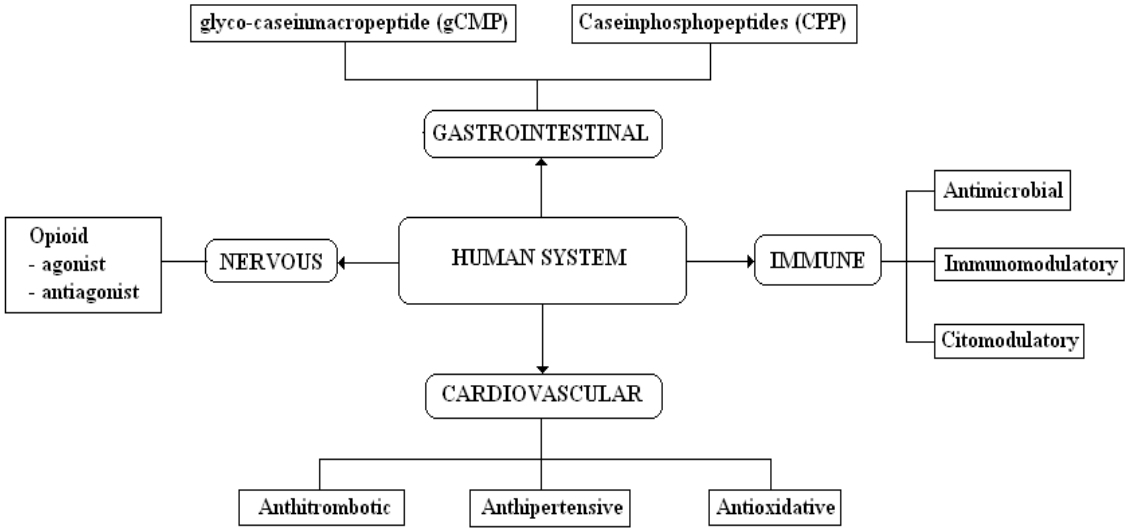

Fig 1. *Physiological functions of milk derived bioactive peptides.*

The primary and secondary structures of major bovine milk proteins are well characterized and the potential bioactivities of peptides released from these proteins are currently a subject of intensive research worldwide. In table 1, a list of milk bioactive peptide sequences in the primary structure of bovine αs_1-, αs_2-, β- and k-CN are reported (Tab 1). Most of the known bioactive peptides are composed of short amino acid sequences: oligopeptides present several advantages in comparison to large-sized molecules in view of a possible therapeutic use because they can more easily pass through the digestive tract and reach the blood circulation to be transported to their target organs.

It must be underlined that even though bioactive peptides have been isolated *in vivo* in individuals following ingestion of caseins (CNs) or of milk-containing foods [25,26], their pharmacodynamic and pharmacokinetic parameters, as well as their bioavailability, remain to be established.

Currently the enzymatic hydrolysis of the whole protein represents the most important way to produce bioactive peptides. Even if many of the known bioactive peptides have been produced using gastrointestinal enzymes as pepsin and trypsin, others enzymes from bacterial

Bovine casein	Peptide	Amino acid sequence	Bioactivity
α_{s1}-CN	23-24	FF	Anthipertensive
	23-27	FFVAP	Anthipertensive
	102-109	KKYKVPQ	Anthipertensive
	144-149	YFYPEL	Antioxidant
	142-147	LAYFYP	Anthipertensive
	157-164	DAYPSGAW	Anthipertensive
	194-199	TTMPLW	Anthipertensive-Immunomodulating
	90-95	RYLGYL	Agonistic opioid
	90-96	RYLGYLE	Agonistic opioid
	91-96	YLGYLE	Agonistic opioid
	1-23	RPKHPIKHQGLPQEVLNENLLRF	Immunomodulating-Antimicrobial
	43-58	DIGSESTEDQAMEDIK	Caseinphosphopeptides
	43-55	GSESTEDQAME	Caseinphosphopeptides
	59-79	QMEAESISSSEEIVPNSVEQK	Caseinphosphopeptides
	66-74	SSSEEIVPN	Caseinphosphopeptides
	106-119	VPQLEIVPNSAEER	Caseinphosphopeptides
α_{s2}-CN	174-179	FALPQY	Anthipertensive
	174-181	FALPQYLK	Anthipertensive
	189-197	AMKPWIQPK	Anthipertensive
	189-193	AMKPW	Anthipertensive
	190-197	MKPWIQPK	Anthipertensive
	198-202	TKVIP	Anthipertensive
	1-32	KNTMEHVSSSEESIISQETYKQEKNMAINPSK	Immunomodulating
	164-179	LKKISQRYQKFALPQY	Antimicrobial
	165-203	KKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPY	Antimicrobial
	183-207	VYQHQKAMKPWIQPKTKVIPYVRYL	Antimicrobial
	2-21	NTMEHVSSSEESIISQETYK	Caseinphosphopeptides
	46-70	NANEEEYSIGSSSEESAEVATEEVK	Caseinphosphopeptides
	55-75	GSSSEESAEVATEEVKITVDD	Caseinphosphopeptides
	126-136	EQLSTSEENSK	Caseinphosphopeptides
	138-149	TVDMESTEVFTK	Caseinphosphopeptides
β-CN	1-18	RELEELNVPGEIVESLSS	Cytomodulatory
	105-117	KHKEMPFPKYPVE	Cytomodulatory
	98-105	VKEAMAPK	Antioxidant
	74-76	IPP	Anthipertensive
	84-86	VPP	Anthipertensive
	108-113	EMPFPK	Anthipertensive
	177-183	AVPYPQR	Anthipertensive
	193-198	YQEPVL	Anthipertensive
	193-202	YQEPVLGPVRGPFPI	Anthipertensive
	194-204	GPVRGPFPIIV	Anthipertensive
	114-121	YPVEPFTE	Anthipertensive
	1-28	RELEELNVPGEIVESLSSSEESITRINK	Immunomodulating
	63-68	PGPIPN	Immunomodulating
	191-193	LLY	Immunomodulating
	193-209	LLYQEPVLGPVRGPFPIIV	Immunomodulating/ Antimicrobial
	1-25	RELEELNVPGEIVESLSSSEESITR	Caseinphosphopeptides
	1-28	RELEELNVPGEIVESLSSSEESITRINK	Caseinphosphopeptides
	2-28	ELEELNVPGEIVESLSSSEESITRINK	Caseinphosphopeptides
	60-63	YPFP	Agonistic opioid
	60-64	YPFPG	Agonistic opioid
	60-65	YPFPGP	Agonistic opioid
	60-66	YPFPGPI	Agonistic opioid
	60-70	YPFPGPIPNSL	Agonistic opioid
	114-121	YPVEPFTE	Agonistic opioid
κ-CN	108-110	IPP	Anthipertensive
	35-41	YPSYGLN	Antigonistic opioid
	58-61	YPYY	Antigonistic opioid
	25-34	YIPIQYVLSR	Antigonistic opioid
	158-164	EINTVQV	Antigonistic opioid

Table 1. *Biological active sequences in the primary structure of bovine α_{s1}-, α_{s2}-, β- and κ-CN.*

and fungal sources, have been also utilised. Moreover digestive enzymes and other different enzyme combination of proteinases, including alcalase, chymotrypsin, pancreatin, pepsin, thermolysin and fungal sources, have been used to release bioactive peptides from intact proteins. Beside the conventional proteolytic enzymes for the production of peptides from protein sources, recombinant DNA techniques have also been experimented for the production of specific peptides or their precursors in microrganisms. Kim *et al.*, demonstrated that the trypsin digest of a recombinant human α_{s1}-CN expressed in *Escherichia coli* contains several ACE-inhibitor peptides compared to that arose from the same non recombinant protein [27].

There is now a considerable amount of scientific data to demonstrate that a wide range of milk peptides can regulate specific physiological functions in experimental animals and humans. These functions relate to general health conditions or a reduced risk of certain chronic diseases. It must be underlined that even if the possible beneficial effects of bioactive peptides have attracted increasing interest in recent years, on the other hand, there are also reports suggesting that some food-derived peptides might adversely affect human health. Among these, β-casomorphin-7 (BCM7) [28], a peptide sequence present in the milk protein β-CN, has been suggested to contribute to an increased risk for certain non-communicable diseases, such as autism, cardiovascular diseases and type I diabetes. Some literature reports have proposed possible mechanistic explanations for such associations. Recognising the alleged negative effect of BCM7 on human health, EFSA deemed it necessary to perform a comprehensive review of the published scientific literature in order to assess the relationship of this peptide and related peptides with non-communicable diseases. The EFSA reviewed all available scientific literature and concluded that a cause-effect relationship between the oral intake of BCM7 or related peptides and aetiology or course of any suggested non-communicable diseases cannot be established. Consequently, a formal EFSA risk assessment of food-derived peptides was not recommended [29].

Examples of milk peptides which have exerted physiological effects in animal model or human intervention studies are given in the following.

2.1 Effect on cardiovascular system

2.1.1 Antihypertensive peptides

Blood pressure regulation (and hypertension, when it cannot be ensured) is partially dependent on the rennin-angiotensin system; renin acts on angiotensinogen, releasing angiotensin I that is further converted into the active peptide hormone angiotensin II, a vasoconstrictor, by the angiotensin-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1). A great number of ACE-inhibitory peptides have been isolated from the enzymatic digest of various milk proteins and they are, at present, the most studied group of bioactive peptides. Milk antihypertensive caseins peptides, also known as casokinins, are mainly produced from α_{s1}- and β-CN while only few studies concerning α_{s2}-CN -which accounts ca. 10% (w/w) of the bovine casein system, owing to its poor solubility and difficult purification.

Moreover, to exert an antihypertensive effect after oral ingestion, active peptides must be absorbed in an intact form from the intestine and further be resistant to degradation by plasma peptidases in order to reach the target sites. In fact, it has been demonstrated using monolayer-cultured human intestinal CaCo2 cells that the ACE-inhibitory tripeptide Val-Pro-Pro can be transported intact through the cell layer via paracellular and transcellular routes, although a significant amount of the peptide is hydrolysed to amino acids by intracellular peptidases even if it is also known that Pro-containing peptides are generally resistant to degradation by digestive enzymes [30].

Several bioactive peptides with ACE-inhibitory activity have also been found in cheeses even if their appearance is deeply influenced by proteolysis, but only to a certain degree. For example, an α_{s1}-CN derived antihypertensive peptide isolated from Parmesan cheese by 6 months of ripening could not be found after 15 months [31]. Accordingly, the antihypertensive activity found in long-ripened Gouda cheese was half that found in its medium-aged counterpart [32]. Partially consistent with these findings are those reported by [33], when studying the ACE-inhibitory peptides in Manchego cheese manufactured with various starter cultures; the anti-hypertensive activity decreased within the first 4 months, was maximum by 8 months of ripening and decreased again towards 12 months. The protein fragments responsible for such activity were mainly low-molecular-weight peptides; a specific interest arose regarding β-CN f199–204 and α_{s1}-CN f102–109. Peptides isolated from the Italian cheeses Italico and Gorgonzola, were also found inhibitory of ACE activity [34]. Following isolation from Gouda cheese, Saito *et al.*, [35] analysed the structure of the

antihypertensive features of a few peptides, and concluded that the strongest depressive effect on the systolic blood pressure and the highest ACE-inhibitory capacity are associated to peptides found in 8-month-old cheese.

2.1.2 Antithrombotic peptides

Peptide sequences which inhibit the aggregation of blood platelets and the binding of the human fibrinogen γ–chain to platelet surface fibrinogen receptors have been classified as casoplatelins. Casoplatelins, which are k-CN derived peptides (f106–116, f106–112 and f113–116), are inhibitors of both the aggregation of ADP-activated platelets and the binding of human fibrinogen γ-chain to a specific receptor region on the platelet surface [36]. The mechanisms involved in the clotting of milk, defined as the interaction of k-CN with coagulating enzyme, and in the clotting of blood, defined as the interaction of fibrinogen with thrombin, have been proved to be similar in nature [37]. In addition, structural homologies between the undecapeptide f106-116 from cow's k-CN, on the one hand, and the C-terminal dodecapeptide f400-411 of human fibrinogen γ-chain, on the other, have been duly reported [38]. Indeed, three amino-acid residues (Ile^{108}, Lys^{112} and Asp^{115}) of the aforementioned undecapeptide of k-CN are in a homologous position when compared with the human fibrinogen γ-chain. Furthermore, the k-CN fragment f103-111 can prevent blood clotting through inhibition of platelet aggregation, but is not able to affect fibrinogen binding to ADP-treated platelets. More recently, it was reported that k-caseinoglycopeptides from several animal species are a source of antithrombotic peptides. The sequence of amino acids in f106–171 of sheep's k-CN, known as k-caseinoglycopeptide, was shown to decrease thrombin- and collagen induced platelet aggregation in a dose-dependent manner [39]. The potential physiological effects of these antithrombotic peptides have not been established. Anyway after breast feeding or ingestion of cow's milk-based formulae, antithrombotic peptides derived from human and bovine k-caseinoglycopeptides, respectively have been detected in the plasma of 5-day-old new borns children [40].

2.1.3 Antioxidant peptides

Peptides generated from the hydrolysis of milk proteins are reported to have radical scavenging activity toward superoxide anion, hydroxyl radical and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [41]. The antioxidant peptides exert *in vitro* antioxidant

activity on the reduction of cellular enzymes affecting both cellular catalase activity and glutathione (GSH) content in Jurkat cells [42] and simultaneously GSH reduction and GSH-reductase content increasing in CaCo2 cells [43]. The metal ion chelating actions of milk peptides and inhibition of lipid peroxidation have also been reported in different model systems. For example, the tryptic peptide β-CN f1-25 may protect polyunsaturated fatty acids (PUFA)-rich phospholipid liposomes against long-term iron induced oxidation, mainly due to iron chelation [44]. Taking into account caseinphosphopeptide (CPP) chelating ability, a compared antioxidant activity of CPP and casein hydrolysates in a cell membrane model system have been assayed: iron-induced lipid oxidation was inhibited by both casein hydrolysates and CPPs, with casein hydrolysates being less effective than CPPs [45].

The antioxidant activity of peptides has been attributed to certain amino acid sequences: high levels of His and same hydrophobic amino acid were related to antioxidant potency [46]. So peptides with Pro-His-His sequence showed the greatest antioxidant activity even if in same cases a possible interaction with non-peptide compounds will enhance the bioactivity. Furthermore, there is evidence that the antioxidant effect of individual amino acids is greater when they are incorporated in peptides, suggesting that peptide bond or structural conformation has an influence of this bioactivity [47].

The antioxidant activity of milk peptides will be more fully detailed in chapter two of this PhD thesis.

2.2 Effect on the nervous system

2.2.1 Opioid peptides

Located in the nervous endocrine and in the immune system, as well as in the intestinal tract of mammals, opioid receptors (of the μ-, δ- and κ-type) interact with endogenous or exogenous ligands. The former, called ‘‘typical’’ opioid peptides, e.g. enkephalin, endorphin and dynorphin, all share the typical N-terminal sequence Tyr-Gly-Gly-Phe. The latter, or ‘‘atypical’’ opioid peptides, have agonistic (exorphins, or formons-‘‘food hormones’’) or antagonistic (casoxins) activities, and are characterized by distinct N-terminal sequences. The common structure of endogenous and exogenous opioid peptides (except α-casein opioids) is the presence of a Tyr residue at the N-terminal, followed by another aromatic residue as Phe or Tyr in the third or fourth position. Several authors have compiled information regarding

opioid receptor ligands derived from milk caseins. The major and the first opioid peptides discovered have been produced from β-CN and are the so-called β-casomorphins [28], which are fragments of β-CN between the 60th and the 70th residues, mainly f60–63, f60–64, f60–65, f60–66 and f60–70 [48]; these peptides were characterized as μ-type ligands [49]. More recently, Perpetuo *et al.*, [50] reported that the β-CN tryptic hydrolysis produced the γ-CN, Tyr-Pro-Val-Glu-Pro-Phe-Thr-Glu, possessing *in vitro* opiate-like activity. Orally administered opioid peptides may modulate absorption processes in the gut and influence the gastrointestinal function in two ways: first, by affecting smooth muscles, which reduces the transit time, and second, by affecting the intestinal transport of electrolytes, which explains their anti-secretory properties. The actual physiological effects of milk-derived opioid peptides remain, however, to be confirmed. The β-casomorphins or their precursors have been detected in the duodenal chyme of minipigs, in the plasma of newborn calves and in the human small intestine upon oral administration of casein or milk [51]. Anyway, opioid casein fragments have not been detected in the plasma of adult mammals and, therefore, it is suggested that only the neonatal intestine is permeable to casomorphins. Interestingly, the α_{s1}-CN derived peptide f91–100 has been demonstrated to possess anxiolytic-like stress-relieving properties in animal model and human studies [52]: this peptide has been employed commercially as an ingredient, e.g. for confectionery and soft drinks.

2.3. Effect on the immune system

2.3.1 Immunomodulating peptides

Breast feeding facilitates transmission of passive immunity towards a number of multifunctional factors, which have a direct effect on the neonate's resistance to bacterial and viral infections; caseins are included among these factors. In fact, during enzymatic digestion of human and bovine milk caseins, peptides with immunomodulating capacities are released enhancing immune cell functions, measured as lymphocyte proliferation, antibody synthesis and cytokine regulation [53]. Jolles *et al.*, [54] were the first to report that extracts of trypsin-hydrolysed human milk possess immunostimulating activity. Several other peptides were later isolated from bovine β-CN and from bovine α_{s1}-CN [55]. All these peptides stimulate phagocytic activity of murine and human macrophages *in vitro*, and exert a protective effect against *Klebsiella pneumoniae* infection in mice *in vivo* [56]. Moreover the C-terminal

sequence of bovine β-CN f193–209, containing β-casokinin-10 increased significantly the proliferation of peripheral blood lymphocytes *in vivo* in rats [57]. Of special interest are peptides released during milk fermentation with lactic acid bacteria, as these peptides have been found to modulate the proliferation of human lymphocytes, to down-regulate the production of certain cytokines and to stimulate the phagocytic activities of macrophages [58].

2.3.2 Citomodulatory peptides

Cytochemical studies have provided increasing evidence that milk derived bioactive peptides modulate the viability (e.g., proliferation, differentiation, and apoptosis) of different human cell cultures [59]. These citomodulatory effects has been demonstrated especially in malignant cells whereas normal cells seem to be less susceptible. An apoptotic and anti-proliferative activity in HL-60 cells have been shown for lyophilized extract of Gouda cheese [60] and for bovine skimmed milk digested with *Saccharomyces cerevisiae* [61] while a yoghurt fraction obtained by membrane dialysis demonstrated an anti-proliferative effect on intestinal CaCo-2 and IEC-6 cells [62]. Generally, the current literature on the effects of hydrolysed casein on cell proliferation of various cell lines shows conflicting results. Although there is much scientific evidence showing the anti-proliferative effects of milk peptides, cell growth-stimulating peptides have also been reported [63].

2.3.3 Antimicrobial peptides

The *in vitro* inhibition of pathogenic bacteria strains occurs in the presence of same specific peptide sequence from α_{s1}-, α_{s2}- and β-CN. The fragment of α_{s1}-CN f1–23, also known as isracidin, has demonstrated antibiotic-type activity *in viv*o versus *S. aureus* and *Candida albicans* and it can protect sheep and cows against mastitis [64]. The immunomodulatory peptide derived from bovine β-CN f193–209, was shown to enhance the antimicrobial activity of mouse macrophages [65]. A fragment from α_{s2}-CN, f165–203, known as casocidin-I, can inhibit growth of *Escherichia coli* and *S. carnosus* [66]. Two other peptides were meanwhile isolated from a peptic hydrolysate of α_{s2}-CN, namely f183–207 and f164–179; the former

exhibited higher antimicrobial activity than the latter, although both possessed comparable hemolytic effects [67].

2.4. Effect on gastrointestinal system

2.4.1 Glyco-caseinmacropeptide (gCMP)

During the enzymatic cheesemaking process, rennet or chymosin hydrolyses the peptide bond between residues 105 and 106 of k-CN, and the resulting molecule, known as glyco-caseinmacropeptide (gCMP) is eluted in the whey. gCMP contains residues 106−169 of k-CN and lacks aromatic amino acids and is rich in branched chain ones. Its C-terminal portion is more hydrophilic, as it contains the oligosaccharides that are O-linked to Thr and Ser. Sweet whey, containing not only whey proteins but also the threonine-rich gCMP, is commonly used for the manufacture of milk infant formulae with the aim to avoid a well-known disease in infants fed with whey protein predominant formulae as hyperthreoninemia. Rigo *et al.*, [68] tested an experimental formula based on acid whey without gCMP, against a formula based on whey with gCMP upon Thr metabolism; acid whey formulae are accordingly recommended for feeding preterm infants. So as Fanaro and Vigi emphasized, the removal of gGMP from whey is recommended towards the development of infant formulae closer to human milk [69]. The large gGMP molecule cannot be absorbed as such, so it has to be broken down into smaller peptides before an effect on blood components arises. Moreover a number of beneficial effects displayed by gCMP upon the nutrition system can be claimed as the control of several liver diseases [70] and a satiety effect when administered to starving animals which is similar to that exhibited following cholecystokinin injection [71]. Besides the various bioactivities displayed by gCMP (or smaller peptides derived therefrom), it is also known to allow absorption of calcium, iron or zinc [72].

2.4.2 Caseinphosphopeptides

The term phosphopeptide means a casein-derived phosphorylated peptide which enhances vitamin D-independent bone calcification in rachitic infants. In addition to their presence in whole milk, caseinphosphopeptides (CPPs) have also been claimed to be released by

proteolytic enzymes of lactic acid bacteria during ripening of such cooked curd cheeses as Grana Padano [73] and Comte´ [74]. The phosphate residues, corresponding to ca. 30% of the phosphorus content in milk, are present as monoesters of Ser and occur mainly in clusters; most CPPs share a common feature, i.e. they are constituted by a sequence of three phosphoseryl residues, followed by two glutamic acid residues, SerP-SerP-SerP-Glu-Glu [75]. Furthermore, the negatively charged side chains of the phosphate groups represent the binding sites for minerals; CPPs have been shown to bind to macroelements as Ca, Mg and Fe as well as oligoelements as Zn, Ba, Cr, Ni, Co and Se. Milk and dairy products provide Ca^{2+}, which can form soluble complexes with CPPs, thus avoiding Ca phosphate precipitation, and enhancing intestinal absorption of Ca in the body [76]. Chabance *et al.*, [77] have proven the occurrence of CPPs in the stomach and duodenum following milk ingestion. CPPs have also been found after *in vitro* and/or *in vivo* digestion of α_{s1}-, α_{s2}- or β-CN [78] and certain CPPs were for the first time detected in ileostomy fluid by Meisel *et al.*, [79], which confirms the ability of such peptides to survive gastrointestinal passage to the human distal ileum. Recently, the structural features and the physiological potential of CPPs have been demonstrated by several *in vitro* studies [80,81].

3. Analysis of food proteins and peptides by mass spectrometry-based techniques

The characterization of milk-derived peptides with classical analytical methodologies is severally challenged by the complexity of the milk protein fraction and by the wide dynamic range of relative peptide abundance in both dairy products and by-products. Milk, in fact, is produced from living organisms which entirely reflect the complexity of the biological system from which they derived with the expression of protein and peptides mixtures arising from post-translational processing (phosphorylation, glycosylation), alternative splicing, genetic polymorphism. Moreover, the technological processes used for dairy production further contribute to enhance the heterogeneity of the protein system by inducing proteolysis. Even if the natural biological turnover is arrested, milk proteins may be particularly subjected to further change during the food storage and maturation: for instance, proteolytic phenomena modify the protein content in cheese, yoghurt and fermented foods. Recently, mass spectrometry (MS) has emerged as a leading technique because of its ability to handle the complexities associated with the proteins and peptides arising from induced or native proteolytic phenomena for protemic and peptidomic analysis, respectively. MS provides

specificity, speed and reliability of the analytical response even if the need of an increasingly comprehensive proteomic and peptidomic analysis has requested the development of progressively more advanced technologies that extend the horizon of MS capabilities. Anyway, protein identification using MS is usually carried out both in the form of whole-protein analysis and analysis of enzymatically or chemical produced peptides: these two approaches have been designated as "top-down proteomics" and "bottom-up proteomics", respectively. These separation strategies involve multiple steps as reverse phase, size-exclusion, isoelectring focusing, ion-exchange, etc at the protein and/or peptide level, conducted on-line or off-line MS techniques, and have been recently dealt [82,83].

Briefly, in the "bottom-up" approach, proteins in complex mixtures can be separated before to enzymatic (or chemical) digestion followed by direct peptide mass fingerprinting-based acquisition or further peptide separation on-line coupled to tandem MS. Alternatively, the protein mixture can be directly digested into a collection of peptides ("shotgun" approach), which are then separated by multidimensional chromatography on-line coupled to tandem MS analysis. Although this latter is relatively simple, it results in greatly increased complexity of the generated peptide mixture, requiring highly sensitive and efficient separation. Information is also lost upon the conversion of intact proteins into a mixture of peptides, which can lead to incorrect identifications. Not all peptides resulting from the digestion of a protein can be observed or correctly identified with MS analysis, especially those with diverse or unexpected modifications. Furthermore the limited dynamic range of mass spectrometric analysis only allows for the peptides present at high relative abundance to be preferentially sampled, while information regarding the proteins represented as low abundance peptides in the complex mixture is commonly not obtained. In the "top-down" approach, instead, proteins in complex mixture are fractionated and separated into pure single proteins or less complex protein mixtures, followed by off-line static infusion of the sample into the mass spectrometer for intact protein fragmentation. The application of MS techniques for the qualitative and/or quantitative analysis of the complex protein and peptides mixtures contained in the most food products is playing a decisive role in the understanding of their nature, structure, functional properties, impact on human health and in the definition of the structure/relationship of food proteins and peptides.

4. Mass spectrometric techniques for proteomics and peptidomics

The use of MS for proteomics is not the application of a single technique for all purposes but rather a collection of methodologies. For any MS experiment, consideration should be given to the type of instrumentation, fragmentation method and strategy analysis best suited for a particular individual sample.

Fundamentally, MS measures the mass-to-charge ratio (*m*/*z*) of gas-phase ions. Mass spectrometers consist of: *a)* ion source that converts analyte molecules into gas-phase ions; *b)* mass analyzer that separates ionized analytes based on *m*/*z* ratio; *c)* detector that records the number of ions at each *m*/*z* value. The development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), actually known as the two soft ionization techniques capable of ionizing peptides or proteins, revolutionized bio molecules analysis by MS. The accuracy of ESI-MS measuring proteins/peptides molecular weight is complemented by the capability of MALDI-MS to analyze proteins with a mass grater than 100.000 Da or polypeptides in a complex mixture [84].

The mass analyzer is central to MS technology and four types of mass analyzers have found largely application in proteomics and peptidomics: quadrupole (Q), ion trap (quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), time-of-flight (TOF) mass analyzer, and Fourier-transform ion cyclotron resonance (FTICR). All these vary in their physical principles of ion separation and analytical performances. “Hybrid” instruments have been designed to combine the capabilities of different mass analyzers and include the Q-q-Q, Q-q-LIT, Q-TOF, TOF-TOF, LTQ-FTICR and LTQ-Orbitrap system (this latter is a new type of hybrid referable to the LTQ-Fourier transformation).

Anyway, the analysis of the thousands of millions of MS/MS spectra generated in proteomic studies can be a daunting task, which requires sophisticated algorithms. Over the last decade, many search engines/algorithms have been developed for handling such complex datasets, among which the most popular ones include MASCOT, SEQUEST, OMSSA, X!Tandem and many others. Databases for searching post translational modifications by MS data include UNIMOD, Deltamass, FindMod and those that use MS/MS data for its characterization include SEQUEST, Modificomb and MODi. Although the emergence of automated database searching significantly increases the throughput of data analysis, it should be used with caution due to imperfect searching algorithms and possible errors existing in various databases. Validation of peptide and protein identification results have therefore become a necessary step.

5. Strategies for the characterization of milk bioactive peptides

Recent analytical research on bioactive peptides in food has followed three main directions aimed at developing sensitive and specific methods for (i) tracing the pathway of formation of bioactive peptides from the parent proteins; (ii) identifying the biological properties; (iii) validation of the "positive" properties discovered in natural peptides by design of synthetic peptide analogues. These studies are aimed to explain the structure-activity relationship of peptides, essential information for the design of novel therapeutics or functional foods ingredients. Moreover, with the aim to identify the patterns of peptide formation and the bioactivity of the peptides present in the sample, the newest analytical approaches combine MS with cell biology, immunology, biochemistry and synthetic chemistry [85]. In the specific case of milk-derived protein/peptides, identification of bioactive components can be achieved through towards the following steps:

1. Extraction and purification of peptides present in a food sample (milk, yogurt, cheese) using high-resolution separation techniques or selective methods for enrichment of some class of components (i.e. phosphopeptides).
2. protein structural characterization by MS analysis complemented with the peptides produced "*in vitro*" or "*in vivo*" ones; this peptidomic approach combines a high-resolution separation technique (2-D gel electrophoresis, nanoHPLC) with MS or other micro-analytical techniques for single protein identification, characterization of post-translational modifications and database cataloguing.
3. *In silico* analysis for prediction of possible bioactive sequences encrypted in the protein sequence.
4. Synthesis of pure peptides, or of peptide analogues.
5. Confirmation of bioactivity through appropriate biological or cellular assays.

These 5 points aimed to the assessment of the structure-bioactivity relationship.

6. The intestinal mucosa as a model to study food-organism interaction

The intestine fully differentiated tissues are characterized by an arresting of cells in the quiescent state with an enterocyte turnover within 48-72 h [86]. As the intestine sits at the interface between the organism and its luminal environment, it represents a critical defense

barrier against luminal toxic agents [87]. Thus, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly injured by reactive oxygen species (ROS) endogenously and exogenously generated [88]. Imbalance between the production of ROS and antioxidant defences, plays an important role in the initiation of the oxidative stress [89] and the imposition of a severe oxidant stress typically results in cytotoxicity. The oxidative stress can induce phase transition in regular cell cycle from a quiescent state to a proliferative, apoptotic, or necrotic one. When concentrations of oxidants are very high, the cellular response may activate different pathways and cell death can occur either by necrosis or apoptosis, prevalently [90]. Additionally, unless the concentrations of oxidants are cytotoxic, necrotic cell death is not necessarily an obligatory endpoint of oxidative stress [91]. Adenocarcinoma cells are remarkably resistant to injury by radiation, immunological, and chemotherapeutic agents [92]. As a consequence, these tumor cells are very difficult to treat and proliferate rapidly, even under conditions that may be adverse for normal cells. This is partially due to the endogenous overexpression of heat shock protein (Hsp) family. Hsps are believed to bind and protect critical cellular proteins, preventing their denaturation by adverse factors or conditions. Although Hsp expression can be induced by a variety of stressful stimuli, certain neoplasm, including human intestinal T84, HT-29 and CaCo2 cell lines, express constitutively high levels of Hsps even under non-stress conditions [92]. The anti-apoptotic action of Hsp is due to their stimulation of Akt activity [93]. Moreover cancer cells are characterized by an increase in their intracellular concentration of biomolecules such as ceramides [94]. The intracellular levels of these sphingolipids are elevated in tumour cells after irradiation or therapy with anticancer drugs, such as doxorubicin [95], implicating endogenous ceramides as important mediators of cancer therapy [96]. It has also been shown that exogenous ceramides induce cell death in a variety of human cancer cell types [97]. Interestingly, cancer cells seem to be more susceptible to the toxic effect of exogenous ceramides than normal cells [98,99,100], rendering exogenous ceramides promising novel drugs for chemotherapy. The sphingolipid ceramides can be formed by sphingomyelin breakdown or through *de novo* synthesis. They are intimately involved in normal and cancerous cells death, growth, differentiation and senescence. This latter is a physiological process that leads to irreversible growth arrest, accompanied by characteristic phenotype changes (such as induction of senescence associated β-galactosidase activity, SA-β-gal). Besides to ‘‘replicative senescence’’ resulting from shortening of telomeres at the ends of the chromosomes [101], an ‘‘accelerated senescence’’ pathway not involving telomeres shortening, but other factors as DNA damage has been described [102]. Growth arrest in both

replicative and accelerated senescence induces cell cycle arrest. Food constituents which are known to prevent the development of colorectal cancer have been shown to enhance apoptosis following DNA damage and this may reflect an important mechanism of cancer prevention [103]. These constituents include butyrate [104,105], flavonoids [106] and glucosinate breakdown products from brassicas [107]. Several bioactive peptides derived from milk proteins are potential modulators of various regulatory processes in the body and thus may exert beneficial physiological effects such as antioxidant activity [108]. The antioxidant activity of milk protein hydrolysates has been interestingly described [109-112] and also individual peptides released after hydrolysis from α_s-casein have been shown to possess free radical scavenging activities inhibiting enzymatic and non-enzymatic lipid peroxidation [113].

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CHAPTER I

1. INTRODUCTION

1.1 Buffalo milk and Mozzarella di Bufala Campana Protected Denomination of Origin (PDO) cheese

It is well known that buffalo milk contains twice the amounts of fat than cow: in cattle, milk contains between 3 to 5 %, depending on feeding and breed, while in buffalo milk the average fat content is usually 7 to 8% but may be as high as 13% in some breeds. Buffalo milk fat has a higher melting point compared to the cow milk fat, due to its higher proportion of saturated fatty acids (77:23, saturated:unsaturated) while phospholipids and cholesterol are lower in buffalo milk. Moreover, the content of protein, lactose and ash is somewhat higher in buffalo milk than in cow milk. Buffalo milk lacks or contains only traces of β-carotene (a precursor of vitamin A): this makes milk look very white, as opposed to cattle milk which has a slight yellow shade. In buffalo milk, vitamin A is present instead of its precursor. Physiology of animal, stage of lactation, season, feed, breed, time and milking sequence are some of the factors affecting the fat content of buffalo milk. Of the main buffalo species, the Italian one *(Bubalus bubalis)* has been widely studied in relation to the exclusive use of its milk for the manufacture of high-quality dairy products, among which Mozzarella cheese with Protected Designation of Origin (PDO) mark is the most famous in the world. In 2006, this cheese and other similar products have been associated to a global market of 500 million and 2-3 billion dollars, respectively [1].

The highest quality buffalo mozzarella bears the "Mozzarella di Bufala Campana" trademark. In 1993, it was granted Denominazione di Origine Controllata (DOC) status, in 1996 the trademark received registry number 1107/96 and in 2008 European Union granted Mozzarella di Bufala Campana Protected Geographical Status and PDO indicator.

It implies that any cheese-making process must comply with the rules established by the National Standard for cheese production, including above all:

- milk must be produced in circumstantiates areas within Southern Italy regions (Campania, Lazio, Molise and Apulia) and the approximate area within which the regulations permit it to be transformed into PDO cheese;

- the cheese must contain exclusively water buffalo milk from "Italian Mediterranean breed";
- whole buffalo milk must be fresh, and addition of frozen or quick-frozen buffalo milk is not allowed;
- no milk standardization is allowed while pasteurization is optional;
- culturing is made by adding starter resulting from spontaneously acidified whey of the previuos day "sieroinnesto" within the cheese plant.

The "Mozzarella di Bufala Campana" is a kind of bland, slightly acidic cheese, with no aged cheese flavour. However, it has an unusual ability to stretch, retain fat, melt evenly, and provide a chewy mouth feel. Even if the buffalo milk transformation is provided by PDO indicators, the previously cited properties are not necessarily automatic and are influenced by coagulants, starter cultures, seasonality, manufacturing conditions, post-manufacturing handling and storage. Moreover "Mozzarella di Bufala Campana" is characterized by a complex sensory profile depending mainly on the heterogeneity of either milk microflora and/or of natural whey cultures "sieroinnesto" which influence the peculiar characteristics of each cheese produced in different areas, so that it is not possible to define the "typical" sensory profile of PDO Mozzarella cheese as related to technological parameters, season, geographical origin and chemical composition of milk [2].

Due to variable composition of milk and starter, yield and quality of Mozzarella cheese are highly variable. Moreover, the manufacturing parameters and any process step affect the composition and the functional properties of Mozzarella cheese. The texture, for example, is influenced by coagulants concerning parameters as enzyme activity, starter culture composition and lactic acid bacteria survival.

The primary function of the coagulant is to transform milk in a curd that is successively stretched into cheese. Anyway, small amounts of residual enzymes in the curd will be carried out in the cheese and are responsible for proteolysis occurring in the final product altering cheese texture during refrigerated storage brine. For this reason, it is very important to reduce enzyme levels in the curd. The retention of chymosin is affected by curd pH, so cutting the curd at higher pH reduces chymosin level retention and the casein proteolysis in the curd [3].

Because of texture influence on shredding, melting properties and water release during Mozzarella cooking, it is really important to reduce the coagulant activity in the curd and to produce inactivation of chymosin/pepsin attaining temperatures sufficiently high during cooking and stretching. Anyway, taking into account that the enzymes involved are more

sensible to denaturation at higher pH, the monitoring of the whey pH is important to reduce the level of enzymes entrapped in the curd.

Moreover, the uncontrolled composition of the starter cultures "sieroinnesto" used in buffalo Mozzarella cheese production, may influence curd and cheese rheological parameters. So, to the natural starter bacteria composition has been standardized by incubation at predetermined temperature. For these reasons, some fermenting vats with controlled temperature or temperature gradient are actually being used.

1.2 Whey classification and its conversion in ingredients for food manufacture

Whey is produced during manufacture of different types of cheese and casein and four major types of whey may be therefore identified:

1. sweet cheese whey from the manufacture of cheese coagulated by rennet at pH 6.0 to 6.4;
2. acid cheese whey from the manufacture of cheese coagulated at pH 4.5 as a result of lactic acid bacteria;
3. acid casein whey from the manufacture of acid casein (pH 4.5);
4. rennet casein whey from the manufacture of rennet casein (pH 6.6);

In view of the similarities between different types of whey, a simpler classification differentiates acid wheys (acid cheese whey or acid casein whey), from the sweet whey (sweet cheese whey or rennet casein whey).

Whey from different sources shows some variations in composition but the most important type can be taken to contain about 6.5% solids of which about 4.8% is lactose, 0.6% protein, 0.6% minerals, 0.15% lactic acid, 0.25% non-proteinaceous nitrogen compounds and 0.1% fat (after fat separation). Whey therefore contains about 50% of the total solids in the whole milk.

Whey has always posed a considerable disposal problem to cheese factories and large quantities of whey are still either sold for stock feeding, discharged to effluent plants or used as fertilizers. However the past 15 years have seen a change in the philosophy of whey utilization with recognition that whey could be considered as a potentially valuable source of protein and lactose which may be used as ingredients in food manufacture. The conversion of whey into lactose and whey products has increased substantially and a number of new technologies have been applied commercially to convert whey into saleable products. The most important of these are described below [4].

- **Alcohol fermentation:** Even if the production of alcohol from whey is a well-established fermentation process, it is only in the last 20 years that whey alcohol production has become commercially viable. As a carbohydrate source for alcohol production, whey has two major disadvantages because lactose is fermented only by few yeasts and the low concentration of lactose in whey gives rise to low ethanol concentrations and high distillation costs. Anyway, the identification of some yeasts strains as *Kluyveromyces fragilis* with a high efficience in lactose fermentation and the development of less energy distillation, have enabled the commercial production of whey alcohol. Before fermentation, whey has to be first ultrafiltrated or submitted to a heat treated with the aim to its deproteinization.

Whey alcohol is of high quality and can either be used in the manufacture of high grade potable spirits as gin, vodka, or as an industrial product.

- **Demineralization:** The demineratization which is carried out either by ion exchange treatment or by electrodialysys, increases the range of opportunities for whey use as a food ingredient since it will be spray dried. In fact the main use of demineralizated whey powder is in the manufacture of infant foods, where it is used together with skimmed milk powder to give the same ratios of casein/whey protein/lactose/minerals as they are found in human milk.

- **Lactose hydrolysis:** Hydrolysed whey syrups are manufactured by hydrolysing the lactose in whey or deproteinized whey, followed by evaporation to a solid concentration of 60-75%. Approximately 80% of the lactose is hydrolysed in order to avoid lactose crystallization in the resulting syrup. Lactose may be hydrolysed by hot acid or by the enzyme β-galactosidase which is preferred and may be used in a batch process or in immobilized form. Hydrolysed whey products may be made either from whole whey or deproteinized whey obtained as a permeate after an ultrafiltration step. The lactose hydrolysed whey is usually evaporated to 70-75% solids and contains about 10% proteins, while lactose hydrolysed from a deproteinized whey is evaporated to only 60-65% solids and may be used as an alternative to glucose syrup.

- **Lactose manufacture:** One of the oldest methods of whey reutilization is the manufacture of lactose. Whey is first evaporated to a solid concentration of 60 to 65% and then transferred to a crystallization tank where lactose seed crystals are added to promote crystallization. The lactose crystals are recovered by centrifugation, washed to remove proteins and salts, and dried. The liquid by-product remaining after centrifugation is known as liquor mother and contains about 20% proteins in dry matter. It is sold either for animal feed or as an ingredient in pet foods, but its high mineral content precludes use in human foods. The described lactose manufacture is suitable for food use but for pharmaceutical ones it is not necessary to purify

the washed crystals further. With this aim, the crystals are redissolved in hot water and boiled with calcium phosphate and charcoal; this causes the protein to precipitate while coloured compounds are adsorbed by charcoal. The solution is filtered hot and the lactose is crystallized and recovered as described above.

- Methane production: Whey, or deproteinized whey, can be fermented anaerobically to convert the organic matter to methane. The methane produced is equivalent in energy terms to about 0.5 Kg fuel oil for each Kg solid whey converted and the gas can be used to fuel the factory boilers.

- Whey protein production: Whey proteins are superior to most other food proteins in terms of nutritional value and possess a number of physical properties which make them very useful as food ingredients. Commercially, whey proteins are usually manufactured by ultrafiltration and are available as spray dried powders containing between 25 and 75% proteins. In food products, whey proteins perform in a similar manner to egg proteins; they are highly soluble, even at acid pH and they foam and form gels on heating. Whey protein powders are therefore used in the manufacture of bakery and meat products, where their gelation properties are particularly useful. Whey proteins are also extracted from whey using ion exchange chromatography techniques. These products may contain up to 90% proteins and are mainly used in nutritional and dietetic products.

The availability of whey in Italy, the spraying of serum today affects latest 10%. According to official data, 60% is used in the liquid state in cattle feed, a little more 8% is used in the production of ricotta, the remaining portion is used in the manufacture of other dairy products (lactose, protein serum) and a portion of all secondary initiated a process of partial concentration not neglecting the possibility that a good part ends its life cycle dispersed into the environment. The data cited leave out the important impact of the reuse of the whey in the livestock sector with many problems related to the health of animals. In this regard, in a study commissioned by the European community to a group of scientific experts on the health and welfare (AHAW), it has emerged that animal milk byproducts that have been adequately treated to avoid the risk of transmission of infectious diseases, including epizootic aptha (FMD) and paratuberculosis (MAP) can be used on farms [5].

Only a small amount of serum is used as growing medium for the preparation of sierinnesto used as leaven in the production of natural buffalo Mozzarella cheese (PDO), as provided by Article 3 of the letter c DPCM 10/05/1993.

1.3 The whey produced during the processing of buffalo milk in Mozzarella

The production of Mozzarella di Bufala Campana PDO cheese has recorded a strong increase in Regione Campania in the last years due to the rise in request by market, so a large amount of whey has to be disposed off yearly, as about 80% milk volume remains as whey after transformation.

The protein component of buffalo whey contributes to the total nitrogen for 0.67% and consists of a fairly heterogeneous protein mixture represented by albumin (approximately 75%), globulins (about 15%) and peptones (about 10%).

There are also water-soluble vitamins and the characteristic yellow colour of whey is due to the presence of a fair amount of riboflavin or vitamin B_2. Whey contains a not nitrogen fraction protein (NPN) which is the 0.27% of total nitrogen, as well as a small fraction of casein fragments, generated by proteolytic enzymes present in milk or added after treatment technology, respectively as plasmin and chymosin on caseins. These fragments given their small size are not incorporated in the caseous curd.

The composition of whey derived from dairy milk processing, depends on the type of clotting occurred and, the substantial qualitative and quantitative differences emerge relatively the NPN composition and nature of the fragments of casein oligopeptides produces. Regarding buffalo whey, all along the technological process of mozzarella production three types of whey are produced:

- Sweet whey immediately produced after or simultaneously to milk clotting; the NPN is formed of chymosin hydrolysis primary fragments: mainly the κ-CN Phe^{105}-Met^{106} (this is due to secondary β type structure that characterizes the amino acid 99-111 and is well suited to the formation of activated complex with chymosin), the α_{s1}-CN Phe^{23}-Phe^{24} and the β-CN Lys^{28}-Lys^{29} hydrolysis induced by plasmin.
- Acid whey (in this PhD thesis called waste whey). Obtained after maturation of the curd in the sweet whey, the NPN is formed by primary fragments induced by hydrolysis of the lactic microflora used as starter. In particular, the proteolytic activity of lactic acid bacteria (LAB), hydrolyzes the primary fragments producing several peptide fragments thereby increasing the complexity of the peptide fraction.
- Scotta. It's a liquid acid-sugar deprived almost entirely of the thermally coagulated whey protein fraction, which is instead incorporated into the "ricotta". The Ricotta is only produced from sweet whey, as waste whey presents a pH below 5, which represents the pH corresponding to the pI of whey proteins that would be destabilized. Only recently Ricotta has

been included in the register of protected designations of origin and protected geographical indications as Ricotta di Bufala Campana (PDO) by the Commission Regulation (EU) No 624/2010.

In sweet whey and waste whey the most abundant protein component is represented by whey proteins whose solubility is a function of heat treatment involved in the technological process of making cheese while in the scotta they are almost completely absent.

In particular, there is a quantitative reverse on the two main whey proteins α-La and β-Lg: in sweet and waste whey the β-Lg is the most abundant of α-La while in scotta, the α-La which is more heat resistant, focuses on a relative basis compared to β-Lg.

In our regional reality, where most of the production of Ricotta is from cheese whey processing, only sweet whey is used, since waste whey presents a pH below 5, which represents the pH corresponding to the pI of whey proteins that would be destabilized.

It's worth repeating that, traditionally, the sweet and acid whey distinction is made by technology of production of cheese whey: sweet whey is obtained from a cheese made with rennet in its various types of liquid, powder or solid, while acid whey, when coagulation is conducted or with bacterial cultures or acidifying chemicals.

However, even after separation of the valuable whey proteins for the Ricotta production, the whey remaining is still a highly polluting liquid and has high chemical oxygen demand. Therefore, whey has to be considered as a by-product in milk processing and, according to the Italian legislation (D.L. 22/97), it is a "special waste" and its disposal engraves highly on costs. The treatment of dairy wastewater which conforms to environmental regulations is a crucial problem due to its high biological oxygen demand (BOD). The main cause of the BOD in dairy wastewater is due to residual whey which consists mainly of lactose.

2. AIM OF THE SECTION

The present PhD thesis will contribute to determine the structural features responsible of bioactivity and the structure-function relationship of buffalo milk bioactive peptides by integration of the new and complementary peptidomic analytical tecniques above illustrated. For this reason, a combined approach is given by the characterization of the evolution of the proteolysis pattern generated on buffalo milk proteins *in vivo* by action of endogenous proteases, or by action of technological process during milk transformation in mozzarella cheese, with the study of antioxidant properties and citomodulatory effects exerted on an *in vitro* cell culture model as the human colorectal adenocarcinoma cells (CaCo2). CaCo2 cells undergo a process of spontaneous differentiation during the 21-day culture, leading to the formation of a monolayer of cells expressing several morphological and functional characteristics of the mature enterocyte. The CaCo2 cell cultures have been commonly used with *in vitro* assays to assess and understand intestinal nutrients and drug transport at the cellular level. In this work we used the CaCo2 model system because it is closer to what could be the target of action of the peptide mixtures we studied if these preparations will be used in the formulation of functional food.

These information are aimed to push the peptidomics investigation beyond the many studies carried out on selected peptides produced *in vitro* from bovine milk proteins, mainly by purified proteases or microbial cell-wall proteinases. Moreover no study has been carried out on peptides in buffalo milk. Peptides naturally occurring in milk or in cheese whey have not been exhaustively characterized, and their potential bioactive properties have not been studied so far.

The need for the characterization of the peptide fraction in buffalo milk arises from the fact that the production of Mozzarella di Bufala Campana Protected Denomination of Origin (PDO) cheese has recorded a strong increase in Regione Campania in the last years due to the rise in request by market. On the other hand, during the production of Mozzarella, it is expected that a large number of peptides can be released in the whey, for the combined proteolytic effect on milk caseins endogenous enzymes (plasmin, cathepsin D), rennet enzymes (chymosin, pepsin) and lactic acid bacteria exo- and endo-peptidases. The mild manufacturing conditions employed in production of Mozzarella di Bufala cheese could effectively preserve the bioactive molecules in whey, particularly in sweet whey and waste whey. Even in fresh milk the presence of peptide size larger or smaller generated by an early action of endogenous proteases on casein is likely; many of these polypeptides are also precursors of many peptide entities in whey. Milk, of course, must be considered a food of

high commercial value, when compared to the whey left from processing; the characterization of peptides from milk casein origin, however, is highly informative allowing to extrapolate the hydrolytic action of endogenous proteases than to the later action of lactic acid bacteria and the contribution of technological processing in determining the final peptide composition.

The analytical research has followed three main consequential directions aimed to (i) tracing the pathway of formation of bioactive peptides, (ii) identifying their biological properties on CaCo2 cell line cycle modulation and (iii) ascertaining the positive properties discovered in natural peptides by synthetic structural analogues toward the biological assays reiteration.
In particular, we characterized the soluble peptide extracts from river buffalo sweet whey (BSW), waste whey (BWW) and "scotta" (BS), tracing the breakdown pathway generating potential bioactive peptides, starting from raw river buffalo milk (BM). The peptide extracts were characterized with mass spectrometric techniques, such as matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry, this latter either off-line or on-line with reverse phase high performance liquid chromatography (RP-HPLC). In addition, we investigated the antioxidant and cytomodulatory effects of the peptide extracts from BS, BSW, BWW and BS on hydrogen peroxide induced oxidative damage in CaCo2 cell lines [6].
With the aim to explain the molecular mechanism involved in the cytomodulatory and antioxidant response of CaCo2 cells, the more active peptide extracts BWW was further purified by a chromatographic step obtaining 5 peptide subfraction (f1-f5). BWW peptide sub-fractions were characterized by complementary mass spectrometric techniques and we studied the effects of BWW sub-fractions on: A) mitochondrial oxidative stress and expression of heat shock proteins (Hsp 70 and Hsp 90); B) cell cycle and differentiation; C) production of ceramides and senescence. These effects were investigated on CaCo2 cell line in which oxidative damage was induced with hydrogen-peroxide (H-CaCo2), to simulate the response of intestinal cells which are constantly exposed to luminal oxidants during food digestion.

3. MATERIALS AND METHODS

For the determination of peptides produced during buffalo milk manufacturing and for the analysis of their bioactivity, a combined use of extraction, purification, mass spectrometric techniques and biological assay was used.

3.1 Materials

River buffalo raw milk and whey samples from manufacturing of Mozzarella di Bufala Campana PDO cheese were provided by three local dairy farm of the Salerno area (Southern Italy). To prevent undesired peptide hydrolysis, immediately after collection samples were added with 1mM phenyl-methyl-sulfonyl fluoride (PMSF, Sigma, St. Louis, MO) and stored at -20°C until used.

All chemicals and reagents were of analytical grade or better from Sigma. HPLC-grade acetonitrile (AcN), methanol (MetOH) and trifluoroacetic acid (TFA) were purchased from Carlo Erba (Milano, Italy). The 218TP54 reversed-phase column C18 (5 μm, 250 mm x 4.6 mm) was purchased by Vydac (Hesperia, CA). Dulbecco's Modified Eagle's Medium (DMEM), PBS (Phosphate Buffer Saline, composed of 0.1M phosphate buffered saline containing NaCl 0.138M, KCl 0.0027M, pH 7.4), Minimum Essential Medium (MEM) nonessential amino acids, streptomycin, penicillin, L-glutamine, FBS (Fetal Bovine Serum), and Nonidet P40 were obtained from Gibco-BRL (Grand Island, NY). Tissue culture plasticware was furnished from Becton Dickinson (Lincoln Park, NJ, USA). Hydrogen peroxide (H_2O_2) 1M solution was prepared immediately before use. Hydroethydine (HE) was purchased from Invitrogen Srl (Milan, Italy).

3.2 Extraction of peptides from buffalo milk and whey samples

BM, BSW, BWW and BS samples were collected at the proper stage of the technological process of Mozzarella manufacturing. The cream was separated from milk or whey by centrifugation at 4500 *g* at 4°C for 30 min (Haereus Biofuge, Kendro, Germany). In order to harden the cream, the tubes were kept at -20°C for 10 min and finally the cream was scraped off.

Skimmed whey samples (25 ml) were ultra-filtered on Centriprep® cartridges having a 3KDa cut-off membrane (Millipore, Bedford, MA). The permeates, lyophilized and solubilised in 6 ml 0.1% (v/v) aqueous TFA, were purified from saline contaminants and lactose with a Sep-pak C_{18} cartridge (Waters, Milford, MA), previously equilibrated in 0.1% TFA and eluted with 70/30/0.1 AcN/water/TFA (v/v/v). The peptide extracts, were dried using a Savant concentrator (Speed-Vac, Milan, Italy) and stored at -20°C either for structural analysis or for biological assays. Fresh milk was processed as above after skimming and isoelectric precipitation of casein as previously reported [7]. The analytical approach for proteomic and bio-functional characterization of the peptides extract from BM, BSW, BWW and BS is reported in Fig 1.

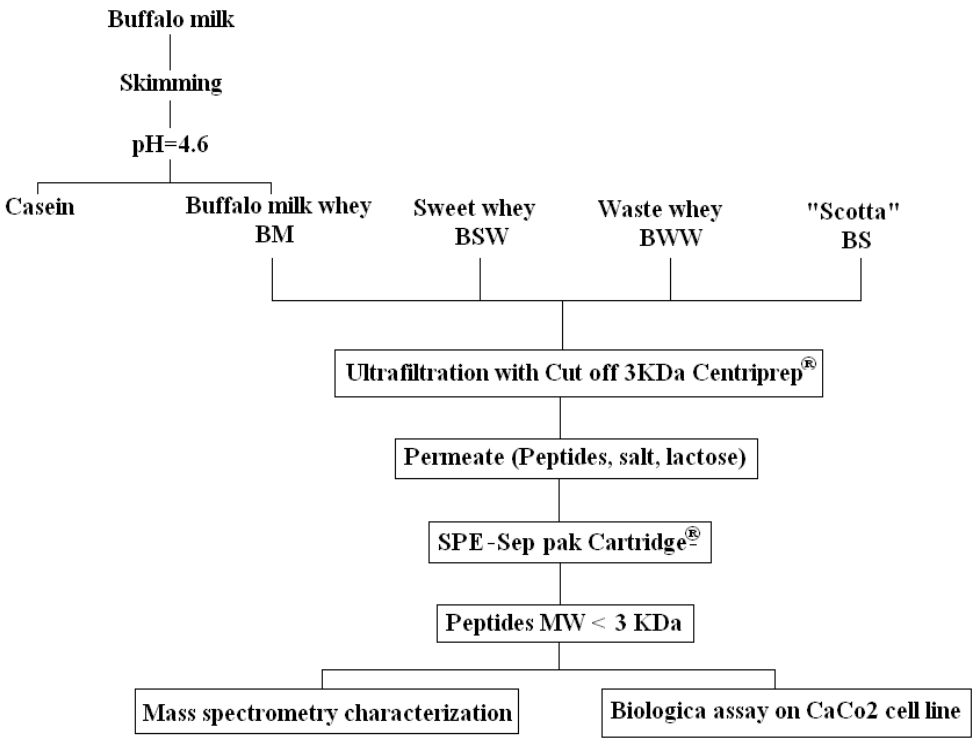

Fig 1. *Analytical approach for proteomic and bio-functional characterization.*

3.3 Matrix-assisted laser desorption ionisation mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF-MS experiments were carried out on a Voyager DE-PRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a N_2 laser (337 nm, 3 ns pulse width) operating both in linear or in reflector positive ion mode, using the Delayed Extraction

technology. Accelerating voltage was 20 kV and tipically 250 laser pulses were acquired for each mass spectrum. In the analysis of sphingolipids laser power was maintained at the lowest possible values in order to prevent in source fragmentation. The peptide or the lipid mixture, were loaded on the stainless steel together with 1 µl of matrix 2,5- dihydroxybenzoic acid - DHB, 10 mg/ml MetOH/0.1% (v/v) TFA- for lipid analysis and α-ciano-4-hydroxycinnamic acid –CHCA, 10 mg in 1 ml AcN 50% (v/v)/ 0.1% (v/v) TFA- for peptides. The external mass calibration was performed with low mass peptide standards (PerSeptive Biosystem). To check repeatability spectra were acquired in triplicate at least. Post-Source Decay (PSD) fragment ion spectra were obtained after isolation of the appropriate peptide precursor using timed ion selection. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflector and the individual segments were combined using the software developed by PerSeptive BioSystems furnished with the instrument. Signals detected in the mass spectra were associated with the corresponding peptides on the basis of the expected molecular weight deduced from the sequence of the casein fractions [8] by using a suitable computer program (GPMAW 5.1, Lighthouse data, Odense, Denmark).

3.4 Liquid chromatography Electrospray Mass Spectrometry (LC/ESI-MS) analysis of BWW peptide extract

The BWW peptide extract was fractionated by HPLC using an HP 1100 modular system (Agilent Technology, Palo Alto). Sample was loaded onto a 218TP52, 5 µm reversed-phase C_{18}, 250 x 2.1 mm column (Vydac, Hesperia, CA). Solvent A was water containing 0.1% TFA (v/v) and solvent B was AcN containing 0.1% TFA (v/v). A linear gradient from 5 to 70% solvent B was applied over 90 min, after 5 min of isocratic elution at 5% solvent B, at a constant flow rate of 0.2 ml/min. UV detection was carried out at 220 nm. The effluents were directly injected, through a 100 µm i.d. fused silica capillary into the electrospray source of a Platform single quadrupole mass spectrometer (Waters, Manchester, UK). The ESI mass spectra were scanned in the positive ion mode, from 1600 to 400 m/z at a scan cycle of 4.9 s/scan and 0.1 s inter-scan delay. The source temperature was 180°C and the capillary and orifice voltages were 3.6 kV and 40 V, respectively. Mass scale calibration was performed using the multiple charged ions from a separate injection of horse heart myoglobin. Mass spectra were elaborated using the software MassLynx 2.0, furnished with the spectrometer.

Tandem MS (MS-MS) data were obtained by using a Q-STAR PULSAR (Applied Biosystems, Foster City, CA) equipped with nanospray interface (Protana, Odense, Denmark). Dried samples were resuspended in 0.1% TFA, desalted by using ZipTip C_{18} microcolumns (Millipore, Bedford, MA), and sprayed from gold-coated "medium-length" borosilicate capillaries (Protana). The capillary voltage used was 800 V. Double-charged ion isotopic clusters were selected by using the quadrupole mass filter and then induced to fragment by collision. The collision energy was 20 to 40 eV, depending on the size of the peptide. The collision- induced dissociation was processed by using Analyst 5 software (Applied Biosystem). The deconvoluted MS-MS spectrum was manually interpreted with the help of Analyst 5 software.

3.5 Large scale fractionation by RP-HPLC of peptide extract from BWW

To assay inhibition properties of simplified peptide sets, BWW peptide extract was fractionated using an HP 1100 modular HPLC apparatus (Agilent, Palo Alto CA, USA). The sample was loaded onto a 218TP54, 5µm reversed-phase column C_{18}, 250 mm x 4.6 mm column (Vydac, Hesperia, CA). Solvent A was water containing 0.1% TFA (v/v) and solvent B was AcN containing 0.1% TFA (v/v). A linear gradient from 5 to 70% solvent B was applied over 60 min with a flow rate of 1 ml/min, after 5 min at isocratic elution (5% solvent B). UV detection was carried out at 220 and 280 nm using a multi-wave length detector. For each run, 100 µl of a 23,5 mg/ml solution of peptide extracts from BWW were injected; eluted peptide fractions were manually collected at 10 min time intervals, obtaining on the whole five different peptide sub-fractions, labelled in chronological order f1-f5. Due to limited sensitivity of CaCo-2 cell growth inhibition assay, sub-fractions collected at the same retention times from three HPLC runs were joined together and used for biological assays. In Fig 2, the analytical approach for f3 proteomic and bio-functional characterization is reported.

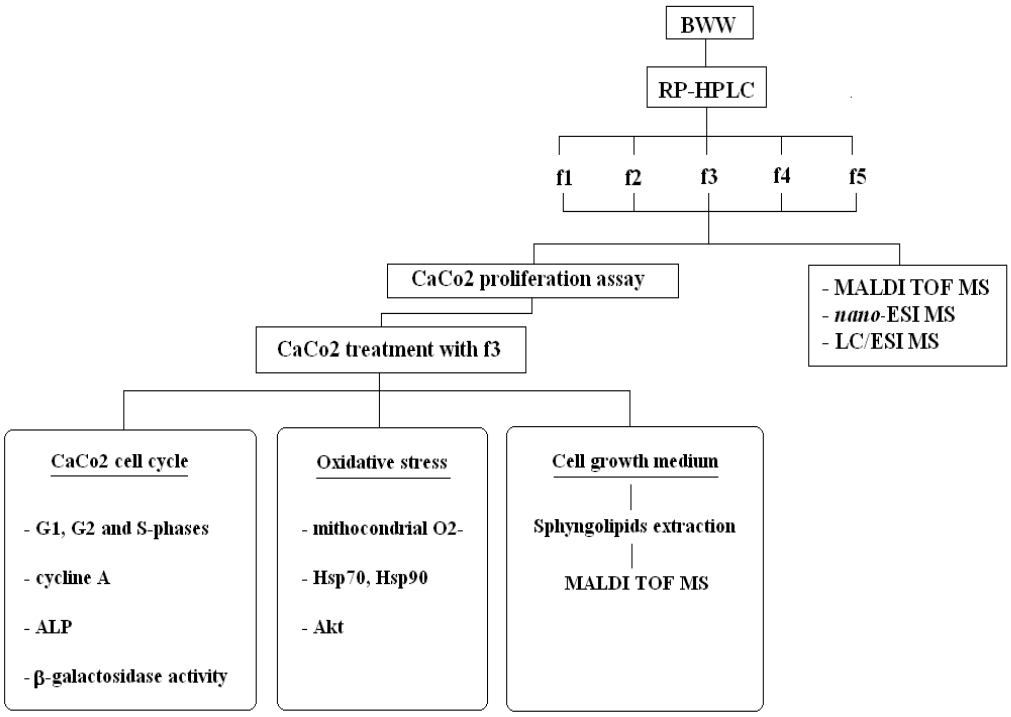

Fig 2. *Analytical approach for f3 proteomic and bio-functional characterization.*

3.6 *nano*- ESI MS and *nano*- ESI MS/MS analysis

Before *nano*-electrospray mass spectrometry (*nano*- ESI MS) analysis, each peptide sub-fractions was purified from residual salts by loading onto $ZipTip_{C18}$ Reversed Phase pre-packed micro-columns (Millipore Bedford MA, USA), previously equilibrated with 0.1% TFA, washing with 0.1% TFA and eluting by aqueous 50% AcN (v/v) containing 0.1% TFA (v/v). Flow direct injection *nano*-ESI MS and *nano*-ESI MS/MS experiments of peptide sub-fractions, manually collected from BWW RP-HPLC, were carried out using an hybrid quadruple-orthogonal acceleration time of flight Q-star Pulsar (PESciex, Toronto, Ontario, Canada) equipped with a *nanospray* source (Protana Inc., Odense, Denmark), operating in positive ion mode. The samples were diluted, doubling the volume, in a solution 2:1 (v/v) H_2O + 5% acetic acid/AcN and introduced in the source trough borosilicate needles, gold coated (Protana Inc., Odense, Denmark). Needle voltage was 800 V and orifice voltage was set at 40 V to minimize fragmentation; air at the pressure of 10 psi was used as "curtain gas". In *nano*-ESI MS/MS experiments, N_2 was used as collision gas; Q0 and Q2 quadrupole voltages were respectively 58.0 V and 9.9 V.

3.7 Extraction of sphingolipids from growth media of preconfluent cells with and without f3 treatment

Spingolipids were extracted form cell culture media of preconfluent cell by adding an equal volume of a mixture of chloroform/methanol 80:20. After vortexing and centrifuging for 5 minutes at 4000 rpm (Minifuge centrifuge, Heraeus, Osterode, Germany), the upper phase was transferred to a new vial for MALDI-TOF-MS analysis.

3.8 Cell culture and cell proliferation assay

CaCo2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in h-glucose MEM containing: 1% (v/v) MEM non-essential amino acids and supplemented with 10% (v/v) decomplemented FBS (Flow, McLean, VA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells (17-21 passages) were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C and were plated in 12 multi-well plates at different densities. After incubation for 4 h in DMEM with 10% FBS, the cells were washed with 1% PBS to remove unattached dead cells.

The starved cells (DMEM without serum) were pre-treated for 30 min with 50 μM H_2O_2 (H-CaCo2) and then incubated with the peptide extracts. To this aim, the dry peptide extracts from BM, BSW, BWW and BS were weighed, dissolved in DMEM without serum and added to the H-CaCo2 cells at the final concentrations of 0,1 or 1 mg/ml.

1.0 x 10^5 H-CaCo2 cells treated with the peptides extracts were seeded in 12 multi-well plates and incubated at 37˚C. After 12 h the cells were counted with a hemocytometric counter and cell proliferation was determined through CyQuant Cell Proliferation Assay Kit (Invitrogen, Milan, Italy) with dye fluorescence measurement at 480 and 520 nm emission maximum.

For the BWW sub-fractions biological assay, the pre-confluent H-CaCo2 (21 passage) were incubated with 0.08 mg/ml of BWW and RP-HPLC purified sub-fractions (f1,f2,f3,f4,f5) for 24 h at 37˚C. All experiments were performed on triplicate cultures. After 24h the cell number and the cell proliferation was determined as already described. Cell proliferation was expressed in percentage of proliferation compared with the control.

The control sample was constituted by H-CaCo2 cells not added of peptide extract (untreated cells). Cell proliferation was expressed as percentage of proliferation compared with the

control sample, which was therefore assumed as 100%. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.

3.9 Flow cytometry analysis of H-CaCo2

H-CaCo2 cells were seeded in 6 multi-well plates at the density of 25 x 10^5 cells/plate. After 12 h incubation with 0.08 mg/ml of peptide extracts from BM and BWW in DMEM without serum at 37°C, and 24 h of incubation with 0.08 mg/ml of f3 sub-fraction, the cells were washed in PBS, pelleted in centrifuged and directly stained in a propidium iodide (PI) solution (50 mg PI in 0.1% sodium citrate, 0.1% Nonidet P40, pH 7.4) for 30 minutes at 4°C in the dark. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). To evaluate cell cycle PI fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 20,000 events for each point were analyzed in at least three different experiments giving a s.d. lower than 5%.

The mitochondrial superoxide anion production was analysed by hydroethidine (HE) staining. The cells were incubated for 1 h at the end of treatment with 20 ng/ml HE stock solution. At the time of processing, cells were scraped, washed twice with PBS and the pellet was added to 1 ml PBS. The hydroethidine-superoxide anion (HE-O) accumulation was determined using the CellQuest software (FACScan, Becton Dickinson) furnished with the cytometer. For each sample, 2 x 10^4 events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

3.10 Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was used as a marker of colonic differentiation degree. The H-CaCo2 cells were washed and lysed with 0.25% sodium deoxycholate essentially as described by Herz et al (1981) [9]. ALP activity was determined using the Sigma Diagnostics ALP reagent (No. 245). Total cellular protein content of samples was determined by the Bradford microassay using the Coomassie Protein Assay Reagent Kit (Pierce). ALP activity was calculated as units of activity for mg of protein.

3.11 Western blot assay

The effect of f3 sub-fraction on expression of Hsp 70, Hsp 90, Akt (also named protein kinase B) and Cyclin A was determined by Western blot. H-CaCo2 treated and untreated (control) were lysed using an ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors containing antipain, bestatin, chymostatin, leupeptin, pepstatin, phosphoramidon, pefabloc, EDTA, and aprotinin (Boehringer, Mannheim, Germany). Equivalent protein samples were resolved on 8-12% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Germany). For immunodetection, membranes were incubated overnight with specific antibodies at the concentrations recommended by the Manufacturer. All antibodies were diluted in Tris buffered saline/Tween 20-1% milk powder. This step was followed by incubation with the corresponding horseradish peroxidase conjugated secondary antibody (antimouse-IgG 1:2000, anti-rabbit-IgG 1:6000; Biosource, Germany). Protein bands were detected by enhanced chemiluminescence (ECL-kit, Amersham, Germany). Total Akt expression was monitored by Western blot analysis with an antibody recognizing Akt independently from its phosphorylation state. For the measure of the activity, Akt was immunoprecipitated with a specific anti-Akt antibody and its activity was measured in an *in vitro* kinase assay using GSK-3a as substrate. Immunoprecipitated Akt kinase was used to phosphorylate glycogen synthase kinase-3a (GSK-3a). GSK-3a phosphorylation was measured by Western blot analysis with a specific anti-phospho-GSK-3a/b antibody. Reactions were carried out according to the instructions of the Akt kinase assay kit from New England Biolabs.

3.12 β-Galactosidase assay

Senescence was assessed by β-galactosidase staining. Cells were washed with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 5 min. After two washes with PBS, the cells were incubated at 37°C for 4 hours in a humidified chamber with freshly prepared SA-β-Gal staining solution (1 mg/ml X-Gal in dimethylformamide, 40 mM citric acid and phosphate buffer, pH 6.0, 5 mM potassium ferrocyanide, 150 mM sodium chloride, and 2 mM magnesium chloride). After one day, cells were washed twice with PBS

at room temperature for 10 min, and staining was visualized and captured using an optical microscope (Carl Zeiss, Inc., USA).

4. RESULTS AND DISCUSSION

4.1 Characterization of peptides in Buffalo Milk and in cheese whey produced during Mozzarella PDO technological process

4.1.1 The peptides of river buffalo raw milk

The peptide extracts from raw BM were analysed in mixture by MALDI-TOF-MS (Fig 3). The mass spectrum clearly showed the presence of two families of casein fragments, those deriving from the N-terminal end of α_{s1}-CN and those from the C-terminal end of β-CN. The simultaneous occurrence of peptides progressively shortened in the same mixture suggested the possible identity of the signals in the spectrum, as it was possible to reconstruct entire sequences on the basis of the differences in molecular masses corresponding to amino acids (Fig 3, inset). For unambiguous assignment of peptide identity, MALDI-TOF-MS PSD analysis and ESI-MS/MS tandem mass spectrometry analysis were carried out.

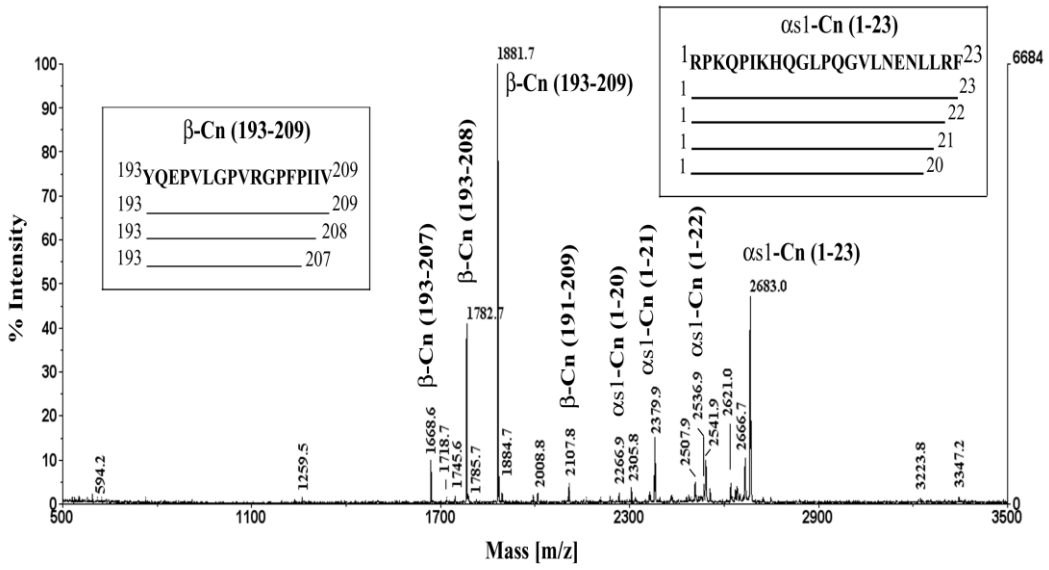

Fig 3. *MALDI-TOF mass spectrum of the low molecular mass peptide fraction (MW‹3KDa) extracted from river buffalo raw milk (BM). In the insets the peptides derived from the main fragments β-CN f193-209 and α_{s1}-CN f1-23 are schematized.*

The peptide α_{s1}-CN f1-23 arose from primary cleavage at the level of the Phe^{23}-Phe^{24} bond, a well known chymosin proteolytic cleavage site [10]. As chymosin is only added at the beginning of the cheese-making process and is not present in raw milk, the peptide bond Phe^{23}-Phe^{24} was probably hydrolyzed by the milk endogenous enzyme cathepsin D [11].

Furthermore, Oomen [12] showed that also the proteolytic action of *L. delbrueckii* spp. *bulgaricus* on α_{s1}-CN produced peptide 1-23 in cow milk. This finding, combined with a possible carboxypeptidase-like enzyme activity producing the shorter peptides α_{s1}-CN f1-22 and f1-21, suggested that α_{s1}-CN N-terminal fragments could be generated by action of extracellular P_I- and P_{III}- proteinases of lactic acid bacteria similarly to what occurs in bovine α_{s1}-CN [13].

The bovine peptide α_{s1}-CN f1-23 presents an antimicrobial activity towards *Staphylococcus aureus* and *Candida albicans* [14]. However, the sequence of cow α_{s1}-CN f1-23 differs from its buffalo homologue in two amino acid substitutions: $His^4 \rightarrow Pro$ and $Glu^{14} \rightarrow Gly$ [15]. Therefore a possible antimicrobial activity for buffalo milk-derived peptide should be confirmed experimentally.

Similarly, for the C-terminal portion of cow β-CN, the finding of the precursor peptide β-CN f193-209, followed by the shorter peptides f193-208 and f193-207, supported the hypothesis of a carboxypeptidase-like activity in BM. Furthermore, β-CN f193-209 is non specifically produced by isolated proteolytic enzymes (unpublished data) indicating that the entire Leu^{192}-Leu-Tyr^{194} sequence is particularly susceptible to hydrolysis possibly because it is located in an exposed protein region.

A multifunctional activity has already been described for the 193-209 region of bovine β-CN, which contains the bioactive sequence β-casokinin-10. Since the C-terminal sequence of β-CN is conserved between river buffalo and cow species, precursors of peptides with immunostimulatory and ACE- inhibitory activities [16] are therefore possibly present also in BM.

4.1.2 The peptides of Mozzarella PDO sweet whey

k-CN is the main substrate of chymosin in cheesemaking, but natural calf rennet contains traces of other proteases, such as pepsin or gastricsin (pepsin C) [17]; furthermore, the microflora of "starter" cultures significantly contributes to proteolysis.

Accordingly, the MALDI-TOF-MS peptide pattern of BSW (Fig 4A) was more complex than that observed for BM peptide extract. Several peptides produced from the C-terminal portion of β-CN, from k-CN (in particular from the k-casein macropeptide, CMP f106-169) and from α_{s1}-CN were identified in BSW peptide extract (Table 1).

The precursor peptide α_{s1}-CN f1-23 previously detected in BM disappeared completely in BSW, although its increase was expected because of the known susceptibility of the Phe^{23}-Phe^{24} bond to chymosin action [18]. This finding could be justified by the secondary proteolysis on the early casein fragment f1-23 which sequentially produced α_{s1}-CN f1-22, f1-21, f1-19; the identification of α_{s1}-CN f24-37 confirmed the cleavage at sites Phe^{23}-Phe^{24} and Val^{37}-Asn^{38}, this latter by action of chymosin [13].

The fragments derived from β-CN C-terminal region were still present in the MALDI-TOF spectrum of BSW peptide extract, although at a lower relative intensity. The precursor peptide f193-209 found in milk was also present in BSW. Identification of β-CN f193-206, f193-207, f193-208 and f195-206, f195-207, f195-208, f195-209 supported the occurrence of secondary proteolysis by action of aminopeptidase and carboxypeptidase removing amino acids sequentially from both the ends of primary peptides.

The peptide β-CN f161-177 was produced by hydrolysis at the level of the residues Gln^{160}-Ser^{161}, as already known for bovine β-CN [19]. Similarly β-CN f57-68 and f58-68 were produced, supporting the hypothesis that, since β-CN is resistant to chymosin action [20] its proteolysis can be ascribed to bacterial hydrolytic enzymes.

Para-k-CN (the N-terminal peptide 1-105 of k-CN generated by chymosin action on casein during milk curding) was scarcely sensitive to proteolysis, whereas several CMP fragments were detected, (Table 1), confirming the CMP sensitivity to proteolysis [21]. As expected by the specificity of chymosin action, significant amount of intact k-CN CMP either glycosylated or non glycosylated (data not shown) were also found. CMP-derived peptides could have a beneficial role in modulating the gut microflora, as the casein macropeptide is known to promote the growth of bifidobacteria mainly because of its carbohydrate content (mainly sialic acid) [22].

Furthermore, CMP and CMP-derived peptides (casoplatelins) may have important biological value as in bovine they exhibited antithrombotic activity [23].

Only the peptides f185-206 (MH^+=2723.8) and f185-207 (MH^+= 2836,1), both with the N-terminal Gln converted to pyroglutamic acid (p-Glu) were identified from α_{s2}-CN (see Table 1).

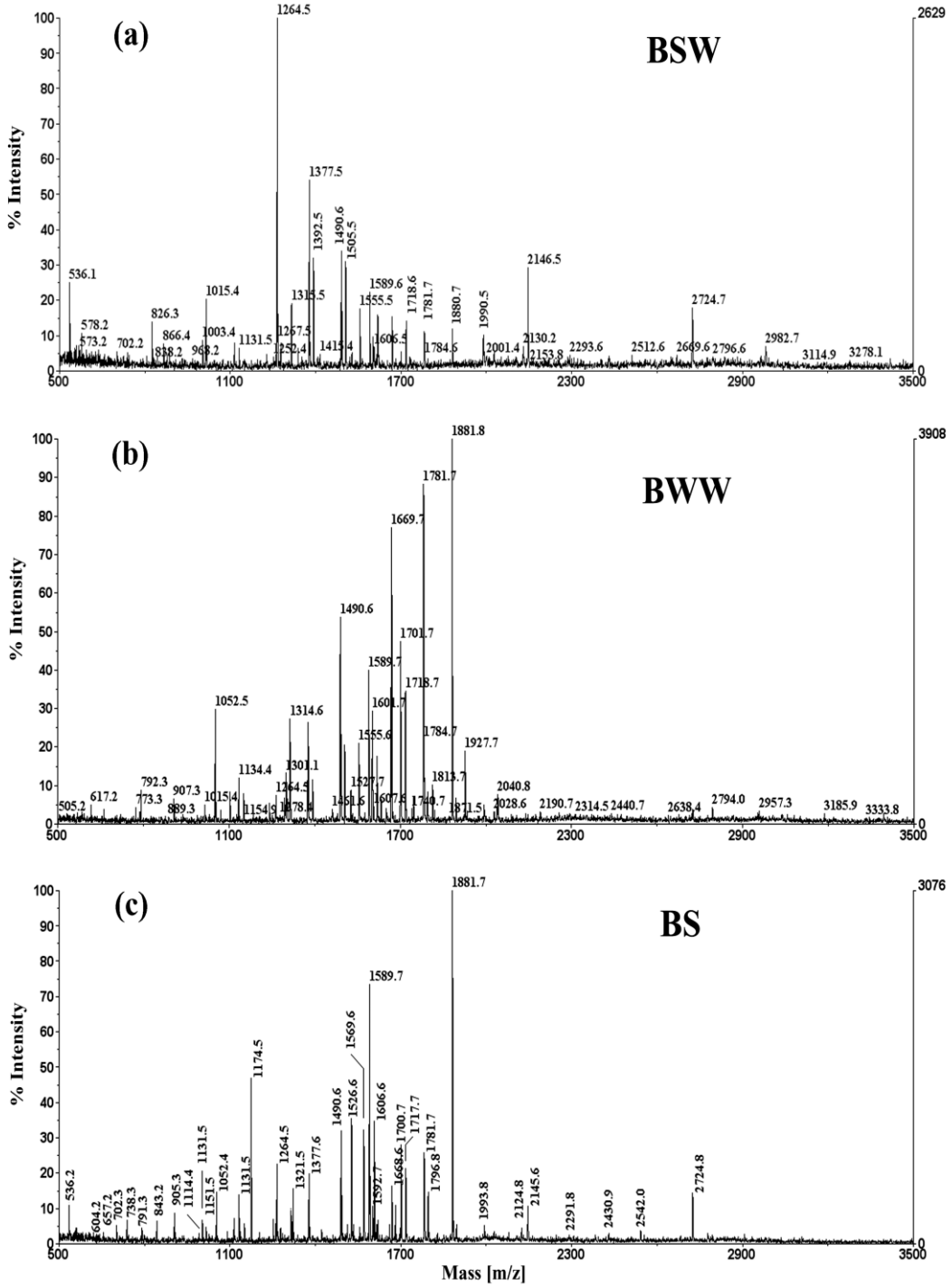

Fig 4. *MALDI-TOF mass spectra of the low molecular mass peptide fractions (MW‹3KDa) extracted from whey at the different stages of Mozzarella di Bufala Campana production: (A) sweet whey (BSW); (B) waste whey (BWW); (C) "scotta" (BS). Assignment of the main mass signals are given in Table 1.*

4.1.3 The peptides of Mozzarella PDO waste whey

As evidenced by MALDI-TOF-MS analysis (Fig 4B), the BWW peptide pattern was very complex because of secondary protease and peptidase action during curd maturation under whey. A multiplicity of peptides from β-, k- and α_{s1}-casein were identified. In the MALDI-TOF spectrum, the most prominent signals arose from the C-terminus of β-CN, probably also because of their high ionization efficiency in MALDI. For this reason, the BWW peptide mixture was also characterized by HPLC/ESI-MS (Fig 5).

A number of peptides derived from β-CN, not present in the BSW peptide extract, were identified in peptide extracted from BWW (Table 1). A drastic progress in the proteolysis of the primary peptide α_{s1}-CN f1-23 was also observed. Several fragments of this peptide were likely produced by the lactic acid bacteria cell-wall envelope enzymes [22].

Furthermore, k-CN fragments either from CMP (f116-127, already identified in BSW) or from para-k-CN, such as f25-40 and f25-45, occurred in BWW. Peptides from para-k-CN, together with the primary peptide f66-79 were identified only in BWW. Infact , the region 66-79 of para-k-CN was particularly exposed to proteolytic attack, as several fragments derived from it. It has to be underlined that para-k-CN following the increase of bacterial proteolytic activity and the pH lowering during curd maturation, becomes sensitive to proteolysis. In bovine, this was already reported by Visser *et al.,* [21] who noted that peptides from para k-CN are generated by action of lactic acid bacteria belonging to *Lactococcus lactis* ssp. *cremoris* AM1.

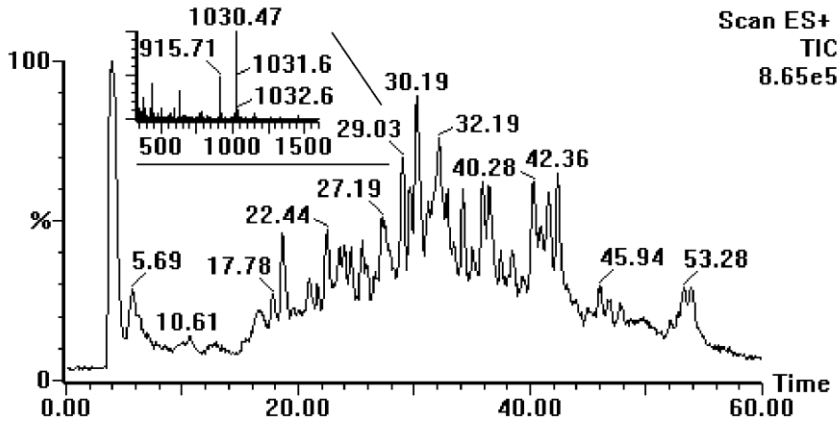

Fig 5. *HPLC/ESI-MS chromatogram of the BWW peptide extract. The total ion current (TIC) chromatogram is shown. Co-elution of several peptides in each chromatographic peaks (inset) emphasizes the great complexity of the peptide fraction.*

Measured mass MH+	***Expected mass***	***Peptide identification***	***BSW***	***BWW***	***BS***
738.7	737.4	αs1-CN f(1-6)		•§	
791.3	790.4	αs1-CN f(18-23)			•
905.3	904.4	αs1-CN f(17-23)			•
1003.4	1002.6	αs1-CN f(1-8)		•	
1018.4	1017.5	αs1-CN f(16-23)			•
1052.4	1051.5	αs1-CN f(17-24)			•
1117.4	1116.6	αs1-CN f(15-23)			•
1131.4	1130.6	αs1-CN f(1-9)		•	
1174.4	1173.6	αs1-CN f(14-23)			•
1188.4	1187.6	αs1-CN f(1-10)		•	
1302.1	1301.7	αs1-CN f(13-23)		•	
1321.5	1320.7	αs1-CN f(14-24)			•
1526.6	1525.9	αs1-CN f(1-13)		•	•
1583.0	1582.9	αs1-CN f(1-14)		•	
1594.4	1592.8	αs1-CN f(24-37)	•		
1683.3	1681.9	αs1-CN f(1-15)		•	
1797.7	1796.0	αs1-CN f(1-16)		•	
2040.7	2039.1	αs1-CN f(1-18)		•	
2154.1	2153.1	αs1-CN f(1-19)	•		
2145.5	2144.0	αs1-CN f(180-199)	•		
2292.6	2291.0	αs1-CN f(179-199)	•		
2379.8	2379.3	αs1-CN f(1-21)	•		
2537.6	2535.4	αs1-CN f(1-22)	•		
2723.7	2722.4	αs2-CN f(185-206) *pyro-Glu*	•		
2836.1	2835.0	αs2-CN f(185-207) *pyro-Glu*	•		
780.1	779.7	β-CN f(169-175)	•	•	•
866.4	866.5	β-CN f(168-175)	•	•	•
995.2	994.5	β-CN f(167-175)	•	•	•
1015.4	1014.5	β-CN f(60-68)		•	
1114.4	1113.6	β-CN f(59-68)		•	•
1135.4	1134.6	β-CN f(196-206)		•	
1227.5	1226.7	β-CN f(58-68)	•	•	•
1264.4	1263.6	β-CN f(195-206)	•	•	•
1314.5	1313.6	β-CN f(57-68)	•	•	•
1377.5	1376.7	β-CN f(195-207)	•	•	•
1392.4	1391.7	β-CN f(194-206)			•
1395.5	1394.8	β-CN f(163-175)		•	
1490.5	1489.8	β-CN f(195-208)	•	•	•
1555.5	1554.8	β-CN f(193-206)	•	•	•
1589.6	1588.9	β-CN f(195-209)	•	•	•
1618.6	1617.9	β-CN f(194-208)		•	
1668.6	1667.9	β-CN f(193-207)	•	•	•
1701.7	1700.9	β-CN f(194-209) *pyro-Glu*		•	
1718.7	1717.9	β-CN f(194-209)		•	
1782.1	1781.0	β-CN f(161-177)	•		
1782.6	1781.9	β-CN f(193-208)	•	•	•
1865.0	1864.0	β-CN f(193-209) *pyro-Glu*			•
1881.7	1881.0	β-CN f(193-209)	•	•	•
1993.8	1994.1	β-CN f(192-209)		•	•
1315.7	1314.7	k-CN f(116-127)	•		•
1415.4	1413.8	k-CN f(116-128)	•		
1773.4	1773.8	k-CN f(153-168)	•		
1903.5	1903.9	k-CN f(151-168)	•		•
1873.7	1873.9	k-CN f(152-169)	•		
2001.3	2000.0	k-CN f(117-135)	•		•
2004.5	2003.0	k-CN f(151-169)	•		
2130.2	2128.1	k-CN f(116-135)	•		•
2629.3	2628.3	k-CN f(25-45)		•	
1194.2	1194.6	k-CN f(70-79)		•	
1282.4	1281.7	k-CN f(69-79)		•	
1083.4	1081.7	k-CN f(66-75)		•	
1396.5	1395.7	k-CN f(66-77)		•	
1495.6	1494.8	k-CN f(66-78)		•	
1608.6	1607.9	k-CN f(66-79)		•	
1934.6	1932.0	k-CN f(25-40)		•	

Table 1. *Identification by mass spectrometry analysis of casein peptides extracted from BSW, BWW and BS produced at the different stages of Mozzarella di Bufala Campana production.*
Expected mass is the mass calculated on the basis of the known casein amino acid sequences.
**For signals simultaneously occurring in different spectra, only one of the measured molecular masses is reported; slight fluctuations on the first decimal place for a signal in different spectra are due to the daily calibration.*
[§] *• indicates the presence of peptide.*

4.1.4 The peptides from "scotta"

"Scotta" is the residual whey resulting from the thermal coagulation of whey proteins during the production of "ricotta" starting from sweet whey. Fig 4C shows the MALDI-TOF spectrum of peptide extracted from BS and the peptides identified are reported in the Table 1. The signals MH^+=2723.8 and MH^+=2145.5 assigned to α_{s2}-CN f185-206 and α_{s1}-CN f180-199 respectively, were found unchanged from BSW to BS, most likely because of proteolytic enzyme inactivation due to thermal treatment applied in the production of "ricotta". The finding that the BSW peptide pattern is quite similar to BS, allowed us to conclude that most of the peptides of BSW are not included in "ricotta" whey protein network and that the peptide content of whey is scarcely affected by mild thermal treatment.

4.2 Functional effects of peptides extracted from BM, BSW, BWW and BS on CaCo2 cells

Recent studies, carried out on several human cancer cell lines, have shown that milk-derived peptides may act on the regulation of cell growth, differentiation and apoptosis [24-27].
Based on these considerations, we decided to evaluate the protective effects of peptides derived from Mozzarella whey samples against H_2O_2-induced DNA damage in CaCo2 cell lines. Starved cells were exposed to 50 μM H_2O_2 for 30 min (H-CaCo2) and then treated for 24 h with 0.1 and 1 mg/ml of peptide extracted from BM, BSW, BWW and BS. Interestingly, we observed that only exposure of H-CaCo2 cells to BWW 1 mg/ml resulted in a 43% reduction in cell proliferation (Fig 6) and decreased mithocondrial superoxide H-CaCo2 anion production analyzed by FACS. The adduct mean fluorescence intensity decreased from 6.2% for the control (H-CaCo2 cells) to 3.7% with BWW peptide extract fraction (Fig 7). To further investigate the effect of peptide extracted from BWW on cell proliferation, the distribution in each phase of the cell cycle was determined by flow cytometric analysis of DNA content.

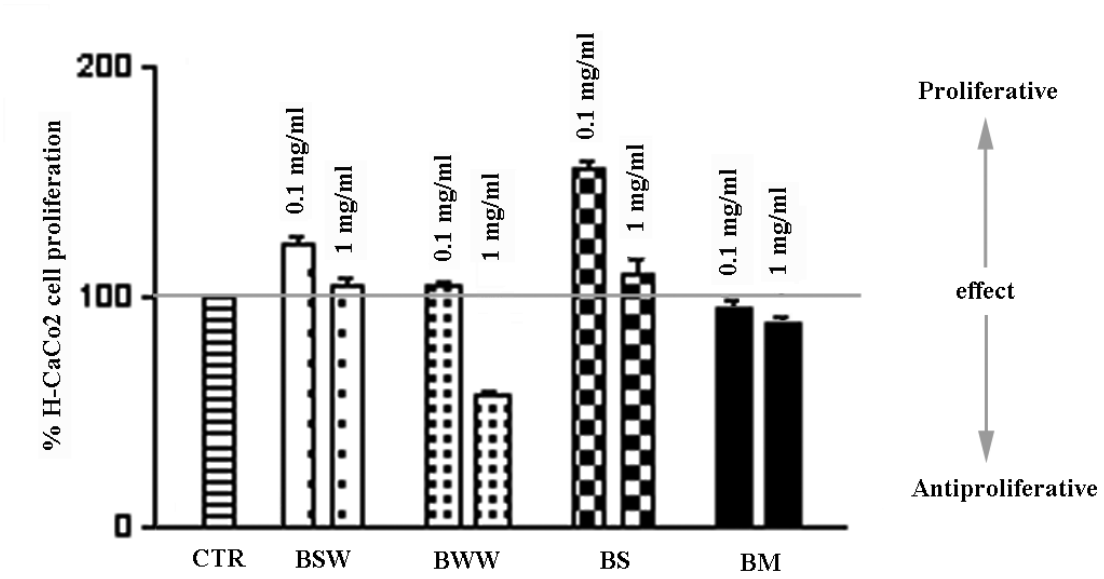

Fig 6. *H-CaCo2 cell proliferation assay. Starved cells were pre-treated for 30 min with H2O2 (50 μM) and then incubated with 0.1 and 1 mg/ml of peptides extracted from BM, BSW, BWW and BS at 37 °C for 12 h. The control sample was constituted by H-CaCo2 cells untreated with the peptide extracts. Cell proliferation measured in treated samples was expressed as percent increase (proliferative effect) or decrease (antiproliferative effect) compared with that of the control sample, assumed as 100%. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.*

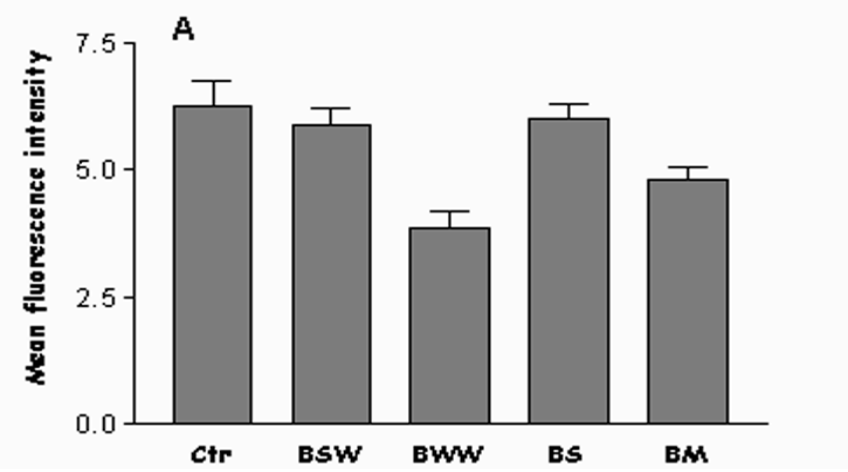

Fig 7. *Flow cytometry analysis of oxidative stress in H-CaCo2 after incubation with peptide extracted from buffalo milk (BM), sweet whey (BSW), waste whey (BWW) and "scotta" (BS). The control represented H-CaCo2 cells untreated with peptide extract. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.*

As reported in Fig 8, a significant reduction ($p < 0.05$) of cells in S phase and an accumulation in the G1 phase were observed in H-CaCo2 cells treated with 0.08 mg/ml BWW for 24 h. Concomitantly a sub-G1 peak in H-CaCo2 cells (data not shown) was detected. These results

suggest that, at the experimental concentration, BWW peptide extract is able to inhibit cell proliferation, interfere with cell cycle and exert a possible pro-apoptotic activity in H-CaCo2 cancer cells.

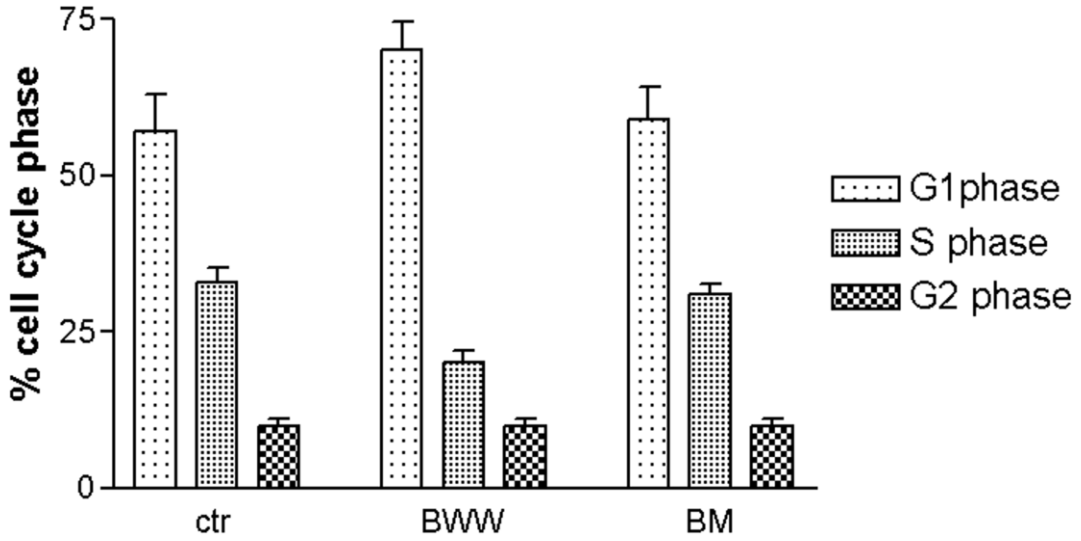

Fig 8. *BM and BWW effects on G1 and S phase of H-CaCo2 cell cycle. All experiments were performed on triplicate cultures and data are the mean ± SD of three experiments.*

It is worth noting that the difference in the biological activity between the peptide extracted from BWW and the other three samples, finds a correspondence in the different peptide composition defined by the structural study (compare the MALDI spectra in Fig 3 and Fig 4). This means that the components responsible for the biochemical effects are specifically produced in the transition from BSW to BWW during curd maturation.

4.3 Mass spectrometry characterization and proliferation effect of BWW sub-fractions on H-CaCo2 cell lines

As evidenced by RP-HPLC analysis (Fig 9A), BWW produced during "Mozzarella di Bufala Campana PDO" manufacturing is a very complex mixture of peptides which derived from hydrolysis of caseins by endogenous proteases (e.g. plasmin), proteolytic enzymes of rennet and endo-/exo-peptidases from lactic acid bacteria. Peptide sub-fractions were obtained by RP-HPLC purification of BWW by collecting eluates at 10 min time intervals.

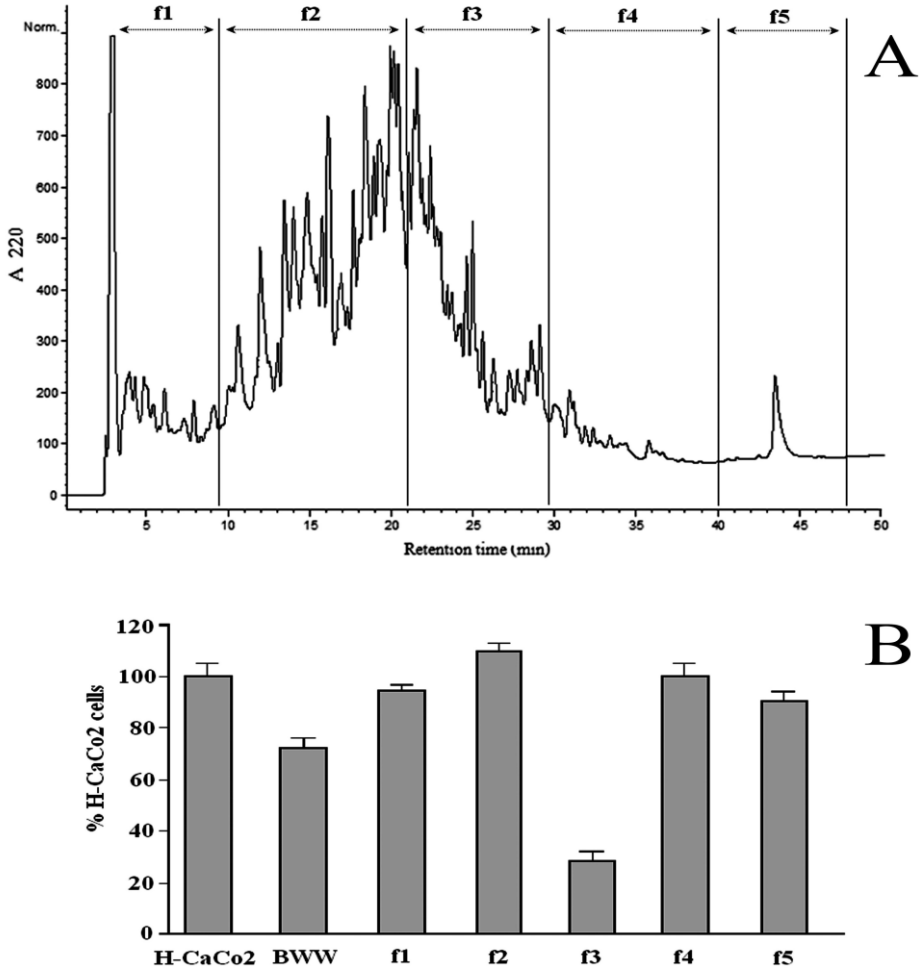

Fig 9. *(A) RP-HPLC fractionation of the 3 kDa-permeate obtained from BWW. Each peptide fraction was collected at 10 min time intervals and termed with f followed by a number. (B) H-CaCo2 cell proliferation assay. The H-CaCo2 were treated for 24h with different RP-HPLC sub-fractions (f1-f5) of BWW. Cell proliferation was expressed as percent compared with the control. Each experimental value is the mean of three different determinations.*

Five peptide sub-fractions (f1, f2, f3, f4 and f5) were tested for 24h in H-CaCo2 cell line to assay proliferation effects (Fig 9B). BWW reduced H-CaCo2 proliferation of about 30% compared with the control sample represented by H-CaCo2 cells untreated with peptide extract. f1, f2, f4 and f5 had no statistically significant effect on cellular proliferation compared to control cells, while only f3 showed a marked antiproliferative effect (67% growth reduction), with respect to the unfractionated BWW (p value > 0,0001). To further investigate its composition and biological effects, f3 was characterized by MALDI-TOF MS (Fig 10). The peptides mainly derived from κ-cn, *g*-CMP (*glyco*-caseinmacropeptide) and β-cn while only three peptides were from α_{s1}- and α_{s2}-CN (Table 2). Peptides identity was confirmed by *nano*ESI-MSMS as shown in Fig 11 where the fragmentation spectrum *nano*ESI-MSMS of double charge ion with m/z=766.9 and molecular weight 1531.8 Da

corresponding to the sequence k-CN f 155-169 is shown. A limited number of peptides remained unidentified. The mass spectrometry analysis allowed us to identify the single peptide components and to hypothesize the presence of active sequences and of their precursors (Table 2) as for the β-CN f57-68 and β-CN f60-68 which are precursors of the agonist opioid *β*-casomorphin 7 and *β*-casomorphin 5 [16]. Very little information is available on the release of BCMs during digestion of β-casein from buffalo milk, so the presence of these peptides is a very interesting information and according to our knowledge it fully complies with the data provided by Petrilli *et al*., [28]: buffalo β-CN simulating gastro-pancreatic digestion using pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, carboxypeptidase B and leucine amino peptidase, does not release BCM7, but only a pro-BCM7 with amino acid sequence corresponding to residues 59-68 of buffalo β-casein. This information leads us to observe that even with the involvement of different enzymatic entities, the gastro-pancreatic process and the proteolysis occurring in BWW, involves peptide similar entities**.**

Recently, it has been reported that casomorphin-agonist peptides derived from the limited proteolysis of caseins, interacting with both opioid [29,30] and somatostatin receptors [31], acted in decreasing cell proliferation. In particular, Hatzglou *et al*. [28] have proved that in T47D human breast cancer cells *in vitro, α*- and *β*-casomorphins inhibit cell proliferation in a dose-dependent manner, by interaction with *δ*- and *κ*-opioid receptors. Opioid and somatostatin receptors are present in the cells of nervous, endocrine and immune system as well as in the intestinal tract of mammals [32], including CaCo2 cells. Therefore, the proliferation decrease we observed in colon cells could be mediated by a direct interaction between opioid precursors contained in BWW peptide extract and the specific opioid and somatostatin receptors [33,34] expressed on CaCo2 cells.

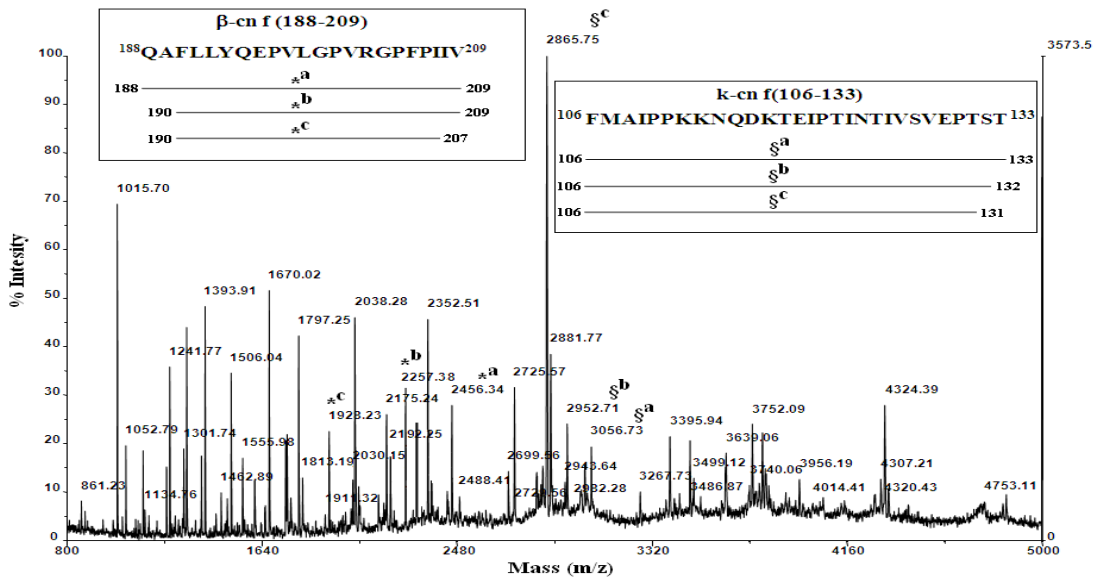

Fig 10. *MALDI-TOF mass spectrum of f3 sub-fraction eluted at 20<tr<30 about. In the insets the peptides derived from the main fragments β-CN f188-209 and κ-CN f106-133 are schematized.*

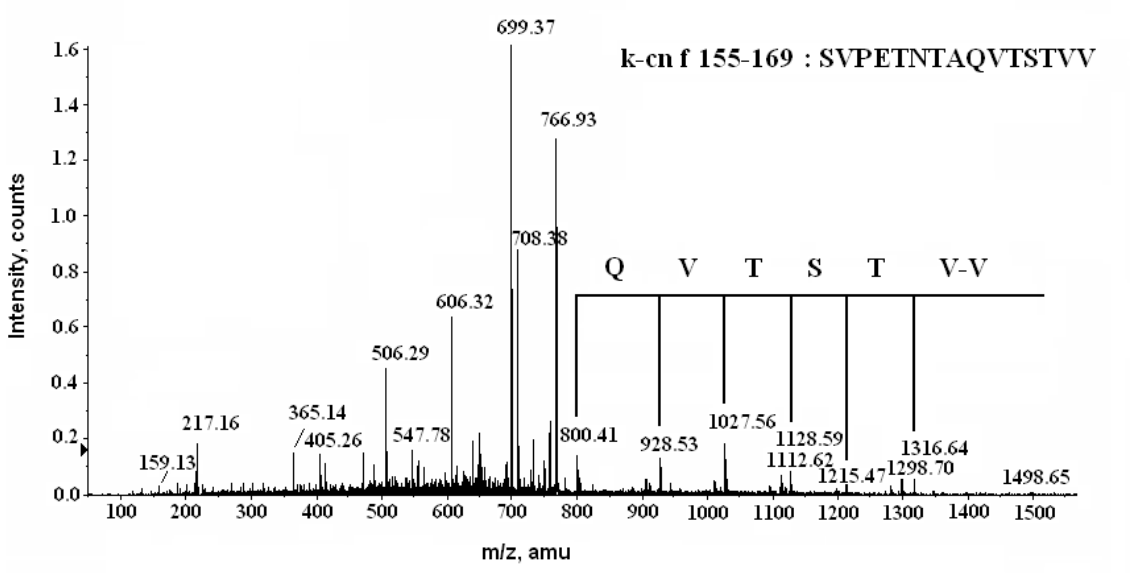

Fig 11. *Nano-electrospray ionization tandem mass spectrometry (nanoESI-MSMS) fragmentation of the double charge ion with m/z = 766.9 and molecular weight 1531.8 Da corresponding to the sequence of k-cn f(155-169) identified in f3 subfraction.*

Measured mass MH^+ *	Peptide identity	Expected mass
1015.08	β-cn f(60-68)	1014.55
1039.04	β-cn f(199-209)	1150.69
1151.20	β-cn f(199-208)	1051.62
1278.24	β-cn f(61-72)	1276.72
1302.14	αs_1-cn f(13-23)	1301.71
1313.28	β-cn f(57-68)	1313.74
1394.06	αs_2-cn f(44-55)	1393.65
1505.34	β-cn f(194-207)	1504.84
1532.27	k-cn f(155-169)	1531.77
1555.30	β-cn f(193-206)	1554.82
1669.37	β-cn f(193-207)	1667.90
1762.38	αs2-cn f(101-115)	1763.95
1796.55	β-cn f(63-79)	1797.02
1927.52	β-cn f(190-207)	2042.14
1943.44	β-cn f(65-80)	1742.99
2256.58	β-cn f(190-209)	2254.29
2455.67	β-cn f(188-209)	2453.39
2724.77	β-cn f(181-204)	2721.45
2865.08	k- cn f(106-131)	2864.54
2952.98	k- cn f(106-132)	2951.57
3055.11	k- cn f(106-133)	3052.62
3413.16	β-cn f(50-80)	3414.82
4926.44	k-cn f(130-169) + 2P + 1 GalNAc 1 Gal 1 NeuAc§	4921.44
4746.36	k-cn f(130-168) + 1P + 1 GalNAc 1 Gal 1 NeuAc	4741.36
4846.92	k-cn f(130-169) + 1P + 1 GalNAc 1 Gal 1 NeuAc	4841.92

Table 2. *Identification by mass spectrometry analysis of casein peptides eluted in f3 sub-fraction after RP-HPLC analysis of the 3-kDa permeate of BWW. Expected mass is the mass calculated on the basis of the known casein amino acid sequences.*
**For signals simultaneously occurring in different spectra, only one of the measured molecular masses is reported; slight fluctuations on the first decimal place for a signal in different spectra are due to the daily calibration.*
§ The carbohydrate part of peptides from g-CMP occurs in the same glycosilated chain composed of only few sugars as N-acetyl Galactosamine (GalNAc), Galactose (Gal) and Neuraminic Acid (NeuAc).

4.4 The antioxidant effect of f3 reduced Hsp 70 and 90 expression and Akt activity

The antioxidant effect of f3 on H-CaCo2 cell line was investigated through analysis of the intracellular redox status of H-CaCo2 cells treated with f3 by evaluating mitochondrial superoxide anion production (Fig 12).

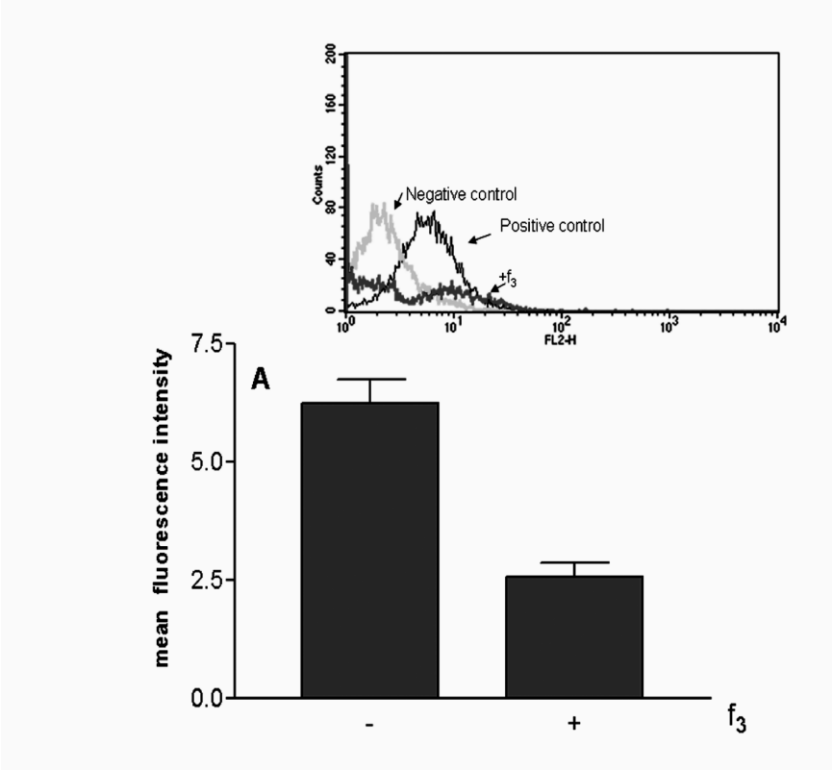

Fig 12. *Flow cytometry measure of oxidative stress on H-CaCo2 without (-) and with(+) f_3 treatment. The intensities of the bands were expressed as percentage respect to the control. For each sample, 2 x 10^4 events were acquired. In the inset was reported fluorescence spectra of bands associated with O-HE analysis, with "negative control" was indicated the H-CaCo2 without HE, the "positive control" was H-CaCo2 with HE and " + f_3" the H-CaCo2 treated with f_3.*

Hydroetidine was used to measure mitochondrial superoxide anion production of vital cells. The adduct mean fluorescence intensity decreased from 6.2% in the control to 2.5% in the cells treated with f3. This indicated a corresponding decrease in the superoxide anion production.

This finding led us to investigate if the decrease of superoxide anion production might influence the expression level of Hsp70 and 90, which are significantly elevated in many cancers, and in the case of some tumor types this is linked with poor prognosis and a muted response to chemotherapy. In the specific case of CaCo2 adenocarcinoma cell line, it has been previously reported that high Hsp levels are expressed constitutively even under non stress conditions [35]. This expression has been associated with enhanced survival to conditions such as oxidant- and thermal- induced stress. In Fig 13 the expression levels of Hsp 70 and Hsp 90 are reported. Fig 13A reports the western blot analysis of Hsp 70 and Hsp 90 in H-CaCo2 cells treated with 0.08 mg/ml of f3 for 24h. Treatment with f3 decreased Hsp 70 and Hsp 90 expression of 36% and 10% respectively (Fig 13B).

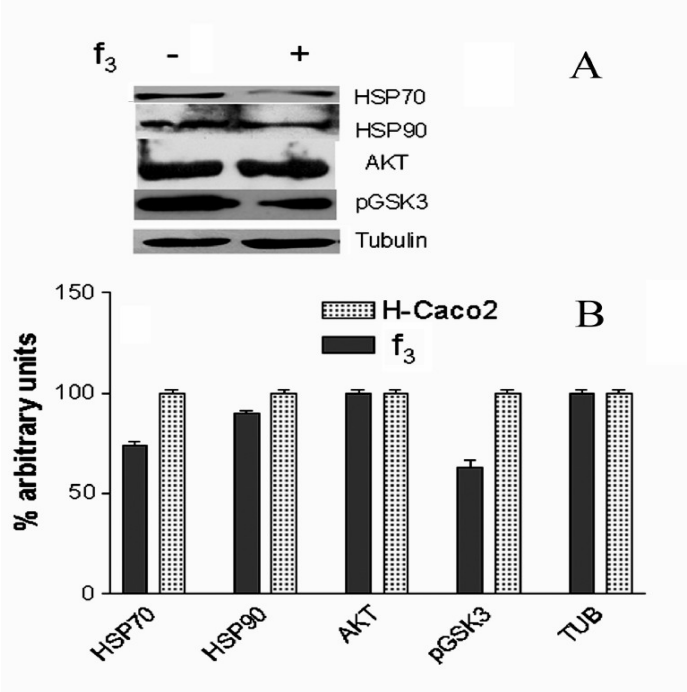

Fig 13. *Western blot analysis of H-CaCo2 without (-) and with (+) f_3 treatment. (A) The bands associated with expression of Hsp70, Hsp90, house-keeping γ-tubulin, Akt and Phospho-GSK-3a/b(Ser21/9) after 24h of treatment with f_3 are visualized. The expression of the house-keeping protein γ-tubulin, was used as loading control. (B) The expression levels of the above proteins are reported as percentage respect to the level of the protein in untreated cells, used as control. Bars show the mean values ± SD. Each experimental value reported is the mean of three different determinations.*

Hsps have a profound impact on the activities of several prosurvival signaling cascades, including those mediated by Akt, a downstream effector of the PI3-K pathway, a ubiquitous lipid kinase involved in receptor signal transduction by tyrosine kinase receptors [36]. There is increasing evidence that the activation of PI3-K/Akt is associated with colorectal carcinoma, and can convert differentiated human gastric or colon mucosa to a less differentiated and more malignant phenotype [37-39].

Akt is overexpressed in several cancers, including those of the colon, pancreas, ovary, and breast [40]. Moreover, Akt phosphorylation in human colon carcinomas is correlated with cell proliferation and inhibition of apoptosis, as well as different clinico-pathological parameters such as invasive grade, vessel infiltration, lymph node metastasis, and tumor stage [41,42].

For this reason, we compared the expression and activity of Akt in H-CaCo2 treated with f3 and control cells (Fig 13A). The treatment with f3 induced no effect on Akt expression whereas the level of p-GSK-3 was decreased of 37% with respect to the control cells (Fig 13B).

4.5 Effect of f3 on lipid synthesis and secretion

We examined whether the Hsp decrease by f3 treatment in preconfluent H-CaCo2 was correlated with production of ceramides. It is well known that several inductors of cell death, including TNFα [43], anthracyclines [44], or irradiation [45] involve ceramide signalling.
Administration of exogenous ceramide also causes cell death in various cancer cell lines [46]. It is noteworthy that many cancer cells have a specific 'sphingolipid–phenotype', including lower endogenous ceramide levels [47] and a higher sensitivity to the effects of exogenous ceramides [48].
So it is well accepted that ceramides function as a second messenger in cells: the increase in their intracellular concentration in response to extracellular signals induces cell arrest and inhibition of growth in cancer cells [49-51].
In Fig 14 the MALDI-TOF mass spectra of the chloroform extracts from the cell growth medium without (Fig 14A) and with (Fig 14B) f3 incubation are reported. A series of sphyngolipid components in the mass range 700-1200 Da was observed in the cells treated with f3, whereas a single component at m/z 1031 Da was found in the control sample. Identified sphyngolipid classes in the sample treated with f3 include ceramides EOH, EOS, AH and cerebrosides EOS, all molecules involved in the process of cell differentiation [43].
To denominate families of sphingolipids, we followed the nomenclature of Motta *et al.,* who designated the three types of fatty acids in sphingolipids as non-hydroxy acids, α-hydroxy acids and ω-hydroxy acids esters linked to linoleate as N, A and EO, respectively [52].
The three types of sphingoid bases, sphingosine, phytosphingosine and 6-hydroxysphingosine found in ceramides and cerebrosides were indicated as S, P and H. For example, a ceramide consisting of a ω-hydroxyacid ester-linked to a molecule of phytosphingosine would be designated as CER EOP, according to Motta *et al.*, [52].

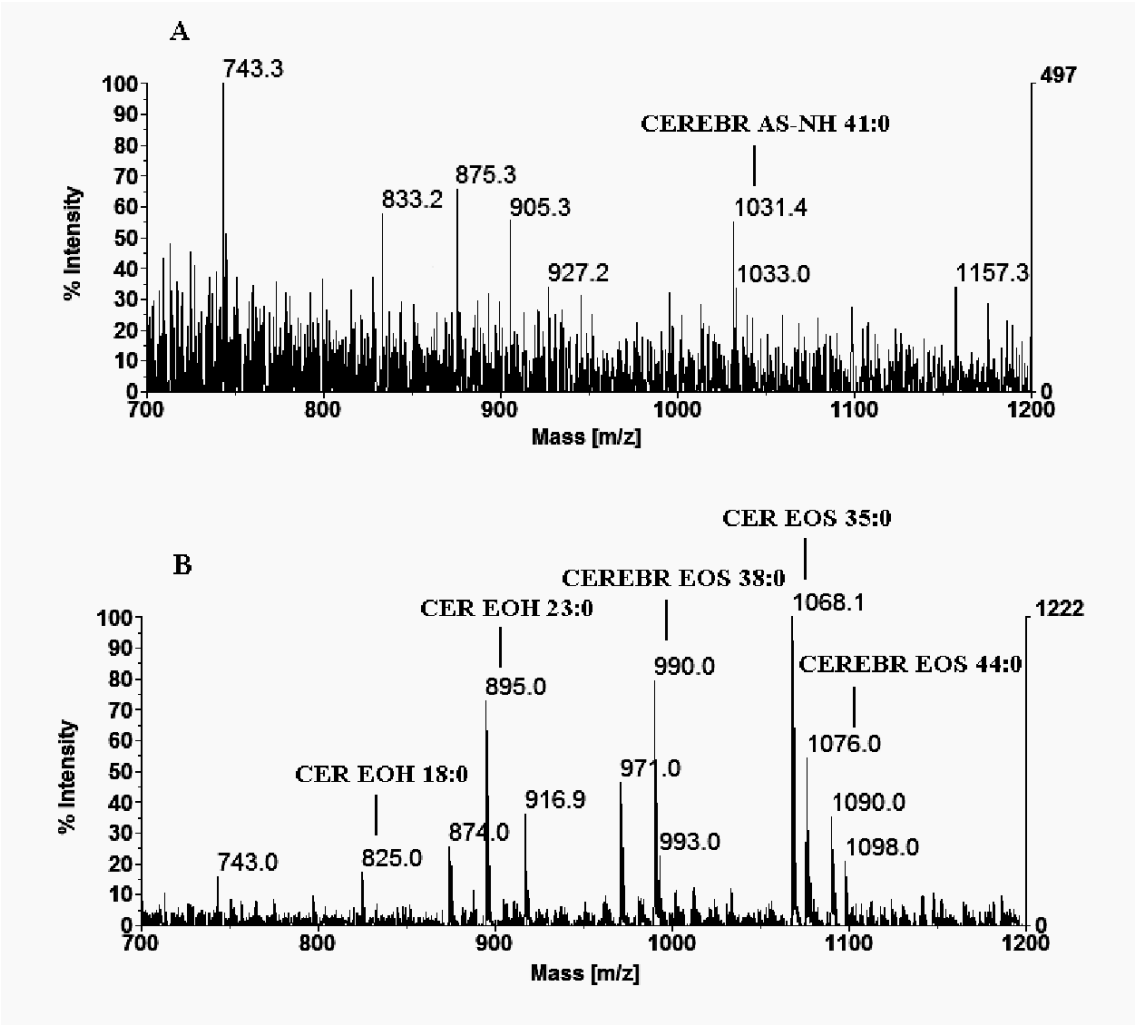

Fig 14. *MALDI- TOF mass spectra of chloroform extracts from the growth serum of pre-confluent cells without (A) and with (B) f3 incubation. A series of sphyngolipid components in the mass range 700- 1200 Da was observed in the cells treated with f3. CER, ceramides; CERBR, cerebrosides.*

4.6 Effect of f3 on H-CaCo2 cells: quiescence, differentiation and senescence

In order to elucidate if the ceramide productions was involved in the regulation of the cell cycle, we analyzed by FACS the percentage of G1/G0, G2/M, and S-phase cells. In Fig 15A the percentage of G1, G2, and S-phase untreated and treated cells with f3 after 24h is reported. The treatment resulted in accumulation of cells in the G1/G0 phases (66%), while concomitantly the S-phase populations decreased (25%). These results suggested that f3 arrest the cell cycle in G1 phase as further confirmed by a 5-fold decrease of cyclin A expression as shown in Fig 15B [53].

These results are associated with increased expression of alkaline phosphatase activity (Fig 15B), a marker of enterocytic differentiation correlated to post-confluent phase. Moreover, cell cycle arrest is accompanied by an increased senescence-associated (beta)-galactosidase (1.5-fold), compared to control (Fig 15C) [54].

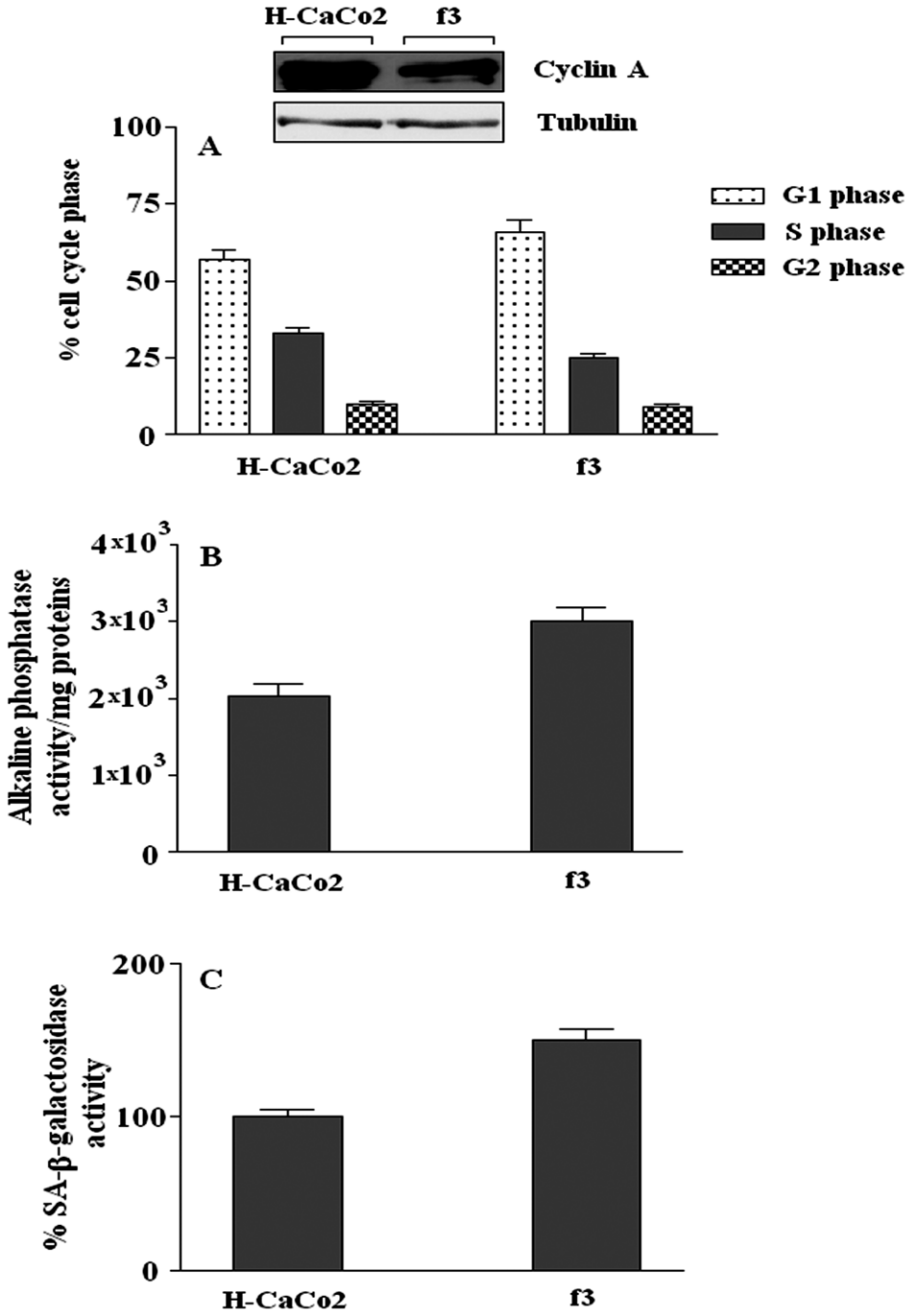

Fig 15. *(A): G1, G2 and S phases cell cycle and cyclin A expression and (B) Alkaline phosphatase activity of H-CaCo2 treated with f3. To evaluate cell cycle, PI fluorescence was collected as FL2 (linear scale). In the inset Western blot analysis with Anti-cyclin A antibody is reported. (B) Alkaline phosphatase activity/mg proteins is showed. ALP activity were measured in homogenates of H-CaCo2 cells (without and with f3 treatment) and expressed as activity per cell proteins. (C) senescence-associated (beta)-galactosidase activity. Bars show mean values ± SD; each experimental value reported is the mean of three different determinations.*

5. CONCLUSIONS

Many of the peptides originated from the degradation of milk proteins have been characterized as biologically active. In this context, the criterion generally used to assess the quality of food proteins, based solely on the nutritional properties of the protein, should be revised on the protein ability to release peptides with biological activity, during food processing. The great complexity and the wide dynamic range of relative peptide abundance in these products severely challenge the capabilities of existing analytical methodologies. A major step forward has been achieved by combining the biochemical and biological approach with the tools of proteomics technologies like addressed in this PhD project.
With regard to the results obtained in this study, we can conclude that:

- ✓ although raw BM has an intrinsic peptide pattern produced by milk endogenous proteases, the proteolysis of buffalo milk proteins, occurring during the production of Mozzarella di Bufala Campana PDO cheese, further increases the complexity and heterogeneity of each peptide extract. The structural characterization allowed us to conclude that milk proteolysis is the result of a complex series of events due to the combined action of either native or endogenous milk proteases, milk clotting enzymes, starter culture, and contaminating microflora. Compared to BM, BSW and BS, only the low-molecular mass peptides in BWW was highly heterogeneous, with a large number of different oligopeptides.
- ✓ the differences in the biological activity among the peptide extracted from BM, BSW, BWW and BS, find a correspondence in the different peptide composition defined by the structural study. Only BWW peptide extract is able to inhibit cell proliferation, interfere with cell cycle and exert a possible pro-apoptotic activity in H-CaCo2 cancer cells. The absence of this effect in the peptide extract from original milk (BM) suggests that the production of specific bioactive compounds occurs specifically during the production process of Mozzarella cheese. Previous studies [38,39] have suggested protective activity of peptides originating either from the bacterial cell-wall or by hydrolysis products of the lactic acid bacteria.
- ✓ the f3 peptide fraction obtained through BWW chromatographic purification not only preserves the same antioxidant and anti-proliferative action on H-CaCo2 cells, but also decreases mitochondrial superoxide anion production and reduces Hsp70 and Hsp90 expression in survival H-CaCo2. The lowered Hsp expression induced by f3 treatment

made these cells significantly more sensitive to the injurious effects of the antioxidant agents. CaCo2 cells, infact constitutively express high levels of Hsps even under non stress conditions which is at the basis of their high resistance to chemical, immunological and chemotherapeutic agents. Moreover, the f3 treatment induces increased secretion of ceramides which resulted in cell cycle arrest, differentiation, and in subsequent 'accelerated senescence" cell death in H-CaCo2. It is known that the intestine undergoes a continuous remodelling process in which ceramides play an important role as messenger molecules.

This finding might envisage future applications of the active peptide fractions for the development of nutraceuticals for prevention of gut disfunctions, for healthing recovery in cases of acute injuries such as damage occurring in celiac disease or other intestinal pathologies and to increase the efficacy of drugs for treatment of gastrointestinal cancer. An intriguing finding was that peptides characterized in f3 are spontaneously produced during the cheese-making process of buffalo Mozzarella cheese, and released by action of the lactic acid bacteria into the waste whey, a natural source with low or null cost, available in large amounts, from which peptides could be easily purified once the appropriate industrial method has been set up. This information could also drive research in the utilization of industrial by-products for the development of dietary supplements for functional foods and of novel drugs for pharmaceutical industry.

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CHAPTER II

1. INTRODUCTION

1.1 Donkey milk

Donkeys provide a kind of milk with nutritional and therapeutic properties known since the time of Herodotus (V century B.C). Taking into account that modern food trends are turning towards alternative products which are more responsive to new market demands, donkey milk represents a novel possibility for food technology science for its promising opportunities.

Although donkeys are less important for milk production than cows, buffaloes, sheep and goats, they have been traditionally important dairy animals in Asia, Africa and Eastern Europe. Donkey milk resembles human milk in many ways and is claimed to have special therapeutic properties: it is becoming increasingly important in Europe because it is recommended as a substitute for cow's milk protein allergy (CMPA) which affects about 3% of children in the first three years of life [1].

Hypoallergenic alternatives to breast milk have been proposed through the years and include soy formulas and extensively hydrolysed milks. The extensive hydrolysed formulas, which are industrial milk-based products treated with proteolytic enzymes, have been recommended as the first choice for the dietoteraphy of CMPA patients [2]. However, administration of these foods, does not completely solve the problem in children with CMPA especially for the presence of possible residual epitopes. Moreover, the unpleasant bitter taste and the poor palatability of the breast milk substitutes can be a significant problem not only for those children suffering from CMPA.

For these reasons, in the last few years, milk from non-bovine mammals (goat, donkey, mare, camel) has been extensively studied to identify the best natural substitute for human milk [3-5]: it seems that milk produced by mono-gastric animal species, such as donkey and mare, is the most suitable.

Donkey milk was found to be a valid alternative to both IgE-mediated and non-IgE mediated CMPA, with favourable effects on palatability in an increasing number of clinical trials [6,7], although the mechanism of this tolerance, likely related to the protein composition, has not been fully clarified yet.

Moreover, a recent clinical study confirmed that feeding with donkey milk is a safe and valid treatment for the most complicated cases of multiple food intolerance, too [6]. In addition to

its use for infants, some researchers have reported that donkey milk has an effect on the osteogenesis process, as well as in arteriosclerosis therapy, for the rehabilitation of patients with coronary heart disease or premature senescence, and in hypocholesterolemic diets [8]. Overall, donkey milk is considered to be highly digestible, rich in essential nutrients and to posses an optimum whey protein/casein ratio, making it suitable not exclusively for the treatment of CMPA.

In the past, donkey breeding was diffuse in Italy. The progressive mechanization of the agricultural activity, the depopulation of rural districts and the consequent reduction of working, has been causing a gradual decrease of donkeys and mules population since the end of the Second World War, with a strong acceleration during 1970s. The fifth Italian Agricultural census (ISTAT, 2006) reports a donkey population of 19325 heads in the year 2000. Nowadays, the donkey breeds officially standardized in Italy are: Amiata, Asinara, Martina Franca, Ragusano and Sardo. However, many other breeds are spread on the national territory, for example Grigio Siciliano, Pantelleria and Romagnolo. Since 1996, the local Breeders' Association of Forlì-Cesena-Rimini area has been promoting the recovery of donkey breeds and it has started a project in order to recognize the breed in the National Stud Book. The Central Technical Committee of Italian Breeders' Association admitted donkey breeds in the Register in 2005 [9].

Nowadays, in Italy, the consumption of donkey milk is gradually increasing thanks to the interest of consumers searching for a natural product, not only to replace human or bovine milk when needed. In the last few years, the number of farms breeding donkeys has notably risen and in 2006 the Italian Breeders Association (AIA) introduced functional controls on donkey milk production.

The various research projects funded at national level in recent years have certainly helped to increase knowledge about donkey milk composition and at the same time have placed the emphasis on its nutritional value. Notwithstanding this renewed growing interest, donkey's milk has not been completely investigated and, in particular, the data available for its protein components are still poor [10-13].

1.2 Donkey milk: composition and commercial regulations

Donkey, as well as horse species, belongs to the *Equus* family, and this phylogenetic relationship emerges in the similarity of their milk composition [14].

Mare milk (which is closely related to donkey milk) has been studied extensively, while little is known about donkey milk. The composition of donkey milk differs considerably from that of the milk of the principal dairying species such as cow, buffalo, goat and sheep, while it is quite similar to that of horse and human ones, as shown in table 1. Overall, in comparison to bovine milk, donkey milk contains less fat fewer proteins and inorganic salts, a relevant percentage of essential amino acids, but more lactose with a concentration close to the one found in human milk: donkey milk has been regarded as the best breast milk substitute thanks to its intrinsic composition.

Furthermore, donkey milk also contains a high amount of lysozyme, which is practically absent in the milk produced by cows, sheep and goats: this enzyme possesses bactericidal properties as it hydrolyzes the polysaccharides of bacterial cell walls and inhibits bacteria development [15].

Species	Total solids	Protein	Casein /Whey ratio	Fat	Lactose	Ash	Gross energy
Donkey[a] (*Equus asinus africanus*)	88.4	17.2	1.3:1	3.8	68.8	3.9	1582
Horse[a] (*Equus caballus*)	102.0	21.4	1.1:1	12.1	63.7	4.2	1883[b]
Man[a] (*Homo sapiens*)	124.0	9.0	0.4:1	38.0	70.0	2.0	2763[b]
Cow[c] (*Bos taurus*)	127.0	34.0	4.7:1	37.0	48.0	7.0	2763[d]
Buffalo[a] (*Bubalus bubalis*)	172.0	46.5	4.6:1	81.4	48.5	8.0	4644[b]
Sheep[c] (*Ovis aries*)	181.0	55.9	3.1:1	68.2	48.8	10.0	4309[d]
Goat[a] (*Capra hircus*)	122.0	35.0	3.5:1	38.0	41.0	8.0	2719[b]

a= values are express as g kg^{-1} milk
b= values are express as kJ kg^{-1} milk
c= values are express as g L^{-1} milk
d= values are express as kJ L^{-1} milk

Table1. *Gross composition of the milk of selected species* [16].

According to the Italian law, donkey milk can only be sold directly from the farm as raw milk (Regio Decreto 1929 n_ 994, paragraph 15, 43).

The EC Regulation n. 853/2004 allows the sale of donkey milk being these animals included in the "other milk species". According to this EC Regulation, donkey milk should have a total bacterial plate count of less than <1,500,000/ml at 30°C. Anyway, if donkey milk is assigned to infants or elderly human beings, it's absolutely necessary that, besides the nutritional requirements, the sanitary aspects are also evaluated. Conte has already discussed the European

indications as inadequate for the control of milk quality in donkeys, demonstrating that in presence of some milk bacteria (*S. aureus*, *S. intermedius*, *S. dysgalactiae*) there is no evidence, in donkeys, of clinical signs and/or increased somatic cell count (SCC) [17].

1.3 Donkey milk protein composition

Even if the content of mature donkey milk protein is lower than that of bovine milk, there is a strong resemblance within the principal classes of proteins as casein and whey proteins. Caseins are the principal class of proteins in cow's milk (about 80% of total milk proteins), while donkey milk contains fewer caseins with a casein/whey protein ratio of 1.3:1 compared to 4.7:1 for cow's milk. So, due to the high content of whey proteins which is slightly less than the amount of casein**s**, donkey milk does not permit the production of cheese. Anyway, the information on the distribution and composition of each part of the class of donkey casein and whey proteins is extremely limited and even susceptible to novel discoveries, perhaps the identification of new components that were originally thought to be non existent in donkey milk, as for example for α_{s2}-CN (see results).

Notwithstanding the renewed growing interest, donkeys' milk has not been completely investigated and, in particular, the data available for its protein components are still poor [10,13].

For these reasons, in our study, we assumed as reference the mare milk protein, where no data in literature can be found for the donkey counterpart. The available knowledge on donkey milk protein structures mainly concerns the primary structure and genetic polymorphism of whey proteins, as α-lactalbumin (α-La) [18,19], β-lactoglobulin (β-Lg) I and II, [10, 20-21] lysozyme [18, 22] and SA (serum albumin) [UniProtKB/Swiss-Prot Q5XLE4].

Regarding donkey casein composition, the occurrence of β-CN and α_{s1}-CN has been reported, by means of IEF and MS-analysis through fitting of their tryptic hydrolysates with the known mare counterparts [23] and the entire β-CN and α_{s1}-CN sequence has been determined [24, 25].

A more recent study [26] was based on N-terminal sequencing and peptide mass fingerprinting of 33 spots excised from 2-DE of donkey milk using horse protein as reference.

1.4 Caseins

Casein micelles are primarily a source of amino acids, calcium, phosphate and bioactive peptides for neonates [27].

The casein fraction of most milk types consists of four gene products: α_{s1}-, α_{s2}-, β- and k-CN, of which the first three are calcium-sensitive. All caseins display a distinct lack of secondary structure, which led Holt and Sawyer [28] to classify them as rheomorphic proteins.

The following sections present the state of art of individual caseins separately by referring to those of mare for the above reasons, focusing the attention on the donkey ones when literature data are available.

1.4.1 α_{s1}-CN

Mare α_{s1}-CN amino acid sequence has been derived from its cDNA (GenBanck Acc. No AAK83668) sequence by Lenasi *et al.,* [29].

The protein consist**s** of 205 amino acid**s** and (without post-translational modifications) it presents a molecular weight of 24614.4 Da. Two smaller isoforms of α_{s1}-CN have been identified in equine milk, which are probably due to alternative splicing processe**s** during transcription leading to casual exons skipping events involving exon 7 and 14 result from the skipping of exons [13].

Equine α_{s1}-CN contains six potential phosphorylation sites [29], five of which are in very close proximity (Ser^{75}, Ser^{77}, Ser^{79}, Ser^{80}, Ser^{81}) forming a phosphorylation centre.

The phosphorylation modification levels of the native isoforms of equine α_{s1}-CN induce an hight heterogeneity: Matéos *et al*., [30] identified 36 different isoforms with several phosphate groups ranging from two to six or eight (depending on whether exon 7 was skipped), which present a complex pattern on 1D and 2D electrophoresis.

Recently, Cunsolo *et al.,* [25] identified in the dephosphorylated casein fraction of an individual milk sample collected in Eastern Sicily from a donkey belonging to Ragusano breed at middle lactation stage, four α_{s1}-CN isoforms with experimentally measured Mr of 23658, 23786, 24278 and 24406 Da with two minor components with experimentally Mr of 25142 and 25272 Da. The variant B (197 amino acids; Mr 23786 Da) and variant B1 (196 amino acids; Mr 23 658 Da) differ for the presence of a glutamine residue at position 83 in the full-length component and present the amino acid substitutions Gln^{8} →His and His^{115} →Tyr

with respect to the mare's α_{s1}-CN. The other two components also named variant A (202 amino acids; Mr 24406 Da) and variant A1 (201 amino acids; Mr 24278 Da), which also differ in the presence of a glutamine residue at position 88 in the full-length component, show the insertion of the pentapeptide HTPRE between Leu^{33} and the Glu^{34}, which accounts for the increase of 620 Da in their molecular masses. The two αs1-CNs variant A and A1 bearing the pentapeptide HTPRE present a less acidic nature (theoretical pIs of 6.12) with respect to the variants B and B1 (theoretical pIs of 6.01).

Taking into account the exon modular structure of mare's α_{s1}-CN, it is reasonable to assume that the pentapeptide HTPRE between Leu^{33} and the Glu^{34} is encoded by the exon 5,which is instead constitutively spliced in mare's α_{s1}-CNs, where it should express the sequence SIPREVRK [29].

On the other hand, the existence of the two isoforms lacking a glutamine residue is probably related to a cryptic splice site occurring at the first codon (CAG) of exon 11 (encoding this glutamine residue) as already reported for ewe, [31] goat, [32] cow and water buffalo [33] α_{s1}-CNs.

The other two minor components at 25142 Da and 25272 Da might correspond to two additional α_{s1}-CN isoforms, according to the results on the characterization of α_{s1}-CN in mare's milk [30].

The existence of different α_{s1}-CN variants also clearly indicates that the donkey α_{s1}-CN polymorphism is typical of equidae milk and reflects the complex exon/intron structure of α_{s1}-CN.

1.4.2 α_{s2}-CN

Similar to human milk in which the α_{s2}-CN have never been identified, mare's milk may not express α_{s2}-CN: the complete amino acid sequence of equine α_{s2}-CN is unknown. Ochirkhuyag [34] published the sequence of α_{s2}-CN N-terminal 15 amino acid residues (Lys-His-Lys-Met-Glu-His-Phe-Ala-Pro-???-Tyr-???-Gln-Val-Leu) and only some of which were later confirmed by Miranda [13].

1.4.3 β-CN

The amino acid sequence of equine β-CN, derived from the cDNA, is reported by Lenasi *et al.,* [29] and revised by Girardet *et al.*, [35], by the insertion of eight amino acids (Glu^{27} to Lys^{34}).

The theoretical molecular mass of this 226 amino acid polypeptide is 25511.4 Da. Two smaller variants of equine β-CN, which probably result from casual exon-skipping during transcription, were reported by Miranda *et al.,* [13].

The 28 N-terminal amino acid residues contain seven potential phosphorylation sites (Ser^{9}, Ser^{15}, Ser^{18}, Ser^{23}, Ser^{24}, Ser^{25}, Ser^{28}) and multi-phosphorylated isoforms of equine β-CN, containing three to seven phosphoserine residues, have been reported, with isoelectric points varying from pH 4.74 to 5.30 [30].

Equine β-CN (Swiss-Prot Accession No. Q9GKK3) also presents several isoforms that contains potentially more phosphate groups than the bovine and human counterparts, i.e. up to 7P and only recently, the location of the phosphorylated residues on the primary structure has been recently determined [36].

In particular, it seems that the *in vivo* phosphorylation follows a sequential way and is not randomly performed: the isoform 4 P was found to be phosphorylated on residues Ser^{9}, Ser^{23}, Ser^{24}, and Ser^{25} and the subsequent phosphate groups on Ser^{18}, Thr^{12} and Ser^{10}, led to the formation of the isoforms 5P-7P, respectively.

The primary structure of a low-*M*r β-CN variant which seems to be specific of the equine species, has been characterized by Miclo *et al.,* [37], and is available in Swiss-Prot databank with the accession number Q9GKK3.

The low-Mr β-CN is apparently absent from the milk of other mammals including ruminants and low-Mr β-CN was isolated from Haflinger mare's milk. This short protein (94 amino acid residues and Mr 10591.6 ±2 Da) is an internally truncated form of the full-length equine β-CN (226 residues).

In particular, in the short β-CN, a large deletion (residues 50-181) is due to a cryptic splice site usage occurring within exon 7 during the course of primary transcripts processing. Seven phosphorylation forms were identified with one to seven phosphate groups with p*I*s ranging between 4.67 and 4.01. The major isoforms carry five and six phosphate groups.

In equine sodium caseinate, the Lys^{55}-Ile^{56} bond of equine β-CN is readily hydrolysed by bovine plasmin whereas no cleavage of a corresponding bond, Lys^{48}-Ile^{49}, in bovine β-CN, occurs [38].

Similarly in bovine β-CN, Lys^{28}-Lys^{29} is readily cleaved by plasmin but the equivalent, Lys^{37}-Leu^{38}, in equine β-CN, is insensitive [39]. Other plasmin cleavage sites in equine β-CN are Lys^{111}-Arg^{112}, Arg^{112}-Lys^{113} and Lys^{113}-Val^{114} [39]. Equine β-CN is readily hydrolysed by chymosin at Leu^{198}-Tyr^{199} [40].

Concerning donkey milk β-CN, Cunsolo *et al.,* 2009 identified in the dephosphorilated casein fraction of a milk sample collected from an individual donkey belonging to the Ragusana breed of Eastern Sicily, two coeluting proteins with Mr values of 25529 and 24606 Da, identified as donkey β-CN [24].

The new donkey β-CN show**s** a mass difference of 923 Da due to the presence of the domain $E^{27}SITHINK^{34}$ in the full length component (Mr 25529 Da) as occurred in the mare's β-CN.

In view of these analogies between donkey and mare β-CN, the full length (226 aa) was termed variant A, whereas the shorter one (218 aa) was termed variant $A^{\Delta 5}$. The two donkey β-CN**s** present nine amino acid substitutions with respect to the mare's β-CN used as reference: L-S^{37}, R-H^{52}, S-N^{81}, P-V^{84}, L-V^{91}, R-Q^{203}, P-L/I^{206}, L-F^{210} and A-P^{219} (amino acid positions are referred to the full-length variant A). These substitutions account for the increase of 18 Da in the Mr of the donkey's β-CN in comparison with the mare's counterparts. About the phosphorylated β-CN pattern, the variant A consists of three isoforms carrying from 5 to 7 phosphate groups, whereas no data were obtained about the phosphorylation level of the short β-CN.

1.4.4 κ-CN

The presence of κ-CN in equine milk was an issue of debate for several years, with several authors reporting its absence [34] even if other studies [41] not only showed its presence, albeit at a low concentration, but also proposed it**s** primary structure.

The equine κ-CN contains 165 amino acid residues and is available in Swiss-Prot databank with the accession number P82187. Before post translational modifications equine κ-CN molecular weight is 18847.7 Da. Although no direct information is available on κ-CN glycosylation, lectin binding studies indicate that equine κ-CN is glycosylated , probably at residues Thr^{123}, Thr^{127}, Thr^{131},Thr^{149}, Thr^{153} [29].

Like mare milk, donkey one could have a κ-CN content so low to make it difficult to identify: donkey κ-CN ha**s** not been structural characterized.

1.5 Non-protein-nitrogen NPN of mares milk

It is widely recognized that the NPN fraction of milk consists primarily of urea, peptides, amino acids and ammonia. NPN constitutes 10-15% of the total nitrogen in mature equine milk which is intermediate between the values for human milk and ruminant milk, 25 % and 5%, respectively.

Anyway, the NPN of equine milk as well as that of donkey milk, has not been studied in detail [42,43].

2. AIM OF THE SECTION

Taking into account that the donkey milk soluble peptides in non nitrogen fraction have never been characterized and their potential antioxidant bioactivity has never been investigated, the aim of this section is to study the low molecular weight peptides (Mr‹3KDa) by a peptidomic approach. Moreover, we have investigated the antioxidant effect of donkey milk peptides (DMPs) on linolenic acid in oxidative stress conditions induced by hydrogen-peroxide (H_2O_2). This study has been carried out on donkey milk samples provided by the broader SELMOL project.

Since the structural characterization of NPN peptides can not be conduced unless reported to the primary sequence of the protein from which they are generated, a proteomics characterization on the casein component has also been carried out.

In particular, the donkey caseome has been characterized coupling one-dimensional (PAGE, UTLIEF) and 2-DE (PAGE → UTLIEF) profiles, stained with either Coomassie Brilliant Blue (CBB) and specific polyclonal antibodies, with structural MS analysis.

3. MATERIALS AND METHODS

3.1 Materials

All chemicals were of the highest commercially available purity and were used without further purification. TEMED, ammonium persulphate, glycine, urea, ammonium bicarbonate (AMBIC), HPLC grade H_2O, formic acid (FA) and CH_3CN were purchased from Carlo Erba (Milan, Italy). TFA, sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide, dithiotreitol, phenyl-methyl-sulfonyl fluoride (PMSF) and linolenic acid (LA) were obtained from Aldrich (St. Louis, MO, USA). Modified trypsin, sequencing grade was Promega (Madison, WI, USA), alkaline phosphatase (AP) was Roche (Mannheim, Germany). Zip-tip C_{18} microcolumns and Centriprep® 10-KDa cut-off membrane were Millipore (Bedford, MA, USA). Sep-pak C18 cartridge were Waters (Milford, MA). Acrylamide, Bis, Ampholine buffers were GE Healthcare Amersham Bioscience (Buckinghamshire, UK). Coomassie Brilliant Blue R 250 and G 250 were purchased from Bio-Rad (Richmond, CA 94804, USA).

All chemicals and reagents were of analytical grade or better from Sigma. HPLC-grade acetonitrile (AcN) was purchased from Carlo Erba (Milano, Italy).

3.2 Donkey milk sampling: casein and native milk peptides extraction

Milk samples collected from 63 donkeys reared in Italy were analysed individually. To prevent undesired peptide hydrolysis, immediately after collection, samples were added with 1-mM PMSF.

Fresh milk was skimmed by centrifugation at 4500 *g* at 4 ◦C for 30 min (Haereus Biofuge, Kendro, Germany). To harden the cream, the tubes were kept at −20 ◦C for 10 min and finally the cream was scraped off.

Each casein sample was prepared by acid precipitation from skimmed milk, followed by centrifugation at 2500 x *g* for 15 min, as described by Aschaffenburg & Drewry [44]. The casein sample was freeze-dried and stored at -20°C before use.

Only 18 on 63 milk samples were used for the extraction of native donkey milk petides (DMPs32-DMPs45), with an ultra-filtration step on Centriprep® cartridges with a 3-KDa cut-off membrane. The permeates, lyophilised and solubilised in 0.1% (v/v) aqueous TFA, were purified from saline contaminants and lactose with a Sep-pak C18 cartridge, previously equilibrated in 0.1% TFA and eluted with 70/30/0.1 AcN/water/TFA (v/v/v). The peptide extracts were dried using a Savant concentrator (Speed-Vac,Milan, Italy) and stored at -20 ◦C either for structural analysis or for biological assay.

3.3 Electrophoretic and immunoblotting analysis

Casein samples (20 g/L) for electrophoretic analysis were dissolved in a 9 M urea solution, containing 2-mercaptoethanol (1 mL/L). Polyacrylamide gel electrophoresis (PAGE) at pH 8.6 was carried out with a vertical electrophoretic apparatus (Protean II, Bio-Rad, Richmond, CA 94804, USA) at 200 V and 6°C for 7 h according to the procedure described by Chianese *et al* [45]. Staining was performed with Coomassie Brilliant Blue R-250.

Ultra-Thin Layer Isoelectric Focusing (UTLIEF) on polyacrylamide gels (0.25 mm) was carried out as previously described [45]. Briefly, the pH gradient in the range 2.5-6.5 was obtained by mixing Ampholine buffers (GE Healthcare Amersham Bioscience, Buckinghamshire, UK), 2.5-5, 4.5-5.4 and 4-6.5 (1.6:1.4:1 by volume). The gel was stained with CBB G-250 as described by Krause *et al.,* [46]. The 2-DE procedure was achieved by combining the first dimension PAGE gel with the second dimension UTLIEF gel; in particular, an unstained strip of gel PAGE, rinsed twice in distilled water and in 9 M urea with mercaptoethanol 0.01% (v/v), was applied along the cathode of prefocused UTLIEF plate.

For immunoblotting analysis, the casein fractions separated either by PAGE, UTLIEF or 2-DE analysis were transferred by capillary diffusion from the gel onto a nitrocellulose membrane (0.45 μm, Trans-Blot, Bio-Rad). Immunodetection was carried out according to the procedure already described by Chianese et al., [45] using rabbit polyclonal antibodies against bovine peptides α_{s1}-CN (187-199) and β-CN (195-199) and porcine κ- and α_{s2}-CN.

3.4 RP-HPLC analysis

Casein samples were fractionated by RP-HPLC on a 214TP54, 5 μm Vydac C_4, 250 x 4.6 mm internal diameter column (Vydac, Hesperia, CA, USA) at a detection wavelength of 220 nm. Solvent A was 0.1% trifluoroacetic acid in ultra pure water (v/v) and solvent B 0.1% (v/v) TFA in acetonitrile. 200 μL of a solution containing 1 mg (casein sample)/mL (solvent A) were loaded onto a C_4 column, equilibrated with solvent A. The elution program involved a gradient from 30 to 50% solvent B in 40 min, then from 50 to 100% B in 2 min, at a flow rate of 1 mL/min. Each eluted casein fraction was manually collected, freeze-dried, and stored at -20°C.

3.5 In-gel digestion of protein spots

In-gel digestion of the protein spots was carried out on selected gel pieces manually excised from the CBB-stained two dimensional electrophoresis gels, following the procedure reported by Mamone *et al*., [47]. Analysis of intact proteins eluted from gels was carried out according to Cohen & Chait [48].

3.6 Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry analysis

MALDI-TOF-MS experiments were carried out on a Voyager DEPRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delay extraction technology and N_2 laser at 337 nm. Mass spectra were acquired both in positive linear or reflector mode and 10 mg/mL SA and CHCA both dissolved in 50% ACN/0.1% TFA, were used as matrices for the analysis of proteins and peptides, respectively. MALDI-TOF analysis of intact proteins were obtained in linear positive ion mode over the m/z range 10000–30000 and were averaged from about 150 laser shots.

Only the mixtures of tryptic peptides were subjected to a desalting/concentration step with Zip-Tip C_{18} microcolumns prior to analysis by MALDI-TOF. Spectra were obtained in reflectron positive ion mode over a *m*/*z* range 600-4000 and were averaged from about 150

laser shots. External calibration was performed by acquiring separate spectra of a mixture of standard peptides (Perseptive Biosystems).

3.7 Electrospray Quadrupole-Time of Flight-Mass Spectrometry

Peptides and proteins were analysed by micro LC ESI Q-TOF MS using a CapLC Waters high-throughput configuration directly connected to a Q-TOF Ultima Mass Spectrometer (Waters Corporation, Manchester, UK). Samples (1 μL) were loaded on 5 mm x 100 μm ID Zorbax^{TM} 300 SB C_{18} trap columns (Agilent Technologies), and the peptides were separated on 15 cm x 100 μm ID Atlantis C_{18} capillary columns at a flow rate of approx. 1 μL/min. Solvent A contained an aqueous 0.1% formic acid solution and solvent B contained 84% ACN in 0.1% FA. The gradient consisted of isocratic conditions at 5% B for 10 min, a linear gradient to 30% B over 40 min, a linear gradient to 100% B over 10 min, and then a linear gradient back to 5% B over 5 min. MS analyses were performed in positive mode using ESI. LC-MS was performed with the Q-TOF Ultima operating in either (continuum) MS mode or in MS/MS mode for data dependent acquisition (DDA) of MS/MS peptide fragmentation spectra.

3.8 TBAR's test

The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress [49].

The biological assay, was performed on 10 μl of each DMPs [16 μg/μl], to which 2 ml of tiobarbituric acid solution (0.37% v/) and TCA (15% v/v) were added and adjust to a final volume with HCl 0.25 N. The sample is first boiled 30 min at 100°C, then cooled in ice and finally centrifuged 5 min at 8000 rpm. A spectrophotometric analysis of the supernatant was carried out at λ 532 nm.

4. RESULTS and DISCUSSION

4.1 PAGE and immunoblotting analysis of donkey casein

The donkeys casein samples were analysed individually. In Fig. 1, the two most common individual donkey casein samples Coomassie Brilliant Blue stained (1A) were compared to the cow counterparts on the basis of their relative net charge at alkaline pH by PAGE analysis. The results obtained after specific immunostaining with polyclonal antibodies against α_{s1}-, α_{s2}-, β- and κ-CN (Fig. 1B, 1C, 1D and 1E, respectively), allowed to detect the four donkey casein fractions.

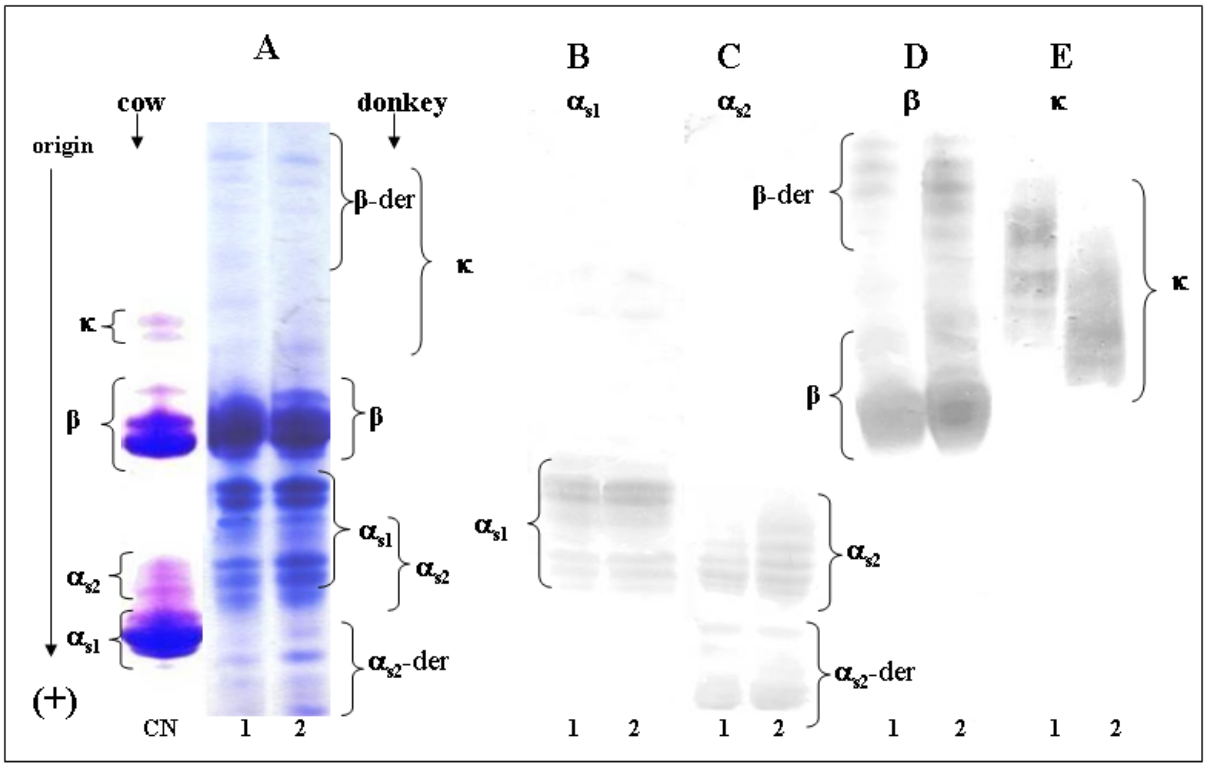


Fig. 1. *PAGE analysis at pH 8.6 of the two common donkey's casein samples (1 and 2) compared to cow casein, after CBB staining (A) and identification of the four casein fractions by immunoblotting with polyclonal antibodies against α_{s1} (B), α_{s2} (C), β (D) and κ-CN(E).*

By comparing the two species with each other, cow and donkey β-CN exhibited a very similar anodic mobility, while donkey α_{s1}- and α_{s2}-CN a lower and reversed anodic mobility than its cow counterpart. The immunostained profiles showed: i) an overlap between α_{s1}- and α_{s2}-CN components; ii) proteolysis of α_{s2}- and β-CN, giving rise to derived peptides having higher

(α_{s2}-CN derived fragments) and lower anodic mobility (β-CN derived fragments) than the parent protein.

Considering the NPN peptide composition of donkey milk, consisting mainly in β-CN and α_{s2}-CN derived peptides generated by plasmin-like specific cleavages (as reported in 4.5 paragraph), it can be supposed that the same enzymes produced the above β-CN and α_{s2}-CN derived insoluble peptides at pH 4.6.

Finally, as shown in CBB stained PAGE analysis (Fig. 1A), it was very difficult to detect the κ-CN components without specific immunostaining (Fig. 1E), likely depending either on the low amount of this fraction in the milk or on its lower reactivity to CBB staining. These results showed: i) the lowest mobility of κ-CN towards the anode compared to the other donkey casein fractions; ii) a different electrophoretic mobility of the two κ-CN (sample 1 > sample 2) likely due to the occurrence of genetic polymorphism at this *locus* (Fig. 1E).

4.2 UTLIEF analysis and immunoblotting of donkey casein

The UTLIEF profiles of the two donkey casein samples, with the four casein fractions labelled in brackets after specific immunostaining, are shown in Fig. 2 (2B, 2C, 2D and 2E). The results underlined a more complex overlapping phenomenon, due to the similar p*I* of casein fractions components, with respect to the above PAGE analysis. In particular, donkey and cow β-CN exhibit a very similar p*I* value, but a different compositional heterogeneity (8-9 components in donkey *vs* 2-3 in cow), due to the different phosphorylation degree of each donkey β-CN component and to the presence of deleted forms, as in mare counterpart [13].

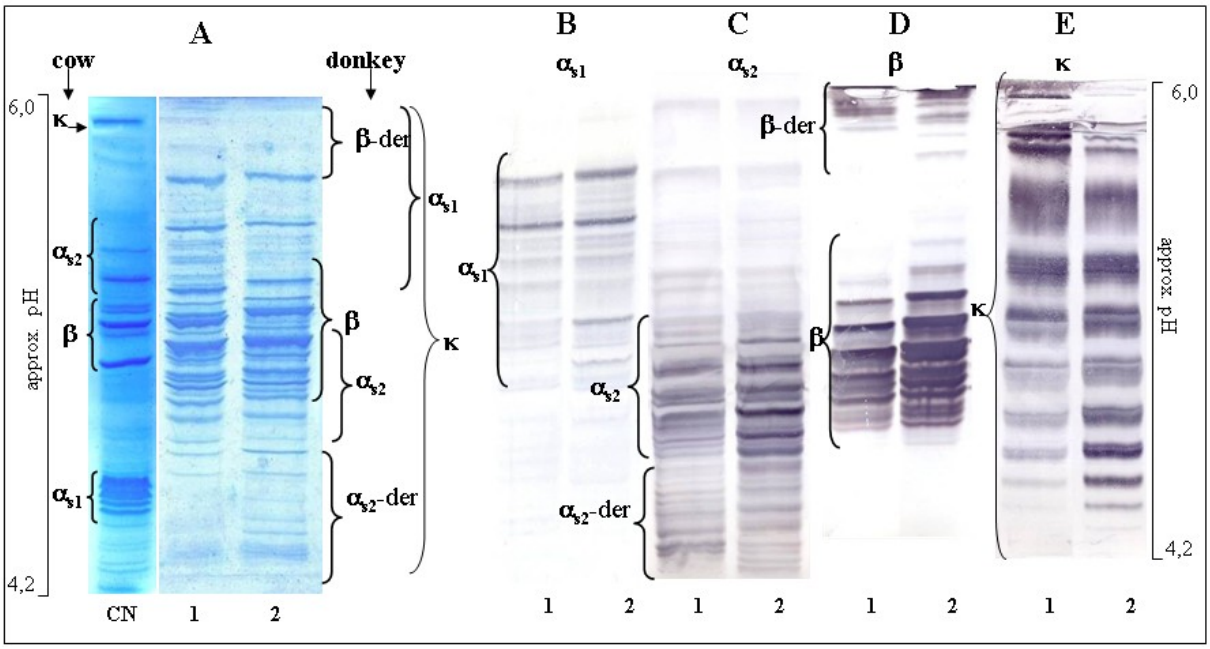


Fig. 2. *UTLIEF analysis of the two common donkey casein samples, 1 and 2, compared to cow casein as reference, after CBB staining (A) and identification of the four casein fractions by immunoblotting with polyclonal antibodies against* α_{s1} *(B),* α_{s2} *(C),* β *(D) and* κ*-CN(E).*

At this regard, Cunsolo *et al*., [24] found three phosphorylated components (5P, 6P and 7P) by MS analysis only in the full-length protein, while no information on the phosphorylation degree has been provided for the shorter β-CN form lacking the β-CN fragment (27-34) [24]. Unlike PAGE, UTLIEF analysis allowed the separation of α_{s1}-CN and α_{s2}-CN focalising at different p*I* values. However, α_{s2}-CN components partially overlapped the β-CN ones, and all these caseins fractions (α_{s1}-CN, α_{s2}-CN, and β-CN) were overlapped by κ-CN components (10-12) focalising throughout the pH gradient (Fig. 2). Concordantly with PAGE analysis (Fig. 1) α_{s2}-CN and β-CN derived fragments focused at lower and higher p*I* values than their parent casein, respectively.

4.3 2-DE analysis (PAGE → UTLIEF)

To resolve the protein mixture components, the most commonly used 2-DE analysis consists in the "vertical" PAGE-SDS separation of in-gel "horizontally" focalised proteins, where the effectiveness of separation depends on the differences among the protein MWs. In ruminants, it is known, casein MW values, unlike these of whey proteins, are very close [50]. For this reason, the 2-DE combination applied for donkey caseome resolution was the "vertical"

UTLIEF analysis of the two donkey caseins in-gel PAGE at pH 8.6 separated (Fig. 3), as already reported for sheep and goat caseome [43,51].

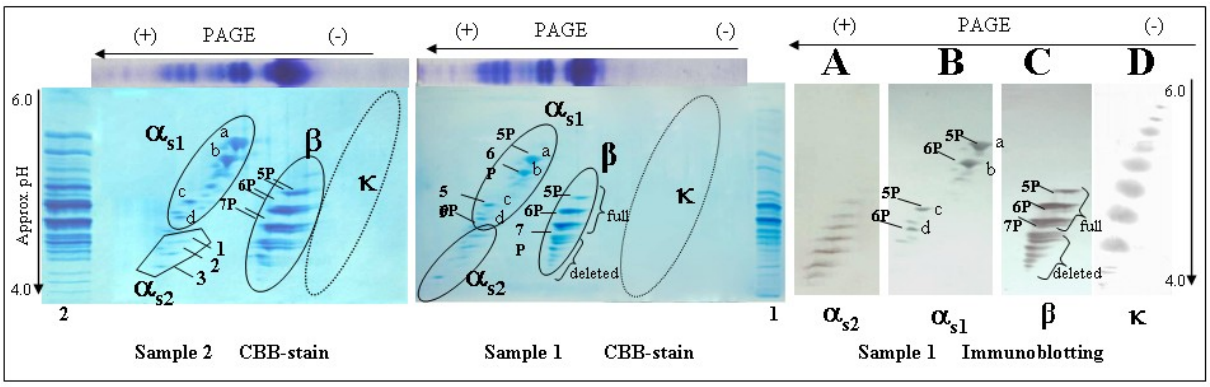


Fig. 3. *2-D separation (PAGE → UTLIEF) of donkey casein samples 1 and 2 showed in Figures 1-2. Staining with CBB and with antibodies preparation raised against α_{s2} (A), α_{s1} (B), β (C) and κ-CN (D).*

By means of specific immunostaining (Fig. 3A, 3B, 3C, 3D) the four zones, each containing a casein family (α_{s1}-, α_{s2}-, β- and κ-CN), were outlined with circles in Fig. 3.

These results allowed to conclude:

- in both samples the α_{s1}-CN composition consisted of two doublet bands (a, b and c, d), having decreasing p*I* values towards the anode;
- in sample 2, the number of β-CN components was doubled (six) with respect to sample 1 (three), likely due to occurrence of genetic polymorphism;
- the most heterogeneous donkey casein fraction was κ-CN, since at least 11 components were specifically immunostained in the 2-D map, focalising throughout the entire working pH range.

4.4 LC/MS analysis of donkey caseins

Comparison of the HPLC profiles of samples 1 and 2 (Fig. 4) showed that the latter was more heterogeneous than the former for the presence of the additional peak 4* eluted at shorter elution time than peak 4, common to the two samples.

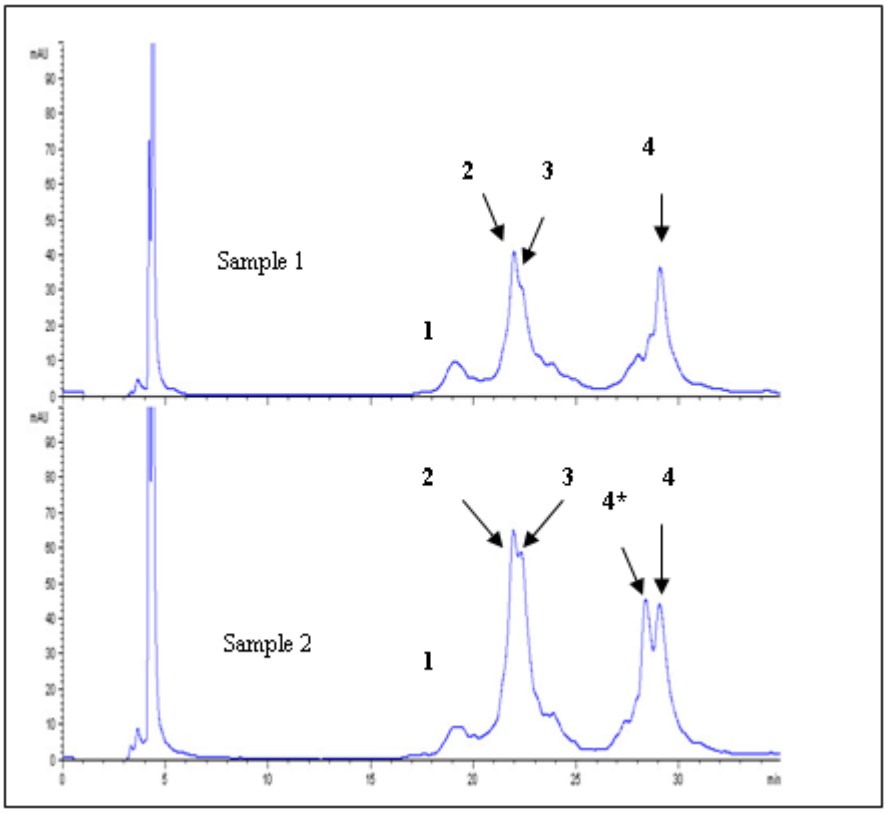


Fig 4. *UV profile (λ = 220 nm) of LC chromatogram of donkey whole casein samples 1 and 2.*

In Table 2, the MW of eluted components, determined by means of LC/ESI/MS analysis either before or after PA action, was reported. These data showed the same eluted components in the four common peaks. In particular in peak 1 β-CN originated by hydrolysis of both full protein length fragments, (226 a.a.) and from its deleted form lacking peptide (27-34) [24], were identified. The MW values of components eluted in peak 2 (Table 2) and those of their derived tryptic peptides (results not shown), both fitting with the reported α_{s1}-CN sequence [25], allowed to conclude the presence of donkey α_{s1}-CN at different phosphorylation degrees (5P and 6P) as well as their counterpart deleted of a Gln residue (-128Da). In the same way in peak 3 donkey α_{s1}-CN, deleted of fragment α_{s1}(34-38; -621 Da) carrying 6P and 5P, was identified together with its deleted form lacking both Gln residue and α_{s1} (34-38) fragment. These results suggested that also in donkey the compositional heterogeneity of α_{s1}-CN is depending on discrete phosphorylation (5P, 6P) and on the presence of non-allelic forms generated by RNA incorrect splicing, as in sheep and goat caseins [32-34].

Retention time (min)	Peak	Molecular mass, Da		Protein identification[a]
		native	dephosphorylated	
19.2	**1**	16959		β-CN fragment (-922 Da) nP[b]
		17039		β-CN fragment (-922 Da) nP[b]
		17119		β-CN fragment (-922 Da) nP[b]
		18042		β-CN fragment nP[b]
		18120		β-CN fragment nP[b]
		18202		β-CN fragment nP[b]
		18279		β-CN fragment nP[b]
21.6	**2**	24761		α_{s1}-CN 6P (-128 Da)
		24681		α_{s1}-CN 5P (-128 Da)
			24283	α_{s1}-CN (-128 Da)
		24890		α_{s1}-CN 6P
		24810		α_{s1}-CN 5P
			24412	α_{s1}-CN
22.1	**3**	24269		α_{s1}-CN 6P (-621 Da)
		24189		α_{s1}-CN 5P (-621 Da)
			23786	α_{s1}-CN (-621 Da)
		24141		α_{s1}-CN 6P (-621 Da) (-128 Da)
		24061		α_{s1}-CN 5P (-621 Da) (-128 Da)
			23662	α_{s1}-CN (-621 Da) (-128 Da)
28.0	**4***	25034		β-CN* 5P (-923 Da)
		25113		β-CN* 6P (-923 Da)
		25193		β-CN* 7P (-923 Da)
			24632	β-CN* (-923 Da)
		26050		β-CN* 6P
		26131		β-CN* 7P
			25570	β-CN*
28.8	**4**	25013		β-CN 5P (-923 Da)
		25099		β-CN 6P (-923 Da)
		25178		β-CN 7P (-923 Da)
			24619	β-CN (-923 Da)
		25938		β-CN 5P
		26023		β-CN 6P
		26101		β-CN 7P
			25542	β-CN

[a] *Protein identification is based on 1 and 2 or more peptides.*
[b] *nP: unknown number of phosphate groups.*

Table 2. *Donkey casein identification in sample 1 and 2 by LC/ESI-MS analysis. The additional peak 4* is only referred to sample 2.*

In peak 4, the full-length β-CN and its deleted form lacking of fragment β(27-34), both having 5, 6 and 7 P/mole, eluted. These forms have been already detected by Cunsolo *et al.,* [24] only in the full-length protein. These data, in addition to p*I* theoretical values of the two β-CN forms (Table 3), allowed us to identify them on the 2-DE map (Fig. 3) as the main and the minor components of donkey β-CN, respectively. In peak 4*, a novel β-CN was found

having a MW 28 mass units higher than the most common β-CN present in peak 4. This showed the occurrence of genetic polymorphism at this casein *locus*.

P/mole	Lenght, a.a.	M_r, Da	Protein	Theoretical Molecular Weight, Da	p*I*
7	226	26101	β-CN 7P	26075	4.74
6	226	26023	β-CN 6P	25997	4.82
5	226	25943	β-CN 5P	25919	4.91
7	218	25178	β-CN 7P (-923 Da)	25151	4.64
6	218	25099	β-CN 6P (-923 Da)	25073	4.72
5	218	25013	β-CN 5P(-923 Da)	24995	4.80

Table 3. *Experimentally measured M_r of full lenght and deleted forms occurring in β-CN. The theoretical molecular weight and isoelectric points are calculated using the algorithm from ExPASy's Scansite Compute pI/Mw program [34].*

Although no HPLC-eluted component was detected to confirm the presence of donkey α_{s2}-CN, it was possible to identify this fraction by MS analysis of the excised spots 1, 2 and 3 from 2-DE map (sample 2 in Fig. 3).

Band	Molecular Mass, Da		Protein identification[a]
	native	dephosphorylated	
1	26829		α_{s2}-CN 10P
		26029	α_{s2}-CN
2	26909		α_{s2}-CN 11P
		26029	α_{s2}-CN
3	26989		α_{s2}-CN 12P
		26029	α_{s2}-CN

Table 3. *MALDI TOF/MS analysis relative to spot 3, 2 and 1 excised from the 2-D gel of donkey's casein sample 2 shown in Fig. 3 A*. A database search using the Mascot software retrieved phosphopetides from in-gel digest of each band with tripsin before and after AP action.*

As an example, in Fig. 5 the MALDI MS spectrum of the component excised from spot 1, before and after AP action, was reported. These procedure (results in Table 4) showed that donkey α_{s2}-CN occurred in spots 1, 2 and 3 with 10, 11 and 12 P/mole respectively. These

assignments were confirmed by MS analysis of tryptic digests (Table 5). The alignment of the tryptic peptides gave a 90% coverage corresponding to a protein 221 a.a. in length, in agreement with the cDNA sequence reported by Ramunno as shown in Fig. 6 [52].

Identification	**Peptide sequence α_{s2} casein**	**Theoretical mass**	**Measured mass**
115-147	IVLTPWDQTKTGASPFIPIVNTEQLFTSEEIPK	3701.25	3702.25
37-63+2P	CSTSCEEATRNINEMESAKFPTEVYSS	3174.29	3177.17
193-219	IVHQHQTTMDPQSHSKTNSYQIIPVLR	3159.59	3161.03
37-63	CSTSCEEATRNINEMESAKFPTEVYSS+2P	3014.29	3015.81
1-22+2P	KHNMEHRSSSEDSVNISQEKFK+2P	2777.85	2777.89
125-148	TGASPFIPIVNTEQLFTSEEIPKK	2647.04	2646.03
89-109	KQLNKINQFYEKLNFLQYLQA	2644.09	2646.83
125-148+1P	TGASPFIPIVNTEQLFTSEEIPKK +1P	2727.04	2728.50
26-46	YVVIPTSKESICSTSCEEATR	2417.71	2419.03
26-46+ 1P	YVVIPTSKESICSTSCEEATR + 1P	2497.71	2499.33
56-72+ 6P	FPTEVYSSSSSSEESAK+6P	2301.88	2301.50
101-114	LNFLQYLQALRQPR	1760.08	1761.10
111-124	RQPRIVLTPWDQTK	1738.03	1738.83
8-22	SSSEDSVNISQEKFK	1684.79	1685.78
10-23	SEDSVNISQEKFKQ	1638.46	1639.64
209-221	TNSYQIIPVLRYF	1613.89	1613.97
34-46+1P	ESICSTSCEEATR+1P	1495.52	1496.50
149-161	TVDMESTEVVTEK	1467.62	1468.60
174-184	LLNKINQYYEK	1425.66	1427.77
34-46	ESICSTSCEEATR	1415.52	1415.50
209-219	TNSYQIIPVLR	1303.53	1304.87
9-18+2P	SSEDSVNISQ+2P	1225.06	1226.54
112-121	QPRIVLTPWD	1224.43	1224.49
115-124	IVLTPWDQTK	1200.41	1200.85
9-18	SSEDSVNISQ	1065.06	1066.67
185-192	FTLPQYFK	1043.24	1043.70
73-80	FPTEREEK	1035.13	1036.68
47-55	NINEMESAK	1035.15	1036.55
1-7	KHNMEHR	951.08	951.61

Table 5. *In-gel α_{s2} –CN tryptic digest of spot 1 excised from the 2-D gel of donkey casein sample 2 shown in Fig. 3, by QTOF/ESIMS analysis.*

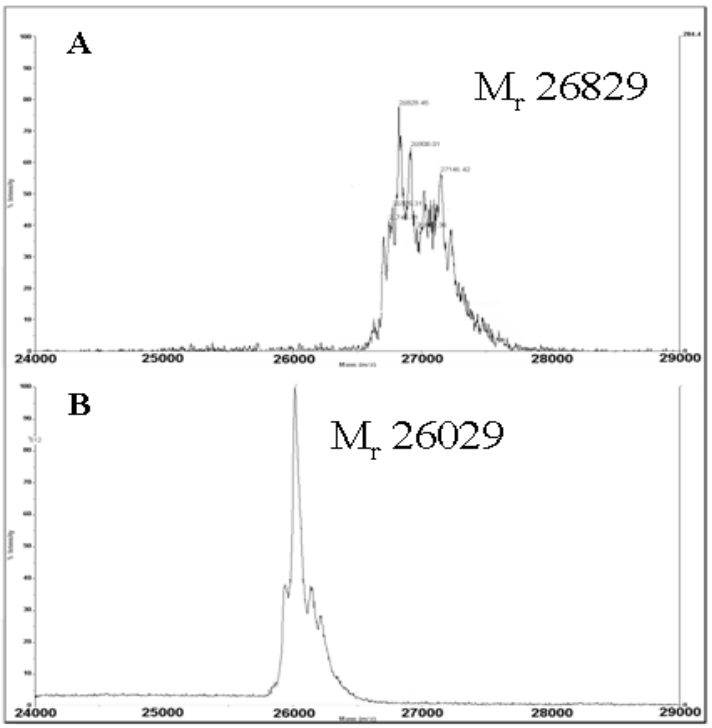


Fig 5. *MALDI TOF/MS spectrum relative to spot 1 excised from the 2-D gel of donkey casein sample 2 shown in Fig. 3 before (A) and after AP action (B).*

10 20 30 40 50 60
KHNMEHRS$_S$**S** EDSVNI**S**QEK FKQEKYVVIP TSKE$_S$ICST**S** CEEATRNINE MESAKFPTEV
70 80 90 100 110 120
Y**SSSSSS**EES AKFPTEREEK EVEEKHHLKQ LNKINQFYEK LNFLQYLQAL RQPRIVLTPW
130 140 150 160 170 180
DQTKTGASPF IPIVNTEQLF TSEEIPKK$_T$V DME**S**TEVVTE KTELTEEEKN YLKLLNKINQ
190 200 210 220
YYEKFTLPQY FKIVHQHQTT MDPQSHSKTN SYQIIPVLRY F

Fig 6. *Primary structure of donkey α_{s2}-CN. Underlined, sequence determined by MS/MS, covering 90% of its cDNA sequence; S residues phosphorylated, in bold; S and T residues only partially phosphorylated, **S** and **T** in bold.*

4.5 DMPs peptidomic characterization

DMPs were extract in solid phase without chemical agents. This method allowed us to extract efficiently low molecular weight peptides and to avoid a possible interference of the precipitating agent used with the TBARS-test. The DMPs mixture was individually characterized by MALDI-TOF MS mass spectra and the peptides identified among the 18 DMP samples are reported in Table 6. Only in DMP 32 peptides from α_{s1}-CN have been

identified: the α_{s1}-CN peptides are produced by a primary proteolysis action of Leu^{125}-Ile^{126} and Glu^{150}-Pro^{151} and by a subsequent amino-peptidase action. This result allowed us to conclude that donkey α_{s1}-CN has a lower susceptibility to proteolysis not involving the conventional N-terminal protein sequence as occurs in other species [53,54].

Measured mass MH^+ *	Identification	Sequence	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
1367,14	β-cn f(41-51)	HEGQQQREVEH	•§			•			•	•	•	•	•	•	•	•	•	•	•	•
1505,07	β-cn f(42-53)	HEGQQQREVEHQ	•		•	•														
1620,23	β-cn f(42-54)	HEGQQQREVEHQD	•	•	•	•	•	•						•						•
1748,34	β-cn f(41-54)	KHEGQQQREVEHQD	•	•	•	•		•						•						
1779,54	β-cn f(40-53)	FKHEGQQQREVEHQ	•						•	•	•			•			•	•	•	
1895,59	β-cn f(40-54)	FKHEGQQQREVEHQD	•	•	•	•	•	•					•	•						
2023,81	β-cn f(40-55)	FKHEGQQQREVEHQDK	•	•	•	•		•						•						
1595,81	β-cn f(176-189)	VAPFPQPVVPYPQR	•				•		•	•	•	•	•	•	•	•	•	•	•	•
1710,84	β-cn f(176-190)	VAPFPQPVVPYPQRD							•	•	•	•	•	•	•	•	•	•	•	•
2137,03	β-cn f(176-194)	VAPFPQPVVPYPQRDTPVQ			•						•				•		•	•		
2207,47	β-cn f(176-195)	VAPFPQPVVPYPQRDTPVQA			•					•	•	•	•	•	•	•	•	•	•	•
2354,97	β-cn f(176-196)	VAPFPQPVVPYPQRDTPVQAF	•	•	•	•		•			•	•	•	•	•	•	•	•	•	
2467,57	β-cn f(176-197)	VAPFPQPVVPYPQRDTPVQAFL	•	•		•		•			•	•	•	•	•	•	•	•		•
2743,7	β-cn f(176-199)	VAPFPQPVVPYPQRDTPVQAFLLY			•												•			
2987,95	β-cn f(176-201)	VAPFPQPVVPYPQRDTPVQAFLLYQD											•			•	•	•	•	•
3497,33	β-cn f(177-207)	APFPQPVVPYPQRDTPVQAFLLYQDPQLGLT			•															
2882,44	β-cn f(200-226)	QDRQLGLTGEFDPATQPIVPVHNPVIV				•														
2689.92	αs2-cn f(23-46)	QEKYVVIPTSKESICSTSCEEATR	•	•	•			•												
2805,01	αs2-cn f(23-47)	QEKYVVIPTSKESICSTSCEEATRN	•	•	•	•		•										•		
2901,19	αs2-cn f(24-49)	EKYVVIPTSKESICSTSCEEATRNIN	•	•	•	•		•												
3030,21	αs2-cn f(23-49)	QEKYVVIPTSKESICSTSCEEATRNIN	•	•	•	•		•												
3383	αs2-cn f(21-49)+1P	FKQEKYVVIPTSKESICSTSCEEATRNIN	•	•	•			•												
2582	αs2-cn f(1-20)+3P	KHNMEHRSSSEDSVNISQEK	•	•	•	•		•												
2502	αs2-cn f(1-20)+2P	KHNMEHRSSSEDSVNISQEK	•	•	•	•														
2889,82	αs1-cn f(128-150)	MKENSQRKPMRVVNQEQAYFYLE					•													
3045,62	αs1-cn f(127-150)	RMKENSQRKPMRVVNQEQAYFYLE					•													
3158,17	αs1-cn f(126-150)	lRMKENSQRKPMRVVNQEQAYFYLE					•													
3271,66	αs1-cn f(125-150)	LIRMKENSQRKPMRVVNQEQAYFYLE					•													

Table 6. *Identification by mass spectrometry analysis of casein peptides extracted from individual DMP. Measured mass is the mass measured by MALDI-TOF MS.*For signals simultaneously occurring in different spectra, only one of the measured molecular masses is reported; slight fluctuations on the first decimal place for a signal in different spectra are due to the daily calibration.* § *• indicates the presence of peptide.*

The αs_2-CN derived peptides have been identified in DMP 28, DMP 29, DMP 30, DMP 31 and DMP 33. The peptides identified are α_{s2}-CN f1-20 1P, α_{s2}-CN f1-20 2P and the primary

peptides α_{s2}-CN f21-49 1P which with a simultaneously action of amino- and carboxy-peptidases produces several other shorter peptides.

Overall, the most abundant peptides come from β-CN in particular both from its C-terminal zone already known for its susceptibility to *Lactococcus sp.* [8] proteases type I, III and from the region zone including 40-55 amino acids.

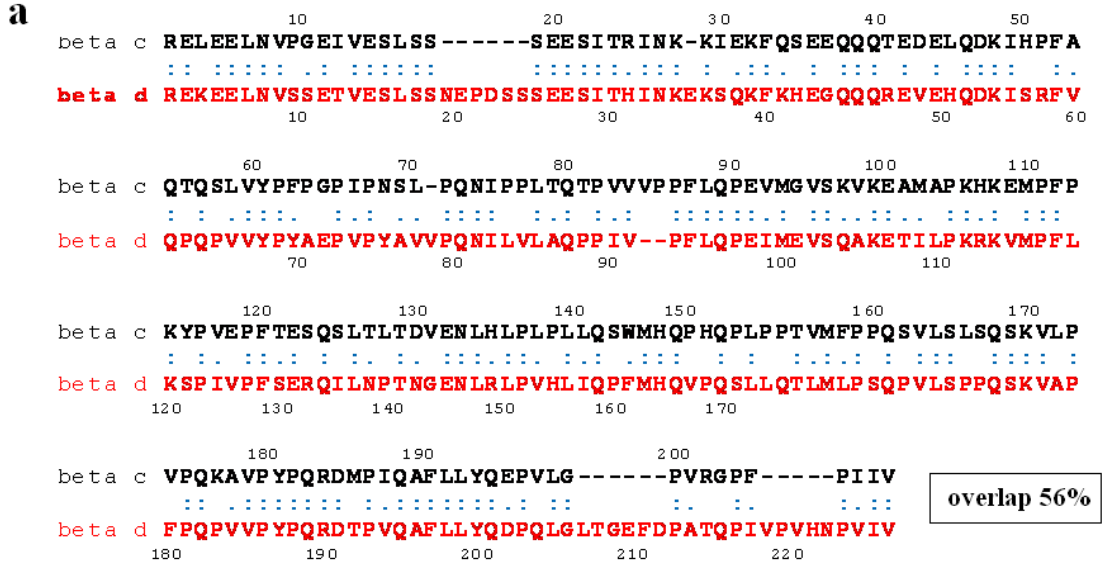
The primary fragment 40-55 is produced by a cleavage site at Lys^{39}-Phe^{40} and Lys^{55}-Ile^{56}. The donkey β-CN Lys^{55}-Ile^{56} has already been demonstrated as a specific plasmin cleavage sites [38], while no information is available for the Lys^{39}-Phe^{40}.

Taking into account the sequence similarities between the amino acids involved in the two cleavage sites, we can deduce that also Lys^{39}-Phe^{40} has been hydrolyzed by plasmin. The primary fragment 40-55 is further hydrolyzed by a combined action of amino- and carboxy-peptidases producing different peptides identified in DMP 28, DMP 29, DMP 30, DMP 31, DMP 33 and DMP 39.

The finding of the precursor peptide β-CN f176-199, followed by its shorter peptides at the C-terminal side, supported the hypothesis of a carboxy-peptidase-like activity in donkey milk. The β-CN C-terminal identified peptides have all been produced by the Lys^{175}-Val^{176} cleavage. This cleavage site is homologous to cow β-CN Lys^{169}-Val^{170} with a common susceptibly to plasmin action. The k-CN is not susceptible to the proteolytic action of endogenous milk enzymes as plasmin neither to the bacterial protease, so no peptides have been tried for this casein. Anyway, a limited number of peptides were unidentified because probably they arose from casein fragment involving genetic variants which have not been characterized.

Cow's β-CN potentially contains a great number of bioactive peptides in its primary sequence. Donkey β-CN produced numerous peptides coming from the entire sequence f40-55 and from its C-terminal sequence beginning from the precursor peptides f176-201. With the aim to identify potential bioactive peptides encrypted in donkey β-CN, an alignment between cow β-CN (UniProtKB P02666-1) and donkey β-CN (UniProtKB P86273-1) has been carried out (Fig 7a).

a



b

COW β-CN		DONKEY β-CN		
peptide	**sequence**	**peptide**	**sequence**	**BIOACTIVITY**
193-202	YQEPVLGPVR	197-214	LLYQDPQLG(LTGEFD)PAT	anthipertensive, immunomodulation
177-183	AVPYPQR	183-190	VVPYPQR	anthipertensive, antioxidant
60-70	YPFPGPIPNSL	67-77	YPYAEPVPYAV	agonistic opioid
60-66	YPFPGPI	67-73	YPYAEPV	agonistic opioid, immunomodulation
60-64	YPFPG	67-71	YPYAE	agonisic opioid
193-209	YQEPVLGPVRGPFPIIV	197-226	LLYQDPQLG(LTGEFD)PATQP I(VPVHN)PVIV	antimicrobial, immunomodulation
191-193	LLY	197-199	LLY	immunomodulation
63-68	PGPIPI	63-68	AEPVPY	immunomodulation
98-105	VKEAMAPK	104-111	AKETILPK	antioxidant
169-176	KVLPVPQK	175-182	KVAPFPQP	antioxidant
170-176	VLPVPQK	175-182	VAPFPQP	antioxidant
15-37	SLSSSEESITRINKKIEKFQSEE+4P	9-25	SSETVESLSSNEPDSSS+ 9P	antioxidant

Fig **7.** *Alignment of the primary sequence of the cow's (UniProtKB P02666-1) and (UniProtKB P86273-1) β-CN (Panel a). Potential bioactive sequence encrypted in donkey β-CN sequence (Panel b).*

The alignment process allowed us not only to ascertain an overlapping of 56% between β-CNs, but to identify the potential bioactive peptides encrypted in the primary sequence of donkey β-CN, as shown in Fig 7b.

However, the immunomodulating peptide f191-193$_{cow}$→f197-199$_{donkey}$ has a completely conserved sequence, while the multifunctional peptide with antihypertensive and antioxidant activity f177-183$_{cow}$→f183-189$_{donkey}$ are not completely conserved sequences. These peptides showed an overlap of 85% differing by a single amino acid substitution $A^{177}{}_{cow}$→$V^{183}{}_{donkey}$.

This substitution should not alter the biological activity because they are both hydrophobic amino acids and it is known that the presence of hydrophobic amino acids in the peptide sequence is a key structural requirement for the antioxidant activity [55].

4.6 Donkey milk peptides antioxidant activity

The explanation of the antioxidant effect of donkey milk peptides (DMPs) on linolenic acid in oxidative conditions (H_2O_2), needs some consideration to introduce the results obtained with the thiobarbituric acid reactive substances (TBARS) test based on the measurement of malondialdehyde (MDA) formed as a consequence of linolenic acid lipid peroxidation.

The antioxidant activity of peptides has been attributed to certain amino acid sequences as well as the peptide presence of peptide bonds or structural conformation arrangement. The presence of high levels of His and of some hydrophobic amino acids was related to peptide antioxidant power: the activity of His-containing peptides is thought to be connected to their hydrogen-donating ability, lipid peroxy-radical trapping, and/ or the metal ion chelating ability of the imidazole group. So, the hydrophobicity of the peptide also appears to be an important factor for its antioxidant activity due to increased accessibility to hydrophobic targets (as for example fatty acids). Furthermore, there is evidence that the antioxidant effect of individual amino acids is greater when they are incorporated in peptides, indicating that the peptide bond or structural conformation has an influence of this activity. Thus, peptide composition can lead to both synergistic and antagonistic effects with regard to the antioxidant activity of free amino acids.

TBARS test is based on the measurement of malondialdehyde (MDA) formed as a consequence of linolenic acid lipid peroxidation. In the presence of hydrogen peroxide, thereby increasing the possibility of formation of hydro peroxides from linolenic acid, only 4 individual DMPs (P31, PP39, P40 and P44) exert a pro-oxidant effect (Fig 8), while all the

other samples exert an antioxidant effect compared to the control. Currently, we have no information about the molecular mechanism involved in the different quantitative production of MDA mediated by DMP. Probably, as every antioxidant including vitamin antioxidants, DMP could react as a redox (reduction-oxidation) agent, protecting against free radicals in some circumstances, promoting free radical generation in others. In particular, DMP has an anti-oxidant function *in vitro* when molecules with a pro-oxidant action as H_2O_2 are concomitantly present.

This information allowed us to conclude that donkey casein peptides effectively protected linolenic acid from oxidation in the presence of H_2O_2. The more interesting antioxidant effect is exerted by P28 and P40: both samples were effective inhibitors of TBARS development when lipid oxidation was promoted by H_2O_2.

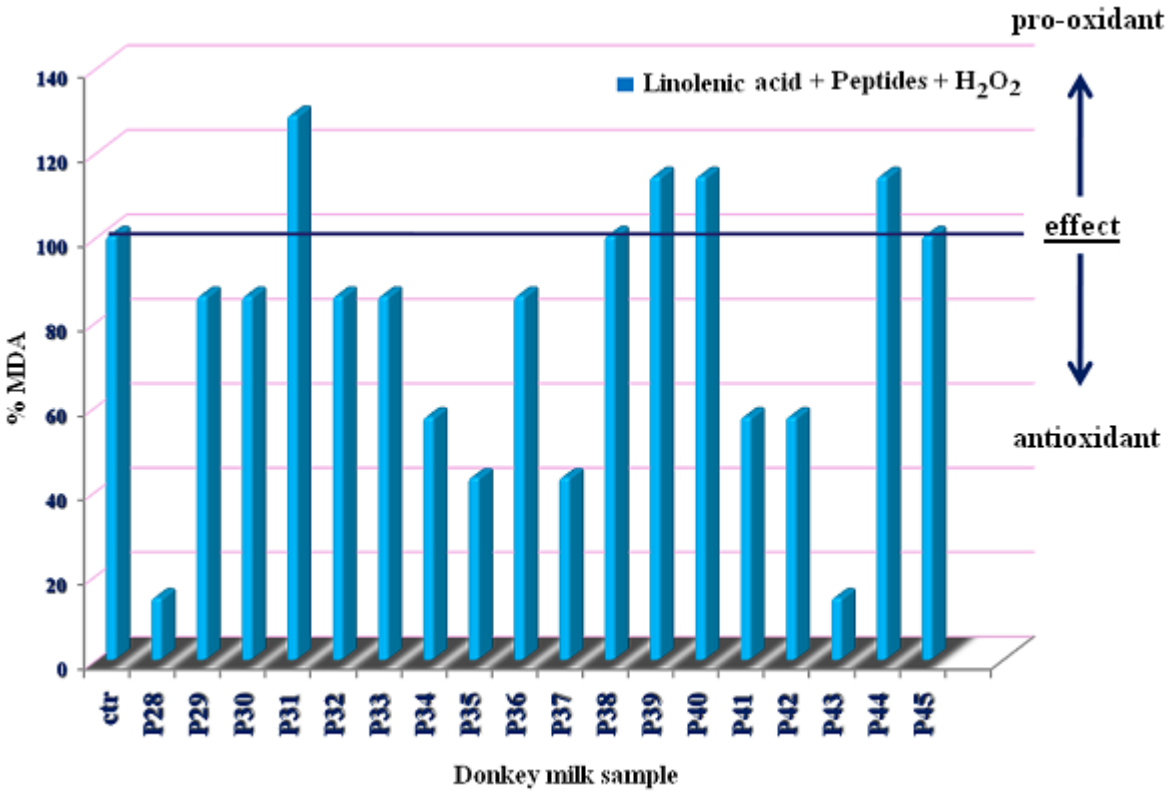


Fig 8. *Antioxidant activity evaluated by TBARS test of peptides extracted from individual DMP and incubated with linolenic acid and the oxidant agent H_2O_2.*

The antioxidant activity found for P28 and P40, may probably be due to an isolated and/or combinated effect of α_{s2}-CN caseinphosphopeptides and of β-CN C-terminal peptides. As shown in table 7, the determination of aliphatic index allowed us to evaluate the relative

Peptide	Sequence	Aliphatic index*	GRAVY§
β-cn f(41-51)	HEGQQQREVEH	26.36	-2.555
β-cn f(42-53)	HEGQQQREVEHQ	24.17	-2.633
β-cn f(42-54)	HEGQQQREVEHQD	22.31	-2.700
β-cn f(41-54)	KHEGQQQREVEHQD	20.71	-2.786
β-cn f(40-53)	FKHEGQQQREVEHQ	20.71	-2.336
β-cn f(40-54)	FKHEGQQQREVEHQD	19.33	-2.413
β-cn f(40-55)	FKHEGQQQREVEHQDK	18.12	-2.506
β-cn f(176-189)	VAPFPQPVVPYPQR	69.29	-0.257
β-cn f(176-190)	VAPFPQPVVPYPQRD	64.67	-0.473
β-cn f(176-194)	VAPFPQPVVPYPQRDTPVQ	66.32	-0.458
β-cn f(176-195)	VAPFPQPVVPYPQRDTPVQA	68.00	-0.345
β-cn f(176-196)	VAPFPQPVVPYPQRDTPVQAF	64.76	-0.195
β-cn f(176-197)	VAPFPQPVVPYPQRDTPVQAFL	79.55	-0.014
β-cn f(176-199)	VAPFPQPVVPYPQRDTPVQAFLLY	89.17	0.092
β-cn f(176-201)	VAPFPQPVVPYPQRDTPVQAFLLYQD	82.31	-0.185
β-cn f(177-207)	APFPQPVVPYPQRDTPVQAFLLYQDPQLGLT	84.84	-0.245
β-cn f(200-226)	QDRQLGLTGEFDPATQPIVPVHNPVIV	104.44	-0.104
αs2-cn f(23-46)	QEKYVVIPTSKESICSTSCEEATR	60.83	-0.575
αs2-cn f(23-47)	QEKYVVIPTSKESICSTSCEEATRN	58.40	-0.692
αs2-cn f(24-49)	EKYVVIPTSKESICSTSCEEATRNIN	71.15	-0.492
αs2-cn f(23-49)	QEKYVVIPTSKESICSTSCEEATRNIN	68.52	-0.604
αs2-cn f(21-49)+1P	FKQEKYVVIPTSKESICSTSCEEATRNIN	63.79#	-0.600#
αs2-cn f(1-20)+3P	KHNMEHRSSSEDSVNISQEK	34#	-1.830#
αs2-cn f(1-20)+2P	KHNMEHRSSSEDSVNISQEK	34#	-1.830#
αs1-cn f(128-150)	MKENSQRKPMRVVNQEQAYFYLE	46.52	-1.270
αs1-cn f(127-150)	RMKENSQRKPMRVVNQEQAYFYLE	44.58	-1.404
αs1-cn f(126-150)	IRMKENSQRKPMRVVNQEQAYFYLE	58.40	-1.168
αs1-cn f(125-150)	LIRMKENSQRKPMRVVNQEQAYFYLE	71.15	-0.977

Table 7. Aliphatic index and GRAVY of the sequences identified in DMPs extracts.

** The aliphatic index of a protein is calculated according to the following formula:*

Aliphatic index *= X(Ala) + a * X(Val) + b * (X(Ile) + X(Leu))*
where X(Ala), X(Val), X(Ile), and X(Leu) are mole percent (100 x mole fraction) of alanine, valine, isoleucine, and leucine. The coefficients a and b are the relative volume of valine side chain (a = 2.9) and of Leu/Ile side chains (b = 3.9) to the side chain of alanine.

§***GRAVY (Grand average of hydropathicity)*** *values were calculated using the ProtParam tool of the ExPASy Proteomics Server. GRAVY value for peptides are calculated according to Kyte and Doolittle as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence* [56].

#*aliphatic index and GRAVY have been determined only for the peptide sequence without taking into account the number of phosphate groups.*

contribution of non-polar amino acids in the peptide sequence, while the GRAVY index provides us information about the total polarity of the peptide: negative GRAVY values are related to the hydrophilic attitude, while positive values to the hydrophobic ones.

Overall, we ascertained that only the peptides produced from the entire sequence of β-CN C-terminal are the most hydrophobic among all the peptides identified in DMPs.

Antioxidant activity of the DMPs hydrolysates seems to be inherent to the characteristic amino acid sequences of peptides identified. Taking into account that these peptides are produced by endogenous donkey milk proteases we can assume that the protease specificity is responsible for milk antioxidant activity.

5. CONCLUSIONS

In previous studies [45,51] one-dimensional electrophoresis did not provide efficient separation of caseins because of their partial overlapping, meanwhile vertical UTLIEF analysis of proteins horizontally in-gel PAGE separated after immunostaining was the most resolving 2-DE. This technique revealed a characteristic protein pattern for the two donkey casein samples consisting in: i) at least four α_{s1}-CN main components: ii) three β-CN components, doubled in sample 2; iii) three α_{s2}-CN components and: iv) eleven κ-CN components.

By means of LC-ESI MS analysis, the heterogeneity of α_{s1}-CN was assigned to either discrete phosphorylation (5, 6 and 7 P/mole) or non-allelic deleted forms generated by incorrect RNA splicing, as already shown in the homologous goat and sheep casein [31-33]. The full length β-CN and its deleted form were both found equally phosphorylated with 5, 6 and 7 P/mole and a novel β-CN variant was found in sample 2. The molecular mass determined by ESI/MS before and after dephosphorylation of the variant showed a MW 28 Da higher than the most common β-CN and the same phosphorylation pattern.

Donkey α_{s2}-CN contained 10, 11 and 12 P, and its structural characterization confirmed for the first time the correctness of the cDNA-derived sequence [52].

Thus, the combined methodologies applied in this study were able to disclose the heterogeneity of each donkey casein fraction due to post-translational phenomena, as well as the occurrence of polymorphism at β-CN *locus*.

The proteomic information allowed us to a deeper structural characterization of donkey milk NPN peptides spontaneously generated by early proteolysis due to endogenous proteases. The peptidomic characterization demonstrate that donkey milk caseins shows an alternative proteolysis susceptibility when compared to proteolysis occurring on buffalo caseins (as demonstrated in Chapter I of this PhD thesis). Endogenous proteases in donkey milk also act on α_{s2}-CN known to be insensitive to the proteolytic action.

Moreover, the NPN donkey casein peptides effectively protected linolenic acid from oxidation in the presence of H_2O_2 demonstrating for the first time the antioxidant activity of donkey milk peptides. This contributes to further expand the already widely recognized donkey milk functional properties.

Overall, the information resulting from proteomic/peptidomic approach may be useful when programming breeding strategies for preservation and selection of donkey biodiversity, aimed

to production of donkey milk for human consumption. In fact, the variability associated with genetic polymorphism complemented with a major or minor complexity in the expression of single casein components may influence qualitative and quantitative donkey milk composition and, as a consequence, its allergenic properties.

The protein fraction is largely responsible for bovine milk allergenicity and both donkey and mare milk have been widely used to replace human milk, especially for children affected by CMPA. Interspecies comparison between donkey and ruminants proteome revealed that donkey caseins resemble human, camel and porcine caseins to a higher degree than ruminants do. Goat milk has been all along used in CMPA patients even if recent studies have shown that donkey milk [6] has a major therapeutic effect compared to goat milk, although the mechanism of this tolerance, likely related to the protein composition, has not been fully clarified. If once donkey milk hypoallergenicity was thought responsible of the α_{s2}-CN absence, similarly to the human milk, in this work we have demonstrated the presence of the α_{s2}-CN in the donkey milk. This means that the donkey milk hypoallergenicity is not caused by the absence of α_{s2}-CN, but there are other milk proteins possibly involved. Therefore, the data obtained by the proposed analytical approach may open new possibilities for understanding the structural basis triggering milk allegenicity, as well as for designing and developing more efficient and safe substitutes of maternal milk. At present, compared with bovine milk, the characterization of donkey milk caseins is at a relatively early stage progress, and only limited data are related to its genetic polymorphism.

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CONCLUDING REMARKS

The analytical strategy developed in this PhD project, allowed us to evaluate the presence, production and bioactivity of peptides in milk and its derived products in two model cases as buffalo milk transformation in Mozzarella di Bufala Campana PDO (Chapter I) and donkey milk (Chapter II).

The two model cases studied have been investigated by the application of proteomic and peptidomic procedures. Taking into account that the use of mass spectrometry for proteomics and peptidomics is not the application of a single technique for all purposes, in this PhD thesis a collection of methodologies, each with strength suited to particular inquires, have been applied with the aim to obtain different information on protein/peptide nature, structure, functional properties, impact on human cell cultures and in the definition of their structure/relationship. Moreover, the integration of proteomic and biochemical methodologies allowed to identify bioactive peptides and to provide molecular basis to elucidate structure-function relationships.

The information acquired allowed us to conclude that milk is a complex biological system with the expression of proteins mixture arising from post-translational process, genetic polymorphism, alternative splicing. The technological process used for dairy production, further enhance the heterogeneity of the protein system by inducing proteolysis which give the formation of peptides mixtures more complex and with a more interesting biological activity on cell cycle modulation than that generated by milk endogenous proteases in milk.

Moreover, the information acquired in the two model cases studied may drive production, utilization and design of bioactive compounds on the basis of their nutritional and economic significance and allowed us to hypothesize that the general criteria used to asses food protein quality, based on proteins nutritional properties, should be revised according to the protein ability to release peptides with biological activity.

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