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DOTTORATO DI RICERCA IN SCIENZE DELLE PRODUZIONI ANIMALI
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AGRARIE E ALIMENTARI

DOCTORAL THESIS

Investigation on donkey milk protein fractions: *in vitro* antimicrobial, antiviral and anti-proliferative activities and casein separation by cation exchange chromatography

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"Spingendo quotidianamente i nostri limiti

riusciamo, a piccoli passi,

a superare le paure che ci vietano

il possesso della nostra esistenza"

Angelo D'Arrigo

Table of Contents

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Horses and donkeys at the first National cattle fair
(beginning of twentieth-century, Catania)

ABSTRACT

This thesis was aimed to study donkey milk protein fractions and their biological properties.

In the first Chapter the donkey's milk gross composition and the related hygienic-sanitary aspects are introduced. The main use of donkey milk in human nutrition, especially in infancy and in patients with cow milk protein allergy (CMPA), is also discussed.

The second Chapter "Antimicrobial compounds in milk" was drafted after an intensive course at the University of Copenhagen: "Functional milk compound – with focus on milk proteins", and presented as the final report of the first year of PhD school. It deals with a review of the antimicrobial activity of milk components, with particular attention to whey proteins, as lactoferrin and lysozyme and their derived bioactive peptides, such as lactoferricin, which play a crucial role in human health and nutrition.

The detailed description of the donkey whey proteins, which exhibit antimicrobial activity is presented in third chapter. Moreover, the antimicrobial activity of fresh, powdered, digested and fermented donkey milk has been discussed.

The Fourth Chapter reports the studies on the antiviral activity of donkey milk and its fractions (skimmed milk, digested milk, casein, whey and low molecular weight whey fraction) that has been tested *in vitro* on an enterovirus, echovirus type 5, in two experimental trials. Among the others, we found that whey proteins showed the strongest inhibitory effects, probably thanks to the synergic actions of lactoferrin, lysozyme and lactoperoxidase.

A preliminary investigation of anti-proliferative effect of skimmed donkey milk and its main protein fractions (casein and whey proteins), carried out through two assay performed *in vitro* on Human Neuroblastoma Cell Lines (SK-N-BE) is presented in the fifth Chapter. In our experimental conditions only the whey proteins, at the highest concentration, have shown anti-proliferative effect on cell growth.

Finally, in the sixth Chapter, the preliminary results obtained from the analysis of the donkey's casein fraction by ion exchange chromatography, are described. By coupling chromatographic and mass spectrometry techniques, we obtained three different fractions from the separation of donkey's casein: pure β-casein, αs-caseins and a pool containing κ-casein and αs-caseins. The survey was carried out with the aim to obtain protein standards to use in quantification analysis.

RIASSUNTO

L'obiettivo principale di questa tesi è stato quello di mettere in luce alcune attività biologiche del latte di asina, con particolare riguardo alle proprietà antimicrobiche, antivirali e anti-proliferative delle sue frazioni proteiche.
Nel primo Capitolo si introduce il latte di asina e se ne descrivono la composizione ed i relativi aspetti igienico-sanitari. Inoltre, il suo utilizzo nell'alimentazione umana, specialmente nell'infanzia e nei pazienti con allergia al latte vaccino è brevemente discusso.
Il secondo Capitolo raccoglie le informazioni acquisite durante un corso intensive seguito presso l'Università di Copenhagen su "Functional milk compound – with focus on milk proteins" e costituisce il report del primo anno di dottorato. Esso riporta un'accurata descrizione sull'attività antimicrobica del latte, con particolare attenzione alle proteine del siero, quali la lattoferrina e il lisozima ed ai peptidi bioattivi da esse derivati, come la lattoferricina, i quali svolgono un ruolo cruciale per la salute umana e la nutrizione.
Il terzo capitolo presenta una dettagliata descrizione delle proteine del siero di latte di asina che hanno mostrato azione antimicrobica nel latte fresco, latte digerito con enzimi proteolitici, ma anche in polvere e fermentato.
Il quarto Capitolo presenta gli studi sull'attività antivirale del latte di asina e le sue frazioni (latte scremato, latte digerito con enzimi gastro-intestinali umani, caseina, proteine del e proteine del siero a basso peso molecolare), che è stata testata *in vitro* su un tipo di enterovirus, echovirus 5, in due prove sperimentali. I risultati ottenuti hanno mostrato un forte effetto inibente sulla replicazione virale delle proteine del siero, probabilmente dovuta al sinergismo tra tra i suoi componenti.
Lo studio sull'attività anti-proliferativa del latte di asina scremato e le sue frazioni proteiche (caseina e siero di latte), eseguito attraverso due prove sperimentali, *in vitro*, su Human Neuroblastoma Cell Lines (SK-N-BE), è presentato nel quinto Capitolo. Solo le proteine del siero alla più alta concentrazione hanno inibito la crescita cellulare.
In fine, nel sesto Capitolo, sono riportati i risultati preliminari riguardanti l'analisi della frazione caseinica mediante cromatografia a scambio ionico. Grazie all'utilizzo combinato di tecniche di cromatografia e spettrometria di massa, sono state ottenute tre frazioni dalla separazione delle caseine del latte di asina: β-caseina pura, αs-caseine e un pool contenente κ-caseina e αs-caseine. Lo scopo della prova sperimentale era quello di ottenere standard proteici da utilizzare in successive analisi quantitative.

Chapter 1

Introduction to Donkey milk

Introduction

The donkey (*Equus asinus*) domestication began about 6000 BC in present-day Libya, starting from one or two subspecies of African wild asses (*E. africanus*). Over the centuries donkeys have spread in Asia, India, South-America and south Europe, being used as beast of burden. Today donkeys still have this role but only in the poorest regions of the world (Bordonaro et al., 2012). During the 20th Century, in Europe donkey population was reduced by 80-90% because of growing mechanization in agriculture that severely affected the use of this specie (Colli et al., 2012).

To date, in Italy, six donkey breeds are already extinct and eight autochthonous breeds (Asinara, Pantesco, Ragusano, Grigio Siciliano, Romagnolo, Amiatino, Sardo Grigio and Martina Franca) are still reared. They have been classified as critically endangered by Food and Agriculture Organization (FAO). Despite this considerable loss, during the last few years, some local breeds and populations are growing thanks to new and rediscovered traditional use of donkeys. This animal can be used for meat (e.g. stew or salame) and milk production (used in human nutrition and in cosmetic industry), for onotherapy (a method of using contact and educational techniques with donkeys to help people with challenges in the relational and emotional areas) and also for recreational purposes such as ecotourism and trekking (Colli et al., 2012; Bordonaro et al., 2011).

General legislative rules regulate the donkey welfare and the commercialization of ass milk (e.g. Regio decreto of the 9th of May 1929, n. 994 -artt. 15 and 43- and Directive 98/58/CE, adopted in Italy by Legislative Decree n.146), but there are not specific rules for donkey breeding and milk production.

This thesis was aimed at the study of donkey milk's protein fractions and its antimicrobial, antiviral, anti-proliferative properties through a detailed review on antimicrobial whey protein properties and *in vitro* experimental tests to verify the antiviral effects of donkey milk fractions (whole, skimmed, digested milk, whey and casein protein fractions) and potential anti-proliferative effects of skimmed donkey milk, whey protein fraction and casein. Finally, a preliminary work was

carried out in order to separate the donkey's casein fractions by cationic exchange chromatography.

1.1 Donkey milk composition

Since ancient times donkey milk (DM) was used as substitute for babies which could not be breastfeed because among different species, equine milk the most similar to human milk (Swar 2011), except for the lipid content (see table1).

Table 1. Gross milk composition from different species. [a]

Species	Total Solids	Milk components				Casein/Whey Protein ratio
		Fat	Lactose	Ash	Protein	
Human[a]	124.0	38.0	70.0	2.0	9.0	0.4:1
Donkey[a]	88.4	3.8	68.8	3.9	17.3	1.3:1
Mare[a]	102.0	12.1	63.7	4.2	21.4	1.1:1
Camel[b]	124.7	38.2	44.6	7.9	33.5	1.68:1
Cow[b]	127.0	37.0	48.0	7.0	34.0	4.7:1
Goat[a]	122.0	38.0	41.0	8.0	35.0	3.5:1

[a] Data from (human, cow, goat and camel) Uniacke-Lowe et al., (2010), (donkey) Salimei et al., (2004), Guo et al., (2007), Tidona et al., (2011a), (mare) Malacarne et al., (2002). Mean value expressed as: [a] g Kg^{-1} and [b] g L^{-1}.

DM composition changes during lactation (about 150 days). The amount produced (0.6-1.66 kg d^{-1}, not including the part sucked by foal) shows a non-linear trend after 45 and 90 days from partum or with a single peak after 90 days (see figure 1). Some authors showed that milk production was affected both by breed and the season of parturition. The donkeys who gave birth in a autumn-winter period yielded more milk (Salimei et al.,2004; Guo et al., 2007; Tidona et. al., 2011a; Cosentino et al., 2012). Moreover a difference was observed between the milked amount in the morning (about 0.67 kg) and that milked in the afternoon (about 1.013 kg) (Tidona et al., 2011a).

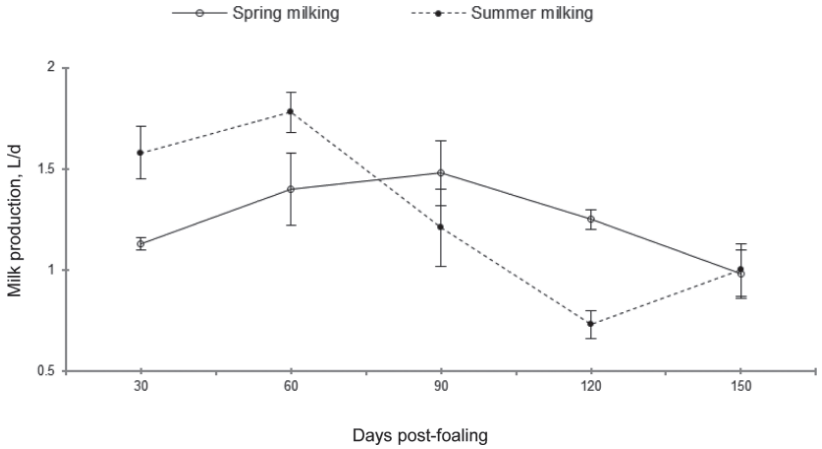

Figure 1. Donkey lactation curve for milk yield, protein and fat percentuage (from Cosentino et al., 2012).

The **pH value** is around 7.2, which is slightly higher than cow milk (6.7), but closer to human (7.3) and mare milk (7.18) (Guo et al. 2007). It decreases in the late lactation, even if the difference is not statistically significant (Guo et al., 2007; Tidona et al., 2011a). The highest value with respect to cow milk might be due to low content of casein and phosphates, which is also common to human and mare milk (Guo et al., 2007; Salimei et al., 2004).

Proteins content ranges from 1.3% to 2.0%, lower than in cow milk (3.2%), and with a low casein/whey ratio (1.04 on average) (Guo et al., 2007). Whey protein fractions are 35-50% of the nitrogen fraction, while in cow milk it represents only 20%. Casein represents about 47 % of crude protein (on average, because the casein amount decreases during lactation) (see figure 2 and 3). The casein/whey protein ratio, which change during the lactation, results to have decreasing value between 1.33 – 0.60 (Tidona et al., 2011a). This low casein/whey protein ratio plays a crucial role in the sensitization to cow milk protein fraction, acting on the allergenic properties.

A relevant heterogeneity was reported for donkey milk protein profiles; in Ragusano donkey it was shown that 35.7% of individual milks had a IEF pattern characterized by the absence of some protein bands with respect to a reference (which consisted of samples with a common protein profile (64.3%) (Criscione et

al., 2009). This observed quantitative polymorphism, especially if it will be confirmed at the genetic level, could influence donkey's milk protein allergenicity.

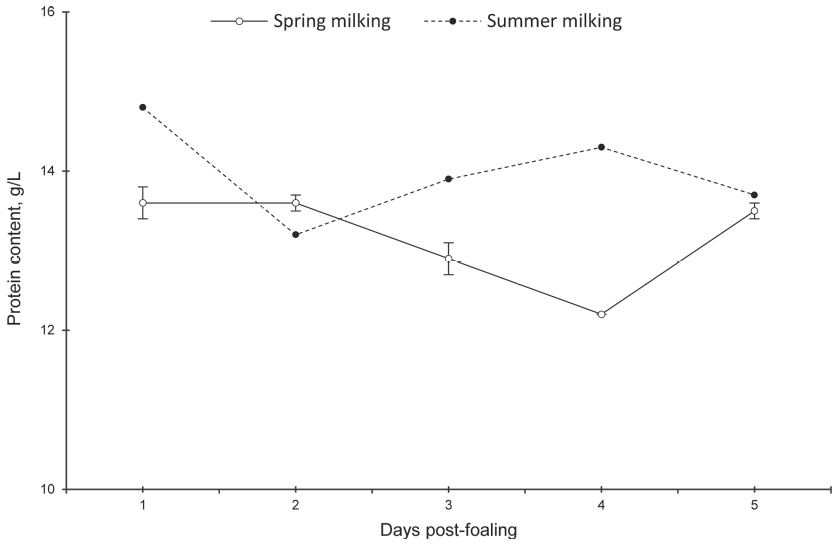

Figure 2. Trend of total amount of protein during lactation (from Cosentino et al., 2012).

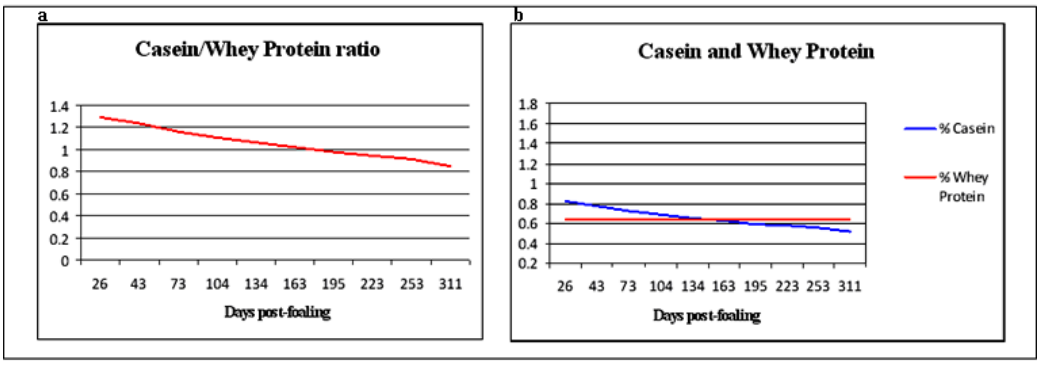

Figure 3. a) Trend of total amount of proteins, casein and whey protein; b) trend of casein/whey protein during lactation (from Tidona et al., 2011a).

The **lipids** amount is low in comparison with the other species. The lipid composition of cow and human milk constitutes 98-99% of triglycerides, 1-2% of phospholipids, sterols, monoglycerides, wax, squalene, carotene and fat-soluble vitamins (A, D, E, K), and traces of free fatty acids. In equine milk the

triglyceride content is lower than in cow and human milk, in mare milk triglyceride content is around 80% (Malacarne et al., 2002).

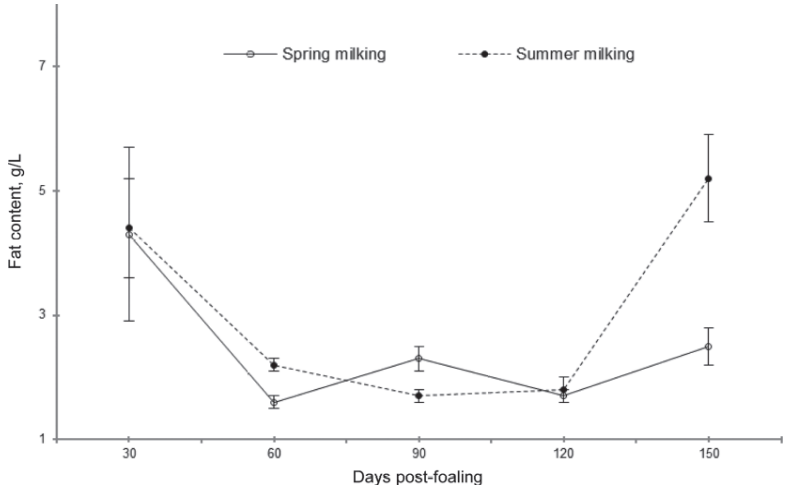

Figure 4. Lipid trend during lactation (Cosentino et al., 2012).

Although the lipid content value in donkey milk could be affected by breed, it is certainly influenced by breeding system, milking technique and interval between milking (Guo et al., 2007). It ranges between 0.03 and 1.18 g Kg^{-1} (see figure 4) (Tidona et al., 2009) with an increasing, not-linear, trend from partum to the end of lactation (Salimei et al., 2004; Guo et al., 2007; Tidona et al., 2011a). That amount is lower than in cow, goat, sheep and human milk. Even if it implies a lipid deficiency in an infant diet, which must be filled by supplements, it is a benefit in the diet therapy to prevent cardiovascular, autoimmune and inflammatory diseases. In particular, the high value of polyunsaturated fat acids (PUFA) (ω6 and ω3) (52.2%), the low ω6 to ω3 ratio, and the advantageous values of atherogenic and thrombogenic indices (see table 2 and figure 5) (Martemucci & D'Alessandro, 2012) tend, in human diet, to lower the level of cholesterol in blood, to prevent the formation of atherosclerotic plaques, removing the risk of coronary heart disease, hypertension and thrombosis (D'amico et al., 2007; Agostino et al., 2007), suggesting the DM as a functional food for infant nutrition, but also for adults that have to follow particular diets (Martemucci. & D'Alessandro, 2012).

Table 2. Fatty acid composition: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA). Mean values influenced by the lactation stage (data from Martemucci. & D'Alessandro, 2012).

Fatty acid composition	%
Saturated (SFA,)	51.98
Unsaturated (UFA)	48.02
Monounsaturated (MUFA)	28.00
Polyunsaturated (PUFA) of which	20.02
PUFA ω3	7.12
PUFA ω6	12.90

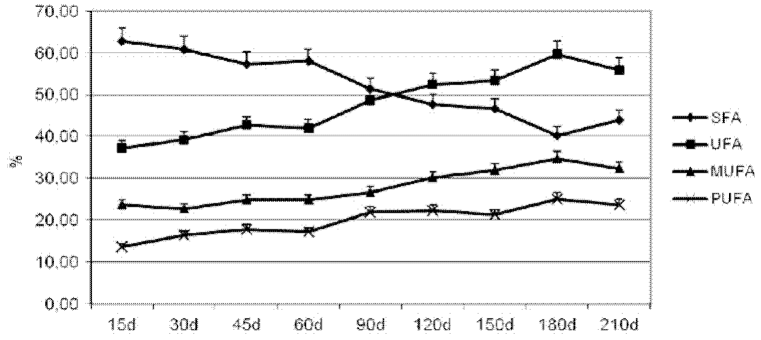

Figure 5. Fatty acids tred during lactation: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA) (from Martemucci. & D'Alessandro, 2012).

Carbohydrates are mainly represented by lactose, which content is generally around 6% (see figure 6) (Salimei et al., 1999; Polidori 1994; Polidori and Vincenzetti 2006). The high lactose content promotes osteogenesis processes improving the intestinal absorption of calcium, phosphorus and influencing the mineral accumulation in bone structure, which is useful for the prevention of osteoporosis (Borrello, 2007). Lactose gives good taste to DM (Iacono et al.,

1992; Monti et al., 2005; Paolicelli 2005), and is also a precious source of galactose, essential for the development of the nervous system.

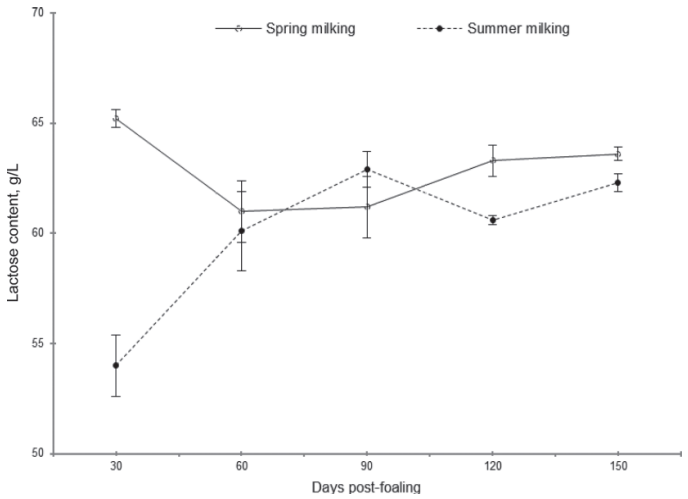

Figure 6. Lactose trend during lactation (from Cosentino et al., 2012).

Vitamins – Although the amount of several vitamins has not yet been detected in donkey milk, it is known that mare milk contains a significantly higher level of vitamin C than cow milk. In DM, with exception for niacin (vitamin B3), the amount of thiamine (vitamin B1), riboflavin (vitamin B2) and cobalamin (B3) is higher than in human milk (see table 3). The level of vitamin E is low in asinine milk (~0.05 mg L^{-1}).

Table 3 – Vitamins in donkey, mare, human and cow milk.[a]

Vitamins	Donkey[1]	Mare[1]	Human[1]	Cow[1]
Vitamin A	0.017[b]	0.093	0.3-0.7	0.32-0.50
Vitamin E	0.051[b]	0.26-0.13	3-8	0.98-1.28
Vitamin C	35-50[c 2]	17.2-147	50-100	0.94
Vitamin B1	0.41[d]	0.3	0.003-0.015	0.37
Vitamin B2	0.64 [d]	0.3	0.38	1.8
Vitamin B3	0.74 [d]	1.4	1.7	0.9
Vitamin B12	1.10 [d]	0.003	0.5	0.004

[a] Data from (donkey[b], mare and human) Salimei et al., (2012), (donkey[c]) Beghelli et al., (20102), (donkey[d] and cow) Uniacke-Lowe (2011). Value are expressed as: [1] mg L^{-1}, [2] mg mL^{-1}.

Mineral –The importance of minerals in human nutrition is well known, because they play a fundamental role in growth and skeletal structure development; nevertheless little information about the mineral composition of DM has been reported in literature so far, Despite some inconsistencies, which might due to differences in breed, stage of lactation and analytical methods applied, mineral fraction represents about 0.39% (mean value) of total solids in donkey milk (Fantuz et al., 2012). Mineral amount is higher in early lactation (0.51 g kg^{-1}), during which the milk is the only source of minerals for the growth of the foal (Salimei et al., 2000; Guo et al., 2007). The reduction observed of Ca, P and Mg, during the lactation, could be explained by the contemporaneous decline of casein amount since those minerals are mainly associated to the casein micelles (Giosuè et al., 2008; Fantuz et al., 2012).

DM mineral content is similar to that of human and mare milk, and lower than that of cow milk (Doreau & Martin-Rosset, 2011; Gaucheron, 2005; Fantuz et al., 2012) (see table 4).

DM contains higher amount of calcium and phosphorus (Belli Blanes, 2001) than HM, even if the amount is still lower than that of cow milk (1.17 g kg^{-1}) (Salimei et al., 2000).

Table 4. Minerals in donkey, mare, human and cow milk. [a]

Minerals	Donkey[b]	Mare[b]	Human[c]	Cow[c]
Ca	330-1140	500-1300	33	122
P	320-650	200-1200	43	119
K	240-747	300-800	55	152
Na	100-268	167-200	15	58
Mg	40-83	40-110	4	12
Fe	0.43-2.64	0.22-1.46	0.20	0.08
Zn	1.23-3.19	0.9-6.4	0.38	0.53
Cu	0.08-0.30	0.2-1.0	0.06	0.06
Mn	trace	0.01-0.05	0.07	0.02

[a] Data from (donkey and mare) Salimei et al., (2012), Csapò et al., (1995); (human and cow) from Park et. al., (2007). Mean values expressed as: [b] mg L^{-1} and [c] mg 100^{-1} g.

1.2 Hygienic-sanitary aspects of donkey milk

Milk, for its gross composition and pH value, is a good medium for the growth of microorganisms. Moreover, the health and hygiene of animal and the temperature of milk can aid pathogen microorganisms' proliferation. In donkey milk, the somatic cell count (SCC) resulted to be lower than in ruminants milk, comparing health animals (Salimei & Chiofalo, 2006; Salimei, & Fantuz, 2010), as well as the total bacterial count: it was reported to be lower (3.66 – 5.87 log CFU mL^{-1}) (Salimei & Chiofalo, 2006; Salimei, & Fantuz, 2010) respect that of the cow milk (7.58 log CFU mL^{-1}) (Tassew & Seifu, 2010). Besides, a lower total bacterial count and a longer shelf-life were found when the mechanical milking was applied (Sorrentino et al., 2010). The low total bacteria count could be explained by the presence of high amount of substances with antimicrobial activities such as lactoferrin and lysozyme (Fantuz et al., 2001).

The European Normative on the food safety and safeguard of the consumers are regulated by Regg. CE 178/2002, 852/2004, 853/2004, 1662/2006 and 1881/2006 (Salimei, & Fantuz, 2010). Moreover, there are hygienic requisites that the farms must observe as well as for the premises and equipments, milking, transportation, and personnel hygiene (Poligneri 2011).

1.3 Donkey milk in human nutrition

Today donkey milk is used both in human nutrition and in cosmetic. It is claimed to have special therapeutic properties due to the particular composition including high levels of whey protein, lactose and minerals and a low amount of fat. Because of its characteristics, DM is a good breast milk substitute, although it needs to be supplemented with about 4% medium-chain triglycerides to reach the right amount of lipids (Salimei, 2012; Swar 2011; Iacono et al., 1992). It has been recommended as a possible milk substitute for babies, children and adults with IgE-mediated cow milk allergy (Tesse et al., 2009; Monti et al., 2007). Moreover DM is used for the treatment of several diseases such as stimulation of the immune system, diarrhoea, gastric disorders (because it contains proteins with antimicrobial activity), prevention of osteoporosis (because it has high amount of calcium), cardiovascular disease, high blood pressure, high cholesterol level and liver problems (because of its high PUFA content, low ω6 to ω3 fatty acids ratio and low energetic value). It is also recommended as an aid in the treatment of cancer patients because its ability to induce release of interleukins (IL) (IL-12, IL-1 beta and IL-10) and tumour necrosis factor-alpha (Tafaro et al., 2007). Moreover, DM induces the release of nitric oxide (NO) from human peripheral blood mononuclear cells (Mao et al., 2009; Tafaro et al., 2007), which, being a strong vasodilator, can be recommended in the prevention of atherosclerosis (Tafaro et al., 2007). The suggested amount of donkey milk per day is 250 mL (Uniacke-Lowe et al., 2010).

1.4 The human immune system

The immune system (IS) has a task to resist infection, protecting the host from potentially harmful microorganisms and cancer cells. It operates in the entire

body, consisting of several molecules, cells and organs with different functions. It can be distinguished into innate immunity and adaptive immunity. The former, also known as non-specific immune system, can act by different ways such as through several kind of cells such as monocytes, granulocytes, leukocytes (white blood cells), phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils and natural killer cells or through physical barriers (mucosal barrier). In particular, the epithelium of small intestine and rectum is rich of Paneth cells, which produce antimicrobial substances such as lactoferrin and lysozyme (Kraehenbuhl et al., 1997; Ganz, 2003). The latter, also known as the specific immune system, recognizes and remembers (immunological memory) specific structures unique for different pathogens. The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation. The cells of the adaptive immune system are special types of leukocytes, the lymphocytes, which operates above all through B and T cells (Chehade & Mayer 2005). Due to the recognition of the antigens, the body is able to develop the oral tolerance, defined as “a state of active inhibition of immune responses to an antigen by means of prior exposure to that antigen throgh the oral route” (Chase, 1946). If recognition fails, food hypersensibility will occurs.

The reaction to food depends on several factors such as age, genetics and intestinal flora (Chehade & Mayer 2005).

1.5 The human immune system in relation to milk protein allergy

Frequently cow, goat and sheep milk have shown proteins cross-reactivity with cow milk proteins (CMP) both *in vitro* and *in vivo,* and several studies have shown that children with cow milk protein allergy (CMPA) synthesized antibodies against casein, β-lactoglobulin and α-lactalbumin (Lara-Villoslada et al., 2005). On the contrary, DM showed to be well tolerated also by children with cow milk protein allergy (CMPA) in terms of clinical tolerability (Carroccio et al., 2000; Muraro et al., 2002; Restani, et al., 2002; Monti et al., 2007). Nevertheless, since DM tolerability (82,6 %) (Monti et al., 2007) did not achieved the 90%, which is

the value required to define an hypoallergenic formula according to FAO and WHO (Alessandri & Mari, 2007).

In the case of CMPA, structural post-translational modifications, such as phosphorylation of αs_2- and β-casein (Bernard et al. 2000) and glycosylation of κ-casein (Pizzano et al., 2005), influenced the IgE binding of these proteins (Bertino et al., 2010). Moreover, bovine α_{s1}-casein had some linear epitopes, with single amino acid substitutions, that drastically reduced the binding capacity of IgE from the sera of patients with CMPA. In DM, α_{s1}- and β-casein showed a considerable heterogeneity due to variable degree of phosphorylation and the presence of genetic variants (Criscione et al., 2009; Cunsolo et al., 2009a; Cunsolo et al., 2009b; Chianese et al., 2010; Vincenzetti et al., 2012). The phosphate group, in particular the phosphoserine residues, could influence the immunoreactivity of casein. However, it is noteworthy that although in donkey milk the risk of allergenicity, due to specific serine residue phosphorylations, is lower than in bovine milk because it contains a low amount of casein protein, it is not possible to exclude possible allergy reaction because there are still potentially phosphorylated serine residues (Vincenzetti et al., 2012). Villoslada-Lara and co-workers (2004), demonstrated that goat milk was less allergenic than cow milk due to its lower content of casein. Then they compared the allergenicity of cow milk (80:20 CN:WP) and cow milk with a modified casein and whey proteins ratio (40:60); their results indicated that the casein/whey protein ratio influence the sensitization capacity of cow's milk (Villoslada-Lara et al., 2005).

Despite that in the past bovine β-lactoglobulin was thought to be primarily responsible for allergic reactions to CM, because this protein is absent in human milk and it is highly resistant to intestinal hydrolysis (Tidona et al., 2011b). Today its role in eliciting allergic reactions has been re-evaluated (Bertino et al., 2010).

Although α-lactalbumin is not considered one of the major CM protein allergens, it has conformational sequence epitopes recognized by specific IgE of CM allergic patients (Natale et al. 2004; Adams et al. 1991). Moreover, the amino acid sequence comprising this region shows only 85.6% similarity (57.1% identity)

between human and bovine α-lactalbumin, while the human and the donkey proteins show 100% similarity (78.6% identity) in the same region. Therefore, the hypoallergenicity of donkey milk could to be explained also by this similarity between human and donkey milk proteins (Bertino et al., 2010), making it a valid substitute of breast and cow milk for feeding allergic children.

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Chapter 2

Antimicrobial compounds in milk

"Antimicrobial compounds in milk" was drafted after an intensive course at the University of Copenhagen: "Functional milk compound – with focus on milk proteins", (August 30th to September 3rd, 2010-09-21), and presented as the final report of the first year of PhD school.

Introduction

Milk contains a wide range of different compounds essential for the health and the growth of the newborn. Its composition varies among species (see table 1) and among individual of the same specie depending of many factors, such as breed, stage of lactation, diet and health of the animal. However the principal constituents of the milk are water, sugar (lactose), lipids and proteins with a minor part of minerals, vitamins, hormones, enzymes and other secondary compounds, many of these in trace level (Fox and McSweeney, 2008).

The major protein fractions in milk include caseins and whey proteins (see table 2). The latter include α-lactalbumin, β-lactoglobulin, immunoglobulins, lactoferrin, proteose-peptide fractions (heat-stable, acid soluble phosphoglycoproteins) that represent the major whey proteins, and minor whey proteins such as transferrin and serum albumin.

Many studies have proved that milk and dairy products contain a great variety of functional molecules, most of them are proteins or bioactive peptides encrypted in the native proteins. Usually, the biologically active peptides consist of 3–20 amino acids, that may be released by enzymatic proteolysis, *in vivo*, during digestion under the action of endogenous gastro-intestinal enzymes and/or during food processing or ripening (Moller et al., 2008).

This review highlights the main antimicrobial compounds in milk, with attention for whey proteins-derivate bioactive peptides.

Table 1. Composition of milk (in %) from different species (Handbook of Milk Composition, by R. G. Jensen, Academic Press, 1995).

Species	Total solids	Protein	Fat	Lactose	Ash
Human	12.6	1.1	4.5	6.8	0.2
Donkey	10.2	1.7	1.2	6.9	0.45
Horse	11.0	2.7	1.6	6.1	0.51
Cow (Holstein)	15.0	3.1	3.5	4.9	0.7
Buffalo	21.5	5.9	10.4	4.3	0.8
Sheep	16.3	5.5	5.3	4.6	0.9
Goat	12.0	3.1	3.5	4.6	0.79
Camel	14.4	3.7	4.9	5.1	0.7

Table 2. Nitrogen fraction composition (g Kg^{-1}) and casein micelles size in equine, human, bovine milk[a] (Uniacke-Lowe et al., 2010)

Protein	**Equine[b]**	**Human[c]**	**Bovine[d]**
Total casein	13.56	2.4	26.0
α_{s1}-Casein	2.4	0.77[b]	10.7
α_{s2} -Casein	0.20	-	2.8
β -Casein	10.66	3.87[b] (>85%)[i]	8.6
κ-casein	0.24	(<15%)[i]	3.1
γ-casein	-	-	0.8
Total whey protein	8.3[e]	6.2[e]	6.3
β-lactoglobulin	2.55	-	3.2
α-lactalbumin	2.37	2.5	1.2
Serum albumin	0.37	0.48	0.4
Proteose peptone	-	-	0.8
Immunoglobulins	1.63[e]	0.96[e]	0.80
IgG1,2	0.38[f]	0.03	0.64
IgA	0.47[f]	0.96	0.14
IgM	0.03[f]	0.02	0.05
Lactoferin	0.58[g]	1.65	0.10
Lysozyme	0.87[g]	0.34l	126x10-6[j]
NPN	0.38[l]	0.485	0.296[k]
Casein micelle size (nm)[h]	2.55	64	182

[a] Where necessary have been adjiusted using density values of 1032 and 1033 Kg m^{-3} for equine (Uniacke-Lowe et al., 2010), human and bovine milk (Neville and Jensen, 1995), respectively, [b] Adapted from Miranda et al., (2004), [c] Adapted from Hambræus (1984), [d] Adapted from Walstra et al. (2006), [e] Park et al. (2006), [f] Hurley (2003), [g] Pagliarini et al. (1993), [h] Malacarne et al. (2002), [i] Hambræus & Lönnderdal (2003), [j] ElAgamy et al. (1996), [k] DePeters & Ferguson (1992), [l] Montagne et al. (1998, 2000).

2.1 Antimicrobial milk compounds

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). These molecules have been shown to exhibit a wide range of bioactivities, including immunostimulating and antimicrobial properties (Kitts and Weiler, 2003). Moreover some regions within the same protein can have multiple activities encompassed within the same or overlapping peptide regions (Moller et al., 2008), so it is complicated to understand the dynamic regulation and origin of bioactivities in milk and whether an activity is due to native protein or to latent encrypted peptides that can be released by proteolysis (Schanbacher et al., 1997).

Peptides may enter milk in different ways: many molecules by direct synthesis and apical secretion into the alveolar lumen by the mammary epithelial cell, other from blood or stromal cells (Schanbacher et al., 1997). Some peptides with antimicrobial appear to be necessary to develop or maintain a healthy mammary gland; in fact during intramammary infection or trauma, the synthesis and secretion of the major milk proteins are diminished while secretion of minor defence or regulatory proteins is enhanced (Talhouk et al., 1996).

Among the wide variety of peptides with different conformations and activity, there are the antimicrobial peptides that have some properties in common, including the affinity for membrane lipids (Epand and Vogel, 1999).

The total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and non-immunoglobulin defence proteins. This is thought to be for their synergy or for the presence of natural bactericidal peptide (Clare and Swaisgood, 2000).

The antimicrobial activity of milk is mainly attributed to immunoglobulins, and to non-immune proteins, such as lactoferrin, lactoperoxidase and lysozyme and in some species are also regarded minor proteins including a folate binding protein. The main anti-microbial compounds in milk are Lysozyme (Lyz) and Lactoferrin

(Lf); they are predominant in human milk, but are both very low in bovine milk, in which Igs form the main defence against microbes (Malacarne et al., 2002).

2.2 Antimicrobial Compounds from whey fraction

2.2.1. β -lactoglobulin

β-Lactoglobulin (β –Lg) is the principal whey protein in the milk.

In bovine milk β –Lg is a typical globular protein that consists of 162 residues per monomer, with a molecular weight of 18 kDa. Ten genetic variants are known of bovine β –Lg but the most abundant variants are β -Lg A and β -Lg B (Farrell *et al* ., 2004) which differ by only at positions 64 (Asp in variant A, Gly in variant B) and 118 (Val in variant A, Ala in variant B) (Farrell *et al* ., 2004).

The proteolytic digestion by trypsin yields four peptide fragments: β–Lg *f*(15–20), *f*(25–40), *f*(78–83) and *f*(92–100). They are negatively charged and thus their bactericidal activity is restricted to Gram-positive bacteria (Pellegrini et al., 2001).

2.2.2 α-Lactalbumin

α –Lactalbumin (α –La) is a globular small calcium metalloprotein containing 123 amino acid residues, with a molecular weight of 14 kDa. It represents about 20% of the protein of bovine milk whey (McKenzie and White, 1991; Brew, 2003). α-La occurs as two, or possibly three, genetic variants (Bell et al., 1981). It shares a high homology with Lysozyme with respect to amino acid sequencesand protein and gene structures (Mc Kenzie and White, 1991).

Pellegrini and co-workers (1999) demonstrated that the digestion with trypsin yielded α-La f(1–5) and α-La f(17–31)S-S(109–114), two peptides with antibacterial activity and another one through digestion with chymotrypsin α-La f(61–68)S-S(75–80). These three fragments are active against Gram-positive bacteria, while only a weak bactericidal activity was revealed against Gram-negative strains (Lopez-Exposito, 2006a): *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Streptococci*, and *C. albicans.*

2.2.3 Immunoglobulins

Immunoglobulins (Ig) are an important defence family proteins for the newborn that finds in milk, protective factors of the gut mucosa against pathogenic micro-organisms. They inactivate bacteria by binding to specific sites on the bacterial surface, in fact, their role is to confer passive immunity to the neonate while its own immune system is developing (Gapper *et al*., 2007). Immunoglobulins seem to act directly in the intestine (Moller et al., 2008).

Three classes of immunoglobulins are commonly found in milk, immunoglobulin G (IgG), A (IgA) and M (IgM); IgG is often sub-divided into two subclasses, IgG1 and IgG2 (Hurley, 2003; Madureira et al., 2007). The relative proportions of the Igs in milk differ considerably between species: in bovine, caprine and ovine milk the predominant species of Ig proteins are members of the IgG subfamily, in particular IgG1(Gapper *et al*., 2007).

2.2.4. Lactoferrin

Lactoferrin (Lf) is an 80-kDa iron-binding glycoprotein of the transferrin family, which is a component of milk. It is also present in exocrine secretions (such as tears, saliva, and the cervical mucosa) (Wakabayashi, 2006).

Lactoferrin has been associated with a wide variety of biologically important functions (Kuwata et al., 1998) as antioxidant, antiviral, anti-inflammatory, immunomodulatory and anti-carcinogenic activity. It also plays a fundamental role in the innate host defense system, because it has antimicrobial activity against a broad range of Gram-positive and Gram-negative pathogens (Valenti and Antonini, 2005). Moreover it seems to promote the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* (Sherman et al., 2004). The antimicrobial activity of lactoferrin seems to be dependent on its iron-free state and of its ability to bind and sequester iron, producing an iron deficient environment that limits microbial growth and also to permeabilize bacterial cell

walls by binding to lipopolysaccharides through its N-terminus. Lactoferrin can also inhibit viral infection by binding tightly to the envelope proteins of viruses. Ellison and Giehl (1991) suggested that Lf and Lyz work synergistically to effectively eliminate Gram-negative bacteria. Lactoferrin binds oligosaccharides in the outer bacterial membrane, thereby opening 'pores' for Lyz to disrupt glycosidic linkages in the interior of the peptidoglycan matrice.

Studies carried out had found that the infection of the target cell may be prevented by direct binding to virus particles, as for *Hepatitis C* virus (HCV), polio- and rotavirus, *herpes simplex* v*iruses* (HSV) and possibly *human immunodeficiency virus* (HIV) (Van der Strate et al., 2001).

It seems that part of the lactoferrin ingested by infants survives passage through the gastrointestinal tract as partially degraded forms and these may influence formation of the intestinal flora, absorption of iron and cell growth (Kuwata et al., 1998) including antimicrobial, antiviral and antioxidant activities as well as immunomodulation, modulation of cell growth. Moreover it has been found that pepsin-hydrolysate of Lf has more potent antimicrobial activity than the native protein (Tomita et al., 1991). Recently oral administration of Lf or its peptide has been recognized to exert various health beneficial effects also in adult animals and humans (Tomita et al., 2002; Teraguchi et al., 2004) against gastric infection with *Helicobacter pylori* (Wada et al., 1999), oral infection with a pathogenic yeast *Candida albicans* (oral candidiasis) (Takakura et al., 2003), and peroral systemic infection with a parasitic protozoon *Toxoplasma gondii* (Isamida et al., 1998). More recently beneficial effects of bLf on rotavirus gastroenteritis were reported by Egashira (2007). Furthermore animal studies have suggested that Lf can inhibit the development and progression of tumors (Wakabayashi, 2006). It seem that lactoferrin acts directly in the intestine (Moller et al., 2008).

Lactoferrin has been found in the milk of a large number of species and its amino acid sequence is known for human, pig, horse, cow, buffalo, sheep, goat, camel and mouse. By comparison, of the amino acid sequence, we can see that the lactoferrin with less amino acid sequence identity are those of human and mouse

milk, while cow, buffalo, goat and sheep lactoferrin share over 90% sequence identity with each other and form an extremely closely related group (Baker and Baker, 2005). The three-dimensional structure shows some subtle differences in human (Anderson et al., 1989), cow (Moore et al., 1997), buffalo (Karthikeyan et al., 1999), horse (Sharma et al., 1998) and camel (Khan et al., 2001) lactoferrin (Baker and Baker, 2005).

Lactoferrin has been also identified in the milk of the African elephant (Stumpf and Welsch, 2004).

Conesa and co-workers (2008) have attempted the isolation of lactoferrin from milk of different species: sheep (Ovis aries), goat (Capra hircus), camel (Camelus bactrianus), alpaca (Lama pacos), Asian elephant (Elephas maximus) and grey seal (Halichoerus grypus), as well as human (Homo sapiens), using an ion-exchange chromatography on SP-Sepharose. They were not able to detect lactoferrin in grey seal milk and, moreover their results confirm that, although lactoferrin is a protein very well preserved among species, some subtle differences in its structure can be highlighted.

Human milk contains a very high level of Lf and therefore there is interest in fortifying bovine-milk-based infant formulas with this precious whey protein (Lönnerdal, 2003).

The Lf hydrolysis by pepsin yields active peptides, among which lactoferricin, which is more bacteriostatic than Lf and its activity is independent of iron status (Mo¨ller et al., 2008).

2.2.5 Lactoferricin

Potent antimicrobial peptides have been derived from bovine Lf, *f*(17–41), and human Lf *f*(1–47) and named, respectively, bovine and human lactoferricin (bLfcin and hLfcin, respectively) which inhibited a number of pathogens (Bellami et al., 1992). Studies demonstrated that an 11-amino acid residue peptide (BL-11)

(RRWQWRMKKLG) within lactoferricin retained a broad spectrum of antimicrobial activity (Kang et al. 1996).

The antimicrobial potency of Lfcin is much greater than that of an equimolar amount of intact lactoferrin (Kuwata, 1998). It has been suggested that the antimicrobial, antifungal, antitumoral, and antiviral properties of bovine Lfcin can be related to the tryptophan/arginine-rich proportion of the peptide (Lopez Exposito, 2006).

A broad spectrum of Gram-negative and Gram-positive bacteria, such as L. *monocytogenes, E. coli, Salmonella enteritidis, Yersinia enterocolitica Camp. jejuni* (Guerrant *&* Bobak 1991), *Staphylococcus aureus, B. cereus* and *Clostridium perfringens* (Cooke, 1990), are susceptible to inhibition and inactivation by bovine lactoferricin while some *Bifidobacterium* species are highly resistant to bLfcin (Bellamy, 1992). In addition to a number of bacteria, Lfcin, and particularly Lfcin B, is also effective at inhibiting the growth of a number of yeasts, molds and filamentous fungi (Chapple, et al., 1998; Dionysius, et al., 1997; Yamauchi, et al., 1993).

The antiviral activity of this peptide is less than that of the mature protein, unlike the antimicrobial effects of Lfcin is (Gifforda, 2005). Though Lfcin moderately inhibits *in vitro* multiplication of a number of viruses, the activity of intact Lf against these same viruses is as much as seven times higher, suggesting that either the size of the molecule is important or that other regions of Lf contribute to the antiviral activity (Di Biase et al., 2003; Andersen et al., 2001).

Although Lfcin is at least partially responsible for the antimicrobial effect against bacteria and fungi, by the formation of pores in the cell wall of fungi and bacteria, this peptide apparently does not seem to be important for the antiviral effect (Van der Strate et al., 2001).

2.2.6 Lactoferrampin

Lactoferricin is not the only bioactive peptide of the lactoferrin; the N1-domain contains a second stretch designated lactoferrampin (Lfampin), which has antimicrobial peptides features (Deber, 1996; Schiffer 1992). Van der Kraan and co-workers (2004) have found the corresponding fragment (f 268–284) and determined its fungicidal activity on yeast *C. albicans*.

2.2.7 Lactoperoxidase

Lactoperoxidase is included in peroxidase protein family. Bovine lactoperoxidase consists of a single peptide chain of molecular mass 70 kDa, a heme, about 10% carbohydrate and a calcium ion strongly bound. It is a major antibacterial agent in colostrum. Its concentration is about 11-45 mg ml^{-1} and 13-30 mg ml^{-1} in colostrum and milk respectively (Korhonnen, 1977).

Although lactoperoxidase is able to oxidize Br^-, I^- and SCN^-, different studies demonstrate that oxidation of SCN^- is involved in the mechanism of the lactoperoxidase-catalyzed antibacterial effect in bovine and other milk, so the hypothiocyanite (OSCN-) is believed the main antibacterial species produced from SCN^- and hydrogen peroxide.

2.2.8 Lysozyme

Lysozyme is a powerful antibacterial protein (Lopez-Exposito, 2008) present in milk and also widely distributed in various biological fluids and tissues including avian egg, plant, bacteria, and animal secretions as tears, saliva (Jolles, 1984). It belongs to a class of enzymes that lyses the cell walls of a range of Gram positive bacteria, as it splits the bond between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan (PG) forming the residues of PG of high and low molecular weight that become the agents responsible for the specific immune cell activation (Hisham, 2001). Lysozyme has been recognized as an important factor in the body's defence against bacteria; beside antimicrobial activity, it has many other functions, including inactivation of certain viruses (Hasselberger, 1978).

Studies on the antimicrobial action of lysozyme, through irreversible heat denaturation at different pHs, suggest that it seems to be decoupled from itself enzyme activity, that is, even at these pH able to disable the enzyme activity of lysozyme, it nevertheless continues to have antibacterial activity, particularly against the lysozyme-sensitive Gram-positive bacteria (Hisham, 2001).

The results of a specific study provide the first direct evidence that the principal bactericidal action of lysozyme against the Gram-positive bacteria *S. aureus* and *B. subtilis* is independent from its catalytic function (Hisham, 2001), and because it has been proven that lysozyme without enzymatic activity still has bactericidal properties, afterwards several studies have been aimed at searching for lysozyme-derived peptides with antibacterial activity (Lopez-Exposito, 2008).

This enzyme, together with other factors, such as lactoferrin and lactoperoxidase, may function in the infant's digestive tract to reduce the incidence of gastrointestinal infections (Businco et al., 2000).

2.3 Antimicrobial compounds from casein fraction

Caseins, the most abundant proteins in milk, are a family of phosphoproteins designed to form spherical, micellar structures in colloidal suspension with calcium phosphate (Marletta et al., 2007). In all species studied so far, four casein genes form a cluster, in which α_{s1}, β, α_{s2} and κ-casein are tightly evolutionarily related and physically and functionally linked (Rijnkels, 2002)

Caseins are primarily considered to have nutritional functions, as source of amino acids and carriers of calcium and phosphate ions; nevertheless they also provide different bioactive peptides released during gastrointestinal digestion of caseinates or during dairy production (Moller et al., 2008).

In fact, *Lb. helveticus,* which possesses high proteolytic activities, is largely used in dairy production becauses induces the release of oligopeptides from the digestion of milk proteins (Trompette et al., 2003).

The casein-derived peptides with antimicrobial activities as far as known are: Kappacin (κ -casein-derivated), Caseidicin, Cp1 and Cp2, Isracidin (α_{s1}-Casein-derivated and α_{s2}-casein-derivated).

Caseinomacropeptide (CMP) derived by κ-Casein hydrolysis, through the enzyme chymosin that cuts the protein between Phe105 and Met106, generating both a hydrophilic phosphorylated and glycosylated C-terminal polypeptide k-casein (106–169) and also a hydrophobic N-terminal para-k-casein polypeptide k-casein (residues 1 to 105). CMP is heterogeneous: in fact three genetic variants of CMP have also been identified, originating from the precursors k-casein variant A, B, and E.

CMP and CMP-derived peptides have been reported to have a variety of biological activities in general and antimicrobial in particular, such as inhibition of influenza virus hemagglutination (Kawasaki, 1993) inhibition of cholera toxin binding (Kawasaki, 1992), and immunomodulating activities (Meisel, 1997). Fractionation of CMP, using reversed-phase high-performance liquid chromatography (RP-HPLC), revealed that the nonglycosylated, phosphorylated form of κ-casein (106–169), namely kappacin, was the active form of the peptide against *Streptococcus mutans,* whereas, the nonphosphorylated peptide, did not inhibit growth of the bacterium, indicating that phosphorylation is essential for antibacterial activity (Malkoski el al., 2001).

Caseicidin was obtained by chymosin-mediated digestion of casein at neutral pH. It exhibited activity against *Staphylococcus spp*., *Sarcina spp*., *Bacillus subtilis*, *Diplococcus pneumoniae* and *Streptococcus pyogenes* (Lahov and Regelson, 1996). A cationic fragment from α_{s2} -casein, f165–203, known as casocidin-I, can inhibit growth of *Escherichia coli* and *S. carnosus* (Zucht et al., 1995).

McCann and coworkers (2006) characterized two different antibacterial peptides, referred to Cp1 and Cp2, isolated from pepsin digestion of bovine sodium caseinate.

Cp1 peptide, which corresponded to residues 99–109 of bovine α_{s1}-casein, is a novel antimicrobial compound and displayed a broader antibacterial spectrum of activity than Cp2 peptide, derived from the C-terminal of bovine α_{S2}-casein (McCann, 2006).

The other fragment of bovine α_{s1}-casein (f1–23), known as isracidin, has demonstrated antibiotic-type activity *in vivo versus S. aureus* and *Candida albicans*; it can protect cow against mastitis (Lahov et al., 1996).

Hydrolysis of α_{s2}-casein with the gastric enzyme pepsin produces two different antibacterial peptides namely α_{s2}-casein f (164–179) and α_{s2}-casein f (183–207) (López-Fandino et al., 2006). Both fragments showed an important activity against Gram-positive and Gram-negative bacteria, but α_{s2}-casein f (183–207) exhibited consistently higher antibacterial activity than f (164–179) (Recio and Visser, 1999).

Recently, new antibacterial peptides have been identified from a chymosin digest of bovine sodium caseinate, all of them originated from the C-terminal of bovine α_{s2}-casein (McCann et al. 2005). In particular the peptide f (165–170), included in the sequence of f (164–179), showed the importance of the residues between position 170 and 181 for antibacterial activity. Similarly, f (203–207) corresponds to the C-terminal hexapeptide of f (183–207) and in this case the shorter form showed higher antibacterial potency than the longer peptide (López-Fandino et al., 2006).

2.4 Minor bioactive milk compounds

Some minor milk compounds, such as the sphingolipids, the proteose peptone 3, bradykinin and kininogen, showed antimicrobial bioactivity.

Sphingolipids and their metabolites, ceramide and sphingosine (SPH), are highly bioactive molecules with multiple beneficial effects on human health as cancer inhibition (Schmelz et al., 2000) and the inhibition of cholesterol absorption (Eckhardt et al., 2001).

Moreover they showed protective capacities against bacterial toxins and infection by bacteria or viruses, even if their concentration in food and consequently also their consumption is low (Vesper et al., 1999). Food sphingolipids bind sites on the intestinal mucosa (Bibel et al., 1992b; Fantini et al., 1997) and, since microbial adherence is often the first step in infection (Ofek et al., 2003), they establish a competition with bacteria and in this way could protect against pathogens. Studies carried out a significantly lower *Escherichia coli* and higher *Bifidobacterium sp*. counts in their faeces if infants consumed milk supplemented with gangliosides, a specific group of sphingolipids (Rueda et al., 1998). Beside this, SPH could also have a direct bactericidal effect (Arikawa et al., 2002; Bibel et al., 1993, 1995) and has been shown to strongly decrease the concentration of several food borne pathogens at low concentration (Sprong et al., 2001, 2002).

2.5 Antimicrobial peptide applications

Milk proteins and biopeptides include techno-functional properties largely exploited by food industries (Bouhallab, 2004). Studies focalize the research both in order to improve the shelf-life of the product and to use them for therapeutic purposes in the field of immunological system disorders, treatment of cancer, infections, cardiovascular disorders (Latham, 1999).

Today the attention is focused on establishing whether these antibacterial peptides produced *in vitro* by the action of gastrointestinal enzymes can also be generated *in vivo*. Research aims to understand their physiological implications once they are released and, once that they are incorporated in the food, understand the influence of the technological processes on their activity. Lactoperoxidase, lactoferrin, and immunoglobulins have already been commercialised (Shah 2000): Lactoferrin is used in food preservation by limiting the growth of microbes: incorporation of bovine Lf into edible films has a great potential to enhance the safety of foods; moreover r it can also be directly used as a spray applied to beef carcases (Taylor et al., 2004). Kappacin may have utility in foods and oral care products to help to lower the risk of dental caries and chronic periodontitis

associated with *Streptococcus mutans* and *Porphyromonas gingivalis*, respectively (Malkoski, 2001).

Compared with antibiotics, antimicrobial peptides have the advantages of being able to kill target cells rapidly and having a broad spectrum of activity, including activity toward some of the most important antibiotic-resistant pathogens in clinics (Bechinger, 1997).

2.6 Conclusions

Antimicrobial milk proteins and biopeptides play a crucial role in human health and nutrition. The interest in bioactive peptides from milk protein is increasing because milk compounds have a great influence on the techno-functional properties, largely exploited by industries both for the application of peptides in the flow-sheet of the food, and therapeutic purposes in the field of the treatment of cancer, infections, immunological system disorders, cardiovascular disorders.

The future applications of bioactive peptides, in fact, look promising in the field of food industry as food preservatives and nutraceuticals and also as natural drug in pharmaceutical industry.

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Chapter 3

Antimicrobial properties of whey proteins in donkey milk

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Antimicrobial properties of whey proteins in donkey milk

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Abstract

Milk is a source of bioactive compounds essential for health and growth of newborns. Donkey milk, rich in lactose and whey proteins, has been proven to be a good breast milk substitute during infancy and an adequate nourishment for patients with cow milk protein allergy (CMPA). This milk is gaining a growing interest for human nutrition because it stimulates the immune-system in

convalescence, regulates gastro-intestinal flora, and prevents inflammatory and autoimmune diseases. The whey protein fraction exhibits a wide range of biological activities, including antimicrobial and anti-inflammatory activity. The aim of this review is to highlight the antimicrobial properties of proteins which constitute donkey's milk whey fraction.

Introduction

In the first months of life, milk provides the several compounds able to fulfil all the nutritional requirements and perinatal passive immunization. In addition, milk is crucial for setting up the oral tolerance to nutrient molecules in the newborn, allowed through the passage of milk and its peptides, which, after digestion, stimulate the mucosa immune system of the infant (Baldi et al., 2005). When the mother is not able to breast-feed, or after weaning, it becomes important finding an adequate alternative nourishment. Cow and goat milk are likely to be used as the main natural substitutes for breastfeeding, as well as extensively hydrolysed formulas (eHF), amino acid formulas (AAF) and soy formulas (SF) which are used as artificial alternatives (Carroccio et al. 2000; Muraro, Giampietro, & Galli, 2002). Nevertheless most of these supplies have scarce palatability and/or could still contain allergens (Høst & Halken, 2004).

Among different species (Table 1), equidae milk is the closest to human milk composition (Swar, 2011) and, in particular, donkey milk (DM) often represent a good breast milk substitute during infancy. It has a particular protein composition, polyunsaturated fatty acids and essential amino acids (Guo et al., 2007) and it is

rich in lactose. DM showed to be well tolerated by children with cow milk protein allergy (CMPA) in terms of clinical tolerability (Carroccio et al., 2000; Muraro et al., 2002; Restani, Beretta, Fiocchi, Ballabio, & Galli, 2002; Monti et al., 2007). Monoclonal and polyclonal antibodies produced against cow milk proteins showed a very mild cross-reaction with donkey milk (Restani, Ballabio, & Di Lorenzo, 2009; El-Agamy, Nawar, Shamsia, Awad, & Haenlein, 2009). However, thorough studies would have to be conducted to achieve more conclusive results using the appropriate management tools defined by FAO (Food and Agriculture Organization) and WHO (World Health Organization) to evaluate foods for allergenicity (Alessandri & Mari, 2007).

Although the mechanism of the reported clinical tolerability has not yet been fully clarified, it is likely related to the protein fraction, in terms of relative percentage and of structural differences of its protein components with respect to bovine milk. (Guo et al., 2007). Indeed donkey milk contains a lower total protein value (about 1.5%) and a low casein/whey proteins ratio (on average 1.04) (Guo et al., 2007; Tidona, Criscione, Guastella, Bordonaro, & Marletta, 2011a; Miranda, Mahé, Leroux, & Martin, 2004) in comparison with other species (Table 1). Both these features play a crucial role in hypoallergenicity.

DM can also exert good effect on gut health because of the high content of lactose, in addiction to giving a good palatability, makes it a good substrate for growing of probiotic bacteria such as *Lactobacillus, L. acidophilus, L. bulgaricu, and L. rhamnosus* (Shah, 2001). On the other hand, the high value of lysozyme (4 mg mL^{-1}) exerts bactericidal action on pathogen or potentially pathogen bacteria (Nazzaro, Fratianni, Orlando, & Coppola, 2010b).

Recently, donkey milk was shown to have positive effects on the regulation of immune response in healthy elderly consumers (Jirillo, Jirillo, & Magrone, 2010). In addition its antimicrobial activity has been tested and proved effective against bacteria and viruses often associated with intestinal infection (Zhang, Zhao, Jiang, Dong, & Ren, 2008; Nazzaro, Orlando, Fratianni, & Coppola, 2010a; Tidona et al., 2011b; Brumini et al., 2013).

The aim of this review is to report antimicrobial properties of whey proteins in donkey milk.

1. Donkey whey proteins with antimicrobial activity

Whey proteins exhibit a wide range of activities influencing different biological functions. The whole whey protein fraction of DM, rich in antimicrobial and bacteriostatic agents, is considered to be responsible for the low bacterial count reported in literature (Chiavari, Coloretti, Nanni, Sorrentino, & Grazia, 2005; Šarić et al., 2012). Due to its peculiar composition, it could play a role in the strengthening of the host immune-defence systems and to prevent oral and gastro-intestinal infections.

Antimicrobial activity in milk is mainly attributed to lactoferrin (Lf), lysozyme (Lyz), immunoglobulins (Ig_s) and lactoperoxidase (LP) (Yamauchi, Wakabayashi, Shin, & Takase, 2006). In DM it has been proposed that Lf, LP and Lyz, working together, could create synergy with potential function in the digestive tract to reduce the incidence of gastrointestinal infections, especially during infancy and

in childood. (Businco, Giampietro, & Lucenti, 2000; Malacarne, Martuzzi, Summer, & Mariani, 2002),

Whey represents about 37 % of the total protein fraction of donkey milk (Salimei et al., 2004). The main proteins are β-lactoglobulin (β-Lg) (29.85%), α-lactoalbumin (α-La), (22.56%), lysozyme (Lyz) (21.03%), immunoglobulins (Ig) (11.5%), serum albumin (SA) (6.2%) and lactoferrin (Lf) (4.48%) (Salimei et al., 2004).

It is noteworthy that some variation in donkey milk composition has been observed during lactation (Tidona et al., 2011a; Guo et al., 2007). In Sicilian Ragusano donkey milk, Tidona et al., (2011a) found a constant reduction of total protein content due to the decrease of casein fraction (about 0.63g 100^{-1} g); as a consequence the casein/whey protein ratio ranged between 1.33 and 0.60 from early to late lactation (Tidona et al., 2011a).

In whey protein fraction, during lactation, β-lactoglobulin, α-lactoalbumin and lysozyme decrease, whereas immunoglobulins increase and lactoferrin maintains around the same amount (Guo et al., 2007; Vincenzetti et al., 2008).

1.1 β-Lactoglobulin

β-Lactoglobulin (β –Lg) is the major whey protein in milk except for in human, camel, lagomorphs and rodent milk (Uniacke-Lowe, Huppertz, & Fox, 2010). It is a globular soluble protein with a molecular weight of 18.400 Da, however at pH 6.7 forms a dimer (Farrell et al., 2004; Kanyshkova, Buneva, & Nevinsky, 2004)

which plays several roles, including the development of the passive immunity together with IgG (Sutton, & Alston-Mills, 2006); moreover, after proteolytic digestion with commercial enzymes, β–Lg yields peptides with bactericidal activity restricted to Gram-positive bacteria (Pellegrini, 2003). In donkey milk two isoforms of 162 (β–Lg I) and 163 (β–Lg II) amino acid residues are known: β–Lg I with its two variants (Mw 18.510 Da) represents about the 80% of total β–Lg (Godovac-Zimmermann, Conti, Sheil, & Napolitano, 1990); whereas β–Lg II (Mw 18.200 Da) presents four variants: A, B, C (Godovac-Zimmermann et al., 1990) and D (Cunsolo, Costa, Saletti, Muccilli, & & Foti, 2007).

β–Lg I A and β–Lg II A exibit 65% of identity. Two and three amino acid substitutions characterize β–Lg II A from β–Lg II B, and both with respet to β–Lg II C.

Despite the low homology between bovine β–Lg and donkey β–Lg I and II, only 51.12% and 45.81% respectively, it is possible to assume that also the donkey's β–Lg peptides, yielded by gastrointestinal digestion, could exert antimicrobial activity as reported by Tidona et al., (2011b).

Though the β-Lg is generally resistant to gastro-intestinal enzymes, in donkey milk the 70% of the β-Lg is digested, that is twice as much in comparison to cow counterpart (Inglistad et al., 2010; Tidona et al., 2011b). This higher degradability of donkey's β-Lg could enhance the yield of bioactive peptides in gut.

The β-Lactoglobulin is absent in human milk and is generally resistant to the gastro-intestinal digestion, probably due to binding of fatty acids in the barrel of the structure. It is considered to be, together with casein fraction, one of the main

causes of CMPA (Restani et al., 2009) and could create an allergic reaction in sensitive subjects (Uniacke-Lowe et al., 2010). In this light, the identification of animals producing milk lacking in β–Lg II protein, (Criscione et al., 2009) appears promising to solve potential residual cases of reactivity (Wal, 2002).

1.2 α –Lactalbumin

α–Lactalbumin (α–La) is a globular small calcium metalloprotein. Its primary physiological function is the lactose-synthesis in the mammary gland. To date, there is no evidence related to a potential antimicrobial activity, even if it shares a striking identity, in terms of amino acid sequence, with lysozyme, which is a known powerful antibacterial agent (Lopez-Exposito & Recio, 2008). Nevertheless, some peptides yielded from bovine α–La by digestion with trypsin and chymotrypsin, showed bactericidal activity restricted to Gram-positive bacteria (Marcus, Olivier, & Haan, 2002; Pellegrini, Thomas, Bramar, Hunziker, & Von Fellemberg, 1999). A protein-lipid complex between human α–La and oleic acid, called HAMLET (human -lactalbumin made lethal to tumor cells), showed effect on inhibiting cancer cells (Svanborg et al., 2003; Permyakov et al., 2012).

Donkey α–Lactalbumin contains 123 amino acid residues and has a molecular weight of 14,222 Da (Giuffrida, Cantisani, Napolitano, Conti, & Godovac-Zimmermann, 1992).

Only one α–La genetic variant with two isoforms characterized by different pIs (4.76 and 5.26 respectively) have been identified so far (Cunsolo et al., 2007;

Vincenzetti et al., 2012). It is resistant to gastric and duodenal enzymes, since the 95% is undigested after a two step *in vitro* test (Tidona et al., 2011b). Even though there is no direct evidence of the biological activity of those peptides, and since the antibacterial activity of digested donkey milk is known (Tidona et al., 2011b), the peptides yielded from the digested 5% could be contributing factor in the healthy effect of donkey milk.

1.3 Immunoglobulins

Immunoglobulins (Igs) in milk are an important defence family of proteins for the newborn protecting the gut mucosa against pathogenic micro-organisms. Igs inactivate bacteria by binding to specific sites on the bacterial surface: their role is to confer passive immunity to the neonate while its own immune system is developing (Gapper, Copestake, Otter, & Indyk, 2007). Three classes of immunoglobulins are commonly found in milk: immunoglobulin G (IgG), which is the principal immunoglobulin in equine colostrum, A (IgA) which is the main form in equine milk (Uniacke-Lowe et al., 2010) and M (IgM); IgG is often sub-divided into two subclasses, IgG1 and IgG2 (Hurley, 2003; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007).

In human, IgG is transferred to the foetus in utero, whereas in donkey IgGs were supplied after parturition only by colostrum and, then, by mature milk. For this reason equidae milk has a higher content of that protein fraction compared with human and bovine (Uniacke-Lowe et al., 2010). In donkey total IgGs content increases in milk during lactation, as reported by Guo et al. (2007).

Immunoglobulins could be important for the gut health because the antimicrobial action is exerted directly in the intestine, similarly to how lactoferrin acts (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008).

1.4 Lactoferrin

Lactoferrin is an 80,000 Da (Vincenzetti et al., 2012) iron-binding glycoprotein of the transferrin family (Wakabayashi et al., 2006). Bovine lactoferrin is associated with a wide variety of biologically important processes such as antioxidant, antiviral, anti-inflammatory, immunomodulatory and anti-carcinogenic activity (Di Biase et al., 2003; Conesa et al., 2008; Alderova, Baroskova, & Faldyna, 2008; Legrand & Mazurier, 2010). Lf also plays a role in the innate host defense system, because it has antibacterial activity against a broad range of Gram-positive and Gram-negative pathogens (Valenti & Antonini, 2005; Möller et al., 2008). The antimicrobial activity of lactoferrin seems to be dependent on its iron-free state and on its ability to bind and sequester iron, producing an iron deficient environment that limits microbial growth (bacteriostatic). Besides, it permeabilizes bacterial cell walls by binding to lipopolysaccharides through its N-terminus (bacteriocidal), and also it inhibits viral infection by binding tightly to the viral envelope proteins. In contrast, Lf is thought to promote the growth of beneficial microflora in the gastrointestinal tract (Liepke et al., 2002; Kim et al., 2004; Sherman, Bennett, Hwang, & Yu, 2004; Baldi et al., 2005).

The amount of lactoferrin in DM (about 0.080 g L^{-1}) was recently assessed by Vincenzetti et al., (2012). This value is similar to that found in mare (0.1 g L^{-1})

(Matèos et al. 2009) and in cow milk (0.02 – 0.2 g L^{-1} (Hennart, Brasseu, Delogne-Desnoeck, Dramaix, & Robyn, 1991), but much lower that in human milk (0.3 – 4.2 g L^{-1}) (Kanyshkova, Buneva, & Nevinsky, 2001). Donkey's milk Lf is quite resistant to the gastric and duodenal juice, and this suggests that its biologic role is not only as a resource of amino acids but it affects antimicrobial activity directly in the gut (Brock, 2002). Besides, part of it is hydrolyzed creating bioactive peptides, such a peptide is known Lactoferricin (Lfcin) and Lactoferrampin (LFampin) produced by commercial pepsin. The first one exerts antimicrobial activity giving various beneficial health effects both in humans and in adult animals (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002; Teraguchi, Wakabayashi, Kuwata, Yamauchi, & Tamura, 2004). This peptide has an higher antimicrobial activity and lower antiviral effects than the mature protein (Gifforda, Huntera, & Vogela, 2005). A comparison *in vitro* of the antiviral action between Lf and Lfcin, on the same virus infection, showed the activity of intact Lf as much as seven times higher, suggesting that either the size of the molecule is important or that other regions of Lf contribute to the antiviral activity (Di Biase et al., 2003; Andersen, Osbakk, Vorland, Traavik, & Gutteberg, 2001). However, Lfcin has not been identified after digestion by human GI enzymes (Furlund et al., 2012).

Lactoferrampin (Lfampin), a sequence in the N1-domain, has antimicrobial properties (Deber & Goto, 1996). Van der Kraan et al., (2004) have found the corresponding fragment: 268–284 and determined its fungicidal activity on yeast *C. albicans*.

The lactoferrin content of donkey milk is 0.37g kg^{-1} (Salimei et al., 2004; Chiavari et al., 2005, Guo et al., 2007), which is a high value if compared with milk from other species, but it is still lower than in human milk (1.65 g kg^{-1}).

Tidona et al. (2011b) found that the antimicrobial activity of native and digested donkey milk against different bacteria, could be due to lactoferrin both intact protein and its peptides yielded by digestion.

1.5 Lysozyme

Lysozyme (Lyz) is a powerful antibacterial protein (Lopez-Exposito et al., 2008) with an important role in the intestinal immune response. It splits the bond between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan leading to fragments with high and low molecular weight, which are the agents responsible for the specific immune cell activations (Hisham, Matsuzaki, & Aoki, 2001).

In donkey milk, Lyz contains 129 amino acids and two variants (A and B) had been described so far (Herrouin et al., 2000).

Lysozyme is resistant to digestion and thermostable: in DM only the 25% of the total protein is digested *in vitro* by gastric intestinal juice (Tidona et al., 2011b). It remains intact even after a thermal treatment (e.g. 63 °C for 30 min) (Coppola, Salimei, & Succi, 2002; Di Cagno et al., 2004; Chiavari et al., 2005).

Although in donkey milk lysozyme is present at a high value (4 mg mL^{-1}), it decreases during lactation (Guo et al., 2007).

Lyz acts as a natural preservative, conferring a lengthy shelf-life; in fact it is employed in the food industry as stabilizer (Paolicelli, 2005) and in cheese making process against the late blowing in hard cheeses (Neviani, 1992).

1.6 Lactoperoxidase

Lactoperoxidase (LP) is an oxidoreductase enzyme with protective function against microorganism infections (Geoffry 2007). It is composed by a heme group, carbohydrate (about 10%) and a calcium ion, covalently bound. LP is a major antibacterial agent in bovine colostrum (de Wit & van Hooydonk, 1996). In donkey milk the amount of lactoperoxidase is about 0.11 mg mL^{-1} (Beghelli, Vincenzetti, Micozzi, Vita, & Polidori 2011; Vincenzetti et al. 2012), closer to human milk (770 mg mL^{-1}) (Shin, Hayasawa, & Lönnerdal, 2001) than bovine milk, in which it is more than 100 lower (0.03±0.1 mg mL^{-1}) (Janet & Tanaka, 2007).

Beghelli et al. (2011), testing anti-oxidant properties of DM, detected a LPO activity equivalent to 0.11±0.027 mg mL^{-1}, similar to the value obtained from human milk.

In an another study a very low peroxidase activity (4.83±0.35; 1.39±0.23; 2.88±0.51 mU mL^{-1}) was found in fresh, frozen and powered DM, respectively (Mariani, 2010). This low activity reveals a small peroxidase concentration in fresh DM (Vincenzetti et al. 2012).

LP is inactivated by high temperature but in raw-fresh milk this enzyme could be of significant interest, because working in synergy with lactoferrin and lysozyme, both being present in high quantity in donkey milk, could enhance the natural preservative action of donkey milk.

2. Antimicrobial activity of fresh, powdered, digested and fermented donkey milk

Fresh and frozen DM show a similar amount of total whey proteins, on the contrary a reduction of lysozyme and beta-lactoglobulin can be observed in powdered milk (Mariani, 2010).

Raw donkey milk, rich in antimicrobial compounds in particular lysozyme and lactoferrin, shows a low total bacterial count when produced under standard conditions (4.24 Log CFU mL^{-1}), and keeps a low value even during storage (Chiavari et al., 2005; Šarić et al., 2012).

Besides, the high content of lysozyme gives to DM a long natural shelf-life and makes not necessary a prolonged heat treatment, which is normally required for maintaining good quality over time. These peculiar features confer to DM high hygienic quality and preserve its nutritional properties.

Moreover fresh DM is a natural resource of probiotic bacteria which are capable to colonize the colon, acting against outcome pathogen bacteria and stimulating the immune system (Nazzaro, Orlando, & Conti, 2012). Eight bacterial belonging to *Lactobacillus* genus have been isolated from donkey milk; they might be used

in fermented milk or yogurt (Nazzaro et al., 2012). Besides, DM, if fermented, will yield metabolites with several healthy functions including antibacterial activity (Nazzaro et al., 2008).

Among metabolites yielded, acetate and lactate inhibit the growth of potential enteropatogens (Fooks, & Gibson, 2002); lactic acid, present at the end of the fermentation, could decrease the pH value in the intestine acting as bacteria antagonist and facilitate calcium absorption (Lan, Lagadic-Gossmann, Lemaire, Brenner, & Jan, 2007). The synergism among natural compound of milk, microorganisms and metabolites, positively influences the intestinal flora composition and the defense mechanisms of the host (Fooks, & Gibson, 2002).

Finally two different probiotic strains, added in lyophilized donkey milk, maintained their viability after milk reconstitution, suggesting the possibility of producing a probiotic infant formula with beneficial properties using donkey's milk as raw material (Vincenzetti et al., 2011).

Besides native proteins, the biological action could be exerted by bioactive peptides: endowed sequences, inactive whilst part of the protein, that after the passage through the stomach and intestine, are partially or totally hydrolyzed devolving into fragments (Hill, Doull, Rutherford, & Cross, 2000; Yalcin, 2006). Protein degradation may be also produced by enzymatic hydrolysis, fermentation conducted by proteolytic bacteria and through food processing or manufacturing (Michaelidou, 2008). Peptides can exert biological activity, in synergism with whole whey proteins and/or whey constituents such as minerals (Bohdan, Akhavan, & Anderson, 2007), by binding specific receptor in the intestinal tract

or in target organs or tissue after absorption into the bloodstream (Bohdan et al., 2007)

In a recent study DM revealed to be a good source of antimicrobial bioactive peptides, released during *in vitro* simulated gastrointestinal digestion processes, able to inhibit the replication of three enterobacteria (Tidona et al., 2011b). β–lactoglobulin and lactoferrin are the highest digested fractions, while α-lactoalbumin and lysozyme the most resistant proteins (Tidona et al., 2011b). However, since part of those proteins remain undigested, continuing to perform their action intact (Tidona et al., 2011b), the antimicrobial activity might result from a synergism of the intact proteins and peptides.

Nazzaro et al (2010a) digested DM with commercial pepsin in order to identify additional components, other than lysozyme, with antimicrobial activity on pathogenic microorganisms. Some chromatographic fractions, obtained from hydrolyzed milk proteins, contained bio-molecules with antimicrobial effect on different bacteria and with a strain-dependent activity within the same species.

3. Conclusions and future perspective

In the light of these recent findings the antimicrobial properties of donkey milk whey proteins seem very promising. DM represents a precious natural breast milk substitute, useful both to strengthen of the host immune-defence systems and to preserve gut health in infancy. Furthermore some recent clinical trials revealed that it stimulates the immune-system, regulates gastro-intestinal flora and prevents inflammatory and autoimmune diseases in children and adults.

Therefore a wider consumption of fresh donkey milk could be definitely advisable. Nevertheless, despite the recent expansion of specialized donkey breeding, DM is still a "niche product", which often can only be retailed in farms. The application of some new technologies, such as lyophilisation and microencapsulation, typically used for controlling the release of flavourings and the production of foods containing functional ingredients (e.g. probiotics and bioactive ingredients) (Ghosh, 2006; Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007) could enable a better exploitation of this product

Since the consumers are more aware of the quality of food in terms of "Healthy foods", it could be a valid perspective to use natural substances such as probiotics, prebiotics and bioactive compounds both to confer benefits in terms of health or nutrition and/or preserve the food. However, various factors have to be taken into account such as the stability, the biological activity and the cost.

Genomics and proteomics could provide cutting edge approaches in this topic. Further studies are needed to evaluate other bioactivities and the potential use of donkey milk as functional food or as bioactive compound to formulate functional food.

In conclusion, considering its nutritional profile, it would be worth to deepen the study of donkey milk components in order to exploit those features and, at the same time, safeguard both donkey breeds and their natural rural environment. In fact, to promote a wider consumption of fresh, powdered and fermented donkey milk might entail a revitalization in breeding of donkey, a specie at serious risk of extinction.

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Table 1. Milk gross composition of different species. [a]

	Human[b]	Donkey[b]	Horse[b]	Camel[c]	Cow[c]	Goat[b]
Total solids	124.0	88.4	102.0	124.7	127.0	122.0
Fat	38.0	3.8	12.1	38.2	37.0	38.0
Lactose	70.0	68.8	63.7	44.6	48.0	41.0
Ash	2.0	3.9	4.2	7.9	7.0	8.0
Protein	9.0	17.3	21.4	33.5	34.0	35.0
Casein/Whey protein ratio	0.4:1	1.3:1	1.1:1	1.7:1	4.7:1	3.5:1

[a] Data from (human, camel, cow and goat) Uniacke-Lowe et al., (2010), (donkey) Salimei et al., (2004), Guo et al., (2007), Tidona et al., (2011a), (horse) Malacarne et al., (2002).

[b] Mean value are expressed as g kg^{-1} milk.

[c] Mean value are expressed as g L^{-1} milk.

Table 2. Whey milk proteins content in different species. [a]

	Human[b]	Donkey[b]	Horse[b]	Camel	Cow[b]	Goat[c]
Total whey protein	97.0	107.0	130.0	127[e]	99.0	3.7-7
β-lactoglobulin	-	29.8	30.7	-	50.8	1.8-2.8
α-lactalbumin	40.3	22.6	28.5	-	19.0	0.6-1.1
Serum albumin	7.7	6.2	4.4	-	6.3	0.3
Immunoglobulins	15.5	11.5	19.6	1.54[d]	12.7	-
Lactoferrin	26.6	4.48	7.0	0.24[d]	1.6	0.12
Lysozyme	5.5	21.0	10.5	0.06[f]	Trace	-

[a] Data from (human, donkey, horse and cow) Salimei et al., (2004), (camel) Shamsia (2009), (goat) Hernández-Ledesma, Ramos, & Gómez-Ruiz (2011).

[b] Mean value are expressed as mg 100 g^{-1} milk.

[c] Mean value are expressed as g L^{-1} milk.

[d] Mean value are expressed as mg mL^{-1} milk.

[e] Mean value are expressed as mg $100mL^{-1}$ milk.

[f] Mean value are expressed as µg ml^{-1} milk.

Chapter 4

Antiviral activity of donkey milk on echovirus type 5

Part of the chapter has been presented at International Symposium on Sheep, Goat and other non-Cow Milk - IDF Dairy Science and Technology Week 2011, Athens, Greece, 16-18 Maggio 2011. Poster n. 32, Session 4. Characteristics and Nutritional Value of Ewe, Goat and Other Non-Cow Milk and Milk Products.
The full paper has been published as a short communication entitled "Antiviral activity of donkey milk protein fractions on echovirus type 5" on International Dairy Journal (2013), 28: 109-111. Moreover, preliminary work has been presented at "III Congresso Lattiero-Caseario – Milano, 28 Ottobre 2012". The article entitled "Attività antivirale del latte di asina su echovirus type 5: uno studio preliminare" has been accepted for publication in the Italian Journal: "Scienza e Tecnica Lattiero-Casearia". The poster has received a prize from scientific Commission AITeL (Associazione Italiana dei Tecnici del Latte), for originality and contribution to science.

Project's outlines

Milk is a healthy food source for energy, proteins, vitamins and minerals. In addition it supplies an array of defence factors, in particular some proteins, which might exert synergic effect when working together (Joslin et al., 2002). Donkey milk is receiving increasing research interest mainly owing to its attractive nutrient and functional compounds, such as lactoferrin and lysozyme, which are present at very high level in DM. Recently, the antibacterial activity of donkey milk and peptides yielded by its hydrolysis (*in vitro* with human gastro-intestinal enzyme or by commercial enzyme) has been described (Nazzaro et al., 2010; Tidona et al., 2011). On the contrary, the antiviral activity of donkey milk proteins have not been investigated.

The aim of the work was to investigate the antiviral activity of donkey milk on echovirus type 5, an enterovirus which infects the gastrointestinal tract of humans. Whole donkey milk, DM digested at pH 2 by human gastro-intestinal enzyme, skimmed DM and donkey milk whey protein fraction were tested at concentrations of 10 mg mL^{-1}. Subsequently, the study was aimed to the comparison of the donkey milk proteins. Casein, whole whey proteins and low molecular weight (below to 30,000 Da) of whey protein fraction were tested on the same cell line infected by echovirus type 5.

4.1 Enterovirus - echovirus

Enteroviruses belong to the family *Picornaviridae* and are viruses that can infect through and/or replicate in human intestine (enteric viruses) (Yezli & Otter, 2011). Enteroviruses resist in several unfavourable environments and in stress conditions such as freezing and acid (Fong & Lipp, 2005); as a consequence they can survive for a long time outside their host. They can contaminate water, consequently vegetables and fruits, and food previously handled (Fong & Lipp, 2005; Furlund, 2012). When ingested, enteroviruses are able to resist to low stomach pH, infect the gastro-intestinal tract (as the primary organ) (Tinari et al., 2005; Pallansh & Roos, 2007), and replicate mainly in the intestine (in the

enterocytes). Then are excreted in faeces of infected individuals and transmitted via faecal-oral route (Furlund, 2012).

Echovirus (Enteric Cytopathogenic Human Orphan Virus) is a small, non-enveloped, single stranded RNA virus, belonging to the *genus* Enterovirus. Echovirus infections have been associated with a wide variety of diseases such as aseptic meningitis, myocarditis, common cold (asymptomatic), infantile diarrhoea, respiratory disease and fever (Hyypia et al., 1997; Siafakas et al., 2005).

4.2 Human Colon Adenocarcinoma Cell Line (CaCo-2)

The human intestinal CaCo-2 cell line has been originally obtained from a human colon adenocarcinoma. It is used as model of the intestinal barrier because, in culture, undergoes a process of differentiation that leads to the formation of a monolayer of cells. Growing as a monolayer, CaCo-2 are able to represent the morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005).

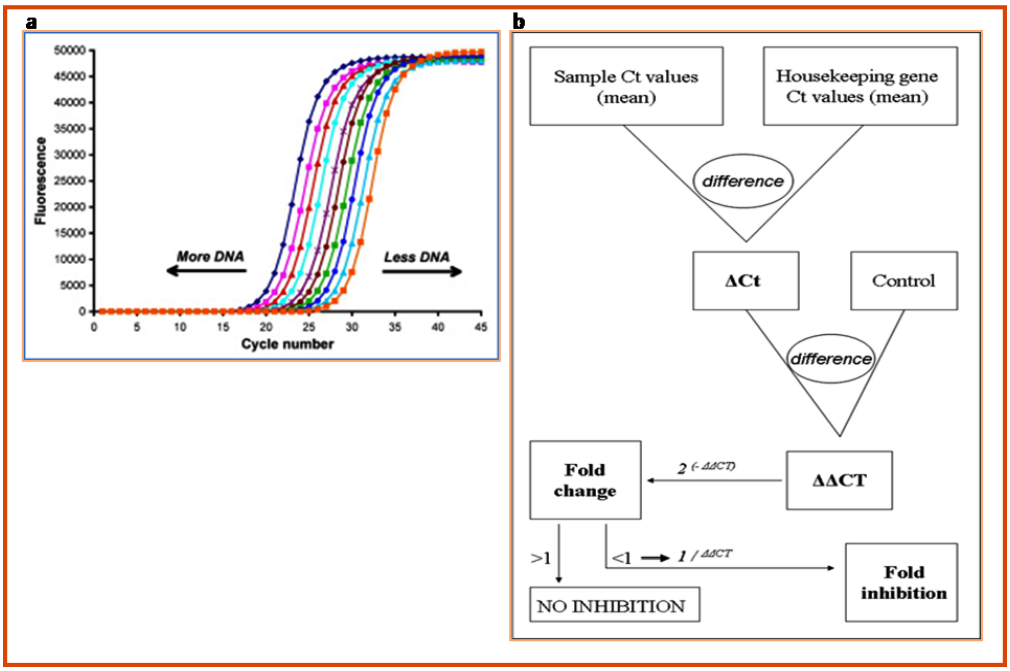
4.3 Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Real-time PCR is a powerful, economical, rapid and high-throughput technique for assaying gene expression and for quantitation of target nucleic acids. It has a high sensibility and works with small volume samples (Yuan et al. 2008). When the quantitation of gene expression has RNA as a starting material, it is necessary to use a process called Reverse Transcription (RT), which translates it for the synthesis of cDNA. Since the results could be skewed by differing amounts of input of nucleic acid templates, quantitative assays use an appropriate endogenous control, which is a gene that normalizes the input amounts. It is an internal standard, mainly housekeeping gene, so called because its synthesis occurs in all nucleated cell types since it is necessary for cell survival (Thellin et al., 1999).

β-actin, a protein essential for the structure and kinetics of the cytoskeleton, is often used as housekeeping gene since it demonstrates stable and constant expression in cell (Thellin et al. 1999; Tafaro et al., 2007).

Real-Time RT-PCR involves calculation based on the cycle number at which logarithm-transformed fluorescence crosses a threshold (Ct). As long as the PCR efficiencies between the target and endogenous control are relatively equivalent, it is possible to avoid the use of standard curves.

The data output are expressed as Cycle thresholds (Ct) and then elaborated (see scheme 1) until fold-inhibition values, which indicate fold-difference of expression levels.



Scheme 1. when fold-change value is above 1, the time that it gets is up-regulated; when that value is below 1, by an additional calculation it gets the fold regulation (the time-fold inhibition of the assay compared to the control).

RNA samples may sometimes be contaminated with chemicals, proteins, and other molecules that can inhibit or interfere with downstream applications. It is important to evaluate its purity by the ratio of the absorbance at 260 and 280nm

(A260/280), which has to be close to 2 (a ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA).

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ATTIVITÀ ANTIVIRALE DEL LATTE DI ASINA SU ECHOVIRUS TIPO 5 : UNO STUDIO PRELIMINARE

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RIASSUNTO – L'attività antivirale del latte di asina è stata investigata sull'echovirus tipo 5, un enterovirus che causa infezioni dell'apparato gastrointestinale nell'uomo. In uno studio preliminare quattro tesi (latte intero, latte scremato, latte digerito a pH 2 con succo gastrico e intestinale umano, proteine del siero) sono state testate alla concentrazione di 10 mg mL^{-1}.
Tutti i campioni hanno mostrato una significativa inibizione della replicazione del virus. Le proteine del siero hanno esercitato il maggior effetto inibente, probabilmente a causa dell'alta concentrazione di alcuni componenti, come la lattoferrina, con ben nota attività antivirale.
I risultati ottenuti mostrano che il latte di asina e le sue sieroproteine sono in grado di inibire la replicazione dell'echovirus tipo 5. Il consumo di latte di asina può dunque contribuire alla naturale prevenzione delle infezioni gastrointestinali sostenute da tale virus.
Parole chiave: latte di asina, sieroproteine, attività antivirale, echovirus.

SUMMARY - **A preliminary study on antiviral activity of donkey milk on echovirus type 5 -** The antiviral activity of donkey milk was investigated for the effect on echovirus type 5, an enterovirus which infects the gastrointestinal tract of humans. Four milk fractions were tested to compare their different activity. Whole milk, skimmed milk, whey protein fraction and whole digested milk to pH 2 with human gastric and duodenal juice were analysed for antiviral activities at concentrations of 10 mg mL^{-1}. All donkey milk fractions showed a significant inhibition of the virus replication. The highest antiviral effect was observed for the whey protein fraction, probably due to the high concentration of lactoferrin, known to have antiviral activity. The findings showed that donkey milk and specifically the whey exerted antiviral effect on echovirus 5 and this may contribute to prevent gastrointestinal virus infection in humans.
Key words: donkey milk, whey proteins, antiviral activity, echovirus.

INTRODUZIONE

Negli ultimi decenni si assiste ad rinnovato interesse per il latte di asina da impiegare nella nutrizione umana, sia per neonati, i quali non hanno la possibilità di essere allattati, sia per bambini e adulti con allergia alle proteine del latte bovino [1, 2]. Questo latte, di elevato valore nutrizionale, ricco in proteine del siero (con un basso rapporto tra caseine e siero proteine) e in lattosio, si caratterizza per ipoallergenicità, palatabilità, migliora l'assorbimento del calcio a livello intestinale [3] e favorisce la crescita dei lattobacilli [4]. Recentemente è stata descritta l'attività antibatterica del latte di asina, anche dopo digestione con enzimi gastro-intestinali [5, 6]; di contro, l'attività antivirale non è stata mai investigata. Scopo di questo lavoro preliminare è stato valutare l'attività antivirale del latte di asina, comparandone diverse frazioni: latte intero, latte intero digerito a pH 2 attraverso succo gastrico e intestinale umani, latte scremato e siero.

Il virus utilizzato è un Enterovirus, echovirus tipo 5, un virus a RNA, che si localizza nell'apparato gastrointestinale, ove causa infezioni opportunistiche. L'infezione colpisce in forma grave soprattutto i bambini nei paesi in via di sviluppo.

MATERIALI E METODI

Lo studio è stato condotto su un campione di latte individuale di razza Ragusana, allevato in Sicilia (Milo – CT). Le diverse tesi sono state ottenute dal latte intero (frazione I): una parte del latte intero è stato digerito (frazione II) con succo gastrico prima (primo step) e succo intestinale dopo (secondo step) di donatori volontari [4]; il latte scremato (frazione III) è stato ottenuto dalla centrifugazione del latte intero a 3.500 x g, per 15', a 4°C e rimozione della fase grassa, prima manuale e poi, per favorirne ulteriormente la scrematura, attraverso filtrazione (0.45 μm). Il siero (frazione IV) è stato ottenuto dalla precipitazione acida (HCl 1M) del latte scremato, seguita da centrifugazione (stesse suddette condizioni). Successivamente le quattro tesi sono state liofilizzate e risospese alla concentrazione di 10 $mgmL^{-1}$ nel terreno di coltura EMEM addizionato di 10% siero fetale bovino (FBS), 12 mL di bicarbonato di sodio ($NaCH_3$) L^{-1}, 10 mL di L-glutammina L^{-1} e 1 mL di Gentimicina mL^{-1}, e quindi testate in tre repliche sulla linea di cellule CaCo2, precedentemente infettate con echovirus 5.

L'RNA virale è stato quantificato attraverso la Real Time Reverse-Transcription-PCR one-step (QuantiTec Probe RT-PCR) usando specifici primers and probe per Enterovirus. I valori di Cycle threshold (Ct) ottenuti sono stati statisticamente analizzati con ANOVA general linear model [7]. La significatività statistica considerata era $P<0.05$.

RISULTATI E DISCUSSIONE

Dal valore del Cycle Threshold di ciascuna replica è stato ottenuto un valore medio per ciascuna tesi. Tale valore esprime l'effetto inibente del campione sulla replicazione del virus. Tutte le tesi testate hanno inibito significativamente la replicazione virale rispetto al controllo. Anche se il valore di inibizione non è risultato significativamente diverso tra le tesi, il siero ha mostrato la maggiore capacità inibente, mentre il latte intero, digerito e scremato hanno fatto registrare valori di Ct comparabili (Grafico 1). La maggiore attività antivirale osservata per il siero può essere attribuita a quei componenti che hanno nota attività

antimicrobica o che coadiuvano tale azione così come la lattoferrina, lattoperossidasi e il lisozima. Il ruolo di quest'ultimo, noto agente antibatterico, di cui è ricco il latte di asina, merita di essere ulteriormente indagato. Di contro in questa prova si è osservato che la digestione con enzimi gastro-intestinali non incrementa significativamente l'azione antivirale del latte di asina, come avvenuto invece con l'attività antibatterica in taluni ceppi (TID).

CONCLUSIONI

In questo studio preliminare il latte di asina ha mostrato un chiaro effetto inibente sulla replicazione dell'echovirus tipo 5, mettendo in luce soprattutto le proprietà antivirali del siero. Ulteriori studi sono necessari per approfondire le conoscenze sull'attività antivirale delle diverse frazioni proteiche del latte e del siero al fine di suggerire una maggiore diffusione del consumo di latte d'asina che, tra l'altro, potrebbe aiutare nella prevenzione delle più diffuse infezioni intestinali virali, almeno quelle sostenute da echovirus tipo 5.

Ringraziamenti:
Poster presentato al 3° Congresso Lattiero-Caseario AITeL. Milano, 28 settembre 2010 "La ricerca e l'innovazione".

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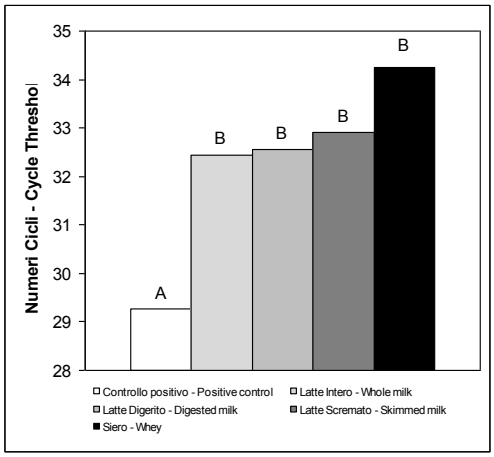
Grafico 1 - Numero di cicli (Ct) delle quattro tesi testate

Graphic 1 - Cycle threshold of the different fractions tested



3° Congresso Lattiero-Caseario - Milano, 28 Settembre 2012

Attività antivirale del latte di asina su echovirus tipo 5: uno studio preliminare

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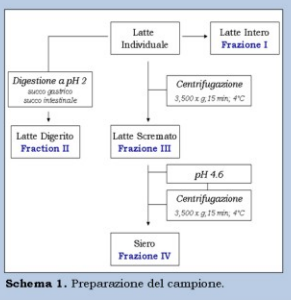
Negli ultimi anni l'attività antimicrobica del latte di asina è stata oggetto di numerosi studi, condotti soprattutto su diverse specie enterobatteriche responsabili di infezioni a carico dell'apparato digerente (1,2). L'attività antivirale, di contro, non è stata investigata.

Scopo del lavoro: valutare l'attività antivirale del latte di asina, sull'echovirus tipo 5, un enterovirus che causa infezioni dell'apparato digerente nell'uomo, comparando latte intero, latte intero digerito *in vitro* con succo gastrico e intestinale umani, latte scremato e siero.

Asini ragusani. Allevamento Asilat – Milo (Ct)

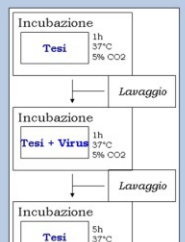
Materiale e Metodi

Un campione di latte individuale prodotto da un'asina di razza Ragusana è stato utilizzato per ottenere 4 frazioni **(Schema 1)**.



Schema 1. Preparazione del campione.

Le quattro frazioni sono state liofilizzate e testate alla concentrazione di 10 mg/mL, in tre repliche **(Schema 2),** sulla linea di cellule CaCo2 precedentemente infettate con echovirus 5 **(Figura 1).**



Schema 2. Fasi dell' infezione

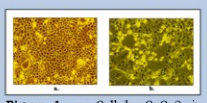


Figura 1. a. Cellule CaCo2 in coltura; **b.** Cellule CaCo2 infettate con echovirus tipo 5

L'RNA virale è stato quantificato attraverso la Real Time RT-PCR (QuantiTec Probe RT-PCR) usando specifici primers and probe per Enterovirus.

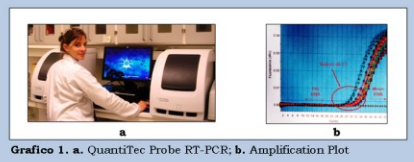


Grafico 1. a. QuantiTec Probe RT-PCR; **b.** Amplification Plot

Dal valore del Cycle Threshold (Ct) **(Grafico 1)** di ciascuna replica è stato ottenuto un valore medio per ciascuna tesi. Tale valore esprime l'effetto inibente sulla replicazione virale. I dati sono stati sottoposti ad analisi statistica (ANOVA general linear model) (3).

Risultati e discussioni

Tutte le tesi hanno inibito significativamente la replicazione virale **(Grafico 2)**.
Il siero ha mostrato il maggiore grado di inibizione.
Il valore di inibizione tra le tesi non è risultato significativamente diverso. In particolare il latte intero (frazione I) e il latte intero digerito (frazione II) hanno mostrato valori di Ct comparabili (32.43 e 32.55 rispettivamente).

Il latte di asina ha mostrato un chiaro effetto inibente sulla replicazione dell'echovirus 5. Il siero ha esercitato la maggiore attività antivirale probabilmente a causa della presenza della lattoferrina e della lattoperossidasi, componenti che hanno una ben nota attività antivirale.

Il ruolo del lisozima, noto agente antibatterico, di cui è ricco il latte di asina, merita di essere ulteriormente indagato. Di contro la digestione con enzimi gastro-intestinali non sembra influenzare significativamente l'azione antivirale del latte.

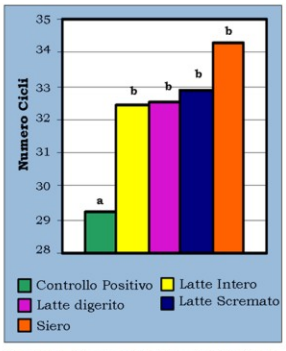


Grafico 2 – Numero di Cycle Threshold (Ct) delle tesi testate

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Short communication

Antiviral activity of donkey milk protein fractions on echovirus type 5

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ABSTRACT

Antiviral activity of Ragusano donkeys' milk proteins was investigated for the effect on echovirus type 5, known to infect the gastrointestinal tract of humans. Three protein fractions were tested; casein (CN), whey protein (WP) and a low molecular whey protein fraction (LWP; <30,000 Da). The antiviral activity of WP and LWP was tested on echovirus type 5 at three concentrations (1, 5 and 10 mg mL^{-1}); CN was assessed only at the lower concentration. All donkey milk protein fractions showed significant inhibition on virus replication at the concentration of 1 mg mL^{-1}, and both WP and LWP fractions showed significant inhibition on the virus replication at all concentrations tested. The strongest antiviral effect was observed for the WP fraction. These findings show that the different whey proteins in donkey milk, probably acting in synergy, exert antiviral activity on echovirus 5 and might contribute to prevent gastrointestinal virus infections in humans.

1. Introduction

Donkey milk is gaining a growing interest in human nutrition due to its distinctive composition and physiological aspects. The low content of total proteins, particularly rich in whey proteins, high level of lactose, together with the peculiar mineral composition makes it more comparable with human milk than is cow milk, a good substitute for newborns who cannot be breast-fed (Salimei et al., 2004), and well tolerated by many persons suffering of cow milk allergy (CMA) or cow milk intolerance (CMI) (Monti et al., 2007).

Antimicrobial activity in bovine milk is mainly attributed to lactoferrin (Lf), lactoperoxidase (LP) (Yamauchi, Wakabayashi, Shin, & Takase, 2006) and lysozyme (Lz). These minor whey proteins, when working together, might exert a synergetic effect (Joslin et al., 2002).

In donkey milk lactoferrin represents more than 2% of the total protein fraction (Guo et al., 2007) and lysozyme is higher than in mare and bovine milk (Malacarne, Martuzzi, Summer, & Mariani, 2002; Salimei et al., 2004). The high content of protective antimicrobial factors in donkey milk suggests its beneficial impact on gut health, particularly for children, the elderly and convalescents, who have a reduced immune defence system.

Donkey milk antimicrobial activity has been reported and proven effective against specific bacteria often associated with intestinal infection (Tidona et al., 2011; Zhang, Zhao, Jiang, Dong, & Ren, 2008); however the antiviral properties have, until now, not been investigated.

Echovirus is a small, non-enveloped, single stranded RNA virus, belonging to the genus Enterovirus of the *Picornaviridae* family, acquired by faecal–oral contamination and infecting the gastrointestinal tract (GIT) as the primary organ (Tinari, Pietrantoni, Ammendolia, Valenti, & Superti, 2005). Infections with echoviruses have been associated with a wide variety of neurological and exanthematic diseases (Park et al., 2011).

The aim of our work was to study and compare the antiviral effect of the different donkey milk protein fractions, casein, whey protein and a low molecular weight of whey fraction, on echovirus type 5.

2. Materials and methods

2.1. Sample preparation

Bulk milk was collected from nine Sicilian indigenous Ragusano donkeys, from a specialised farm in Sicily. All animals were in

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middle lactation stage and the milk was immediately frozen and stored at −18 °C.

Casein (CN) and whey protein fraction (WP) were obtained according to Criscione et al. (2009). Both fractions were dialysed using a dialysis membrane of cut-off 3500 Da (SpectrumLab – Spectra/Por – Membrane 3, Breda, The Netherlands). An aliquot of WP was further filtrated using a 30 kDa cut-off membrane (Millipore Amicon Ultra – ultracell –30 K – Bedford, MA, USA), to obtain the low molecular weight fraction (LWP). Finally, all three donkey milk protein fractions (CN, WP and LWP) were freeze-dried.

The protein content in whole milk and the different fractions were visualized by SDS-PAGE using 4% acrylamide stacking gel and a 12.5% separating gel (Laemmli, 1970).

Prior to the experiments each fraction was diluted in cell culture Eagle's minimal essential medium (EMEM) to final concentrations: 1 mg mL^{-1}, 5 mg mL^{-1} and 10 mg mL^{-1}.

2.2. Cells and virus propagation

Human intestinal epithelial cell line Caco-2 (American Type Culture Collection) was grown at 37 °C in a humidified atmosphere with 5% CO_2 in Eagle minimal essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), 1.2 g mL^{-1} sodium bicarbonate ($NaHCO_3$), 2 mM L-glutamine and 50 mg mL^{-1} gentamicin sulphate (All medium compounds were from Lonza, Basel, Switzerland).

Echovirus type 5 (kindly provided by Dr. Gabriel Aanestad, the Norwegian Institute of Public Health), was propagated in Caco-2 cells at 37 °C in 5% CO_2 until extensive cytopathic effect (CPE).

Infected culture was frozen and thawed three times, centrifuged at 3500 × *g* for 10 min at 4 °C and the supernatant was stored at −80 °C until used. The virus content in supernatant was titered by serial dilution, and tissue culture infective dose 50 mL^{-1} ($TCID_{50}$ mL^{-1}) was calculated according to Reed–Muench (Reed & Muench, 1983).

2.3. Infection assay

Caco-2 cells were grown for five days until 90–100% confluence in three 24-well tissue culture plates. The plates were washed twice with phosphate-buffered saline (PBS) and incubated with sample fractions (CN, WP, LWP) at different concentrations (0, 1, 5 and 10 mg mL^{-1}), in virus growth medium for 60 min at 37 °C in a 5% CO_2. Then the plates were washed twice with PBS and incubated at the same temperature and CO_2 conditions with media containing the corresponding sample at the same concentration as the pre-infection step, supplemented with virus at a Multiplicity Of Infection (MOI) of 1 plaque forming unit (pfu) $cell^{-1}$. After 60 min, virus was removed, the plates were washed twice with PBS and fresh medium containing milk fractions at corresponding concentration was added. The cells were then further incubated for 5 h at 37 °C, to allow for virus replication. Following incubation, cell culture supernatant was discarded, and the cell monolayer was washed twice with PBS. One millilitre extraction lysis buffer was added to the monolayer, and the cells were lysed for 30 min. All protein fractions and controls were run in triplicates.

2.4. Extraction of nucleic acids and real-time PCR

Total nucleic acids were extracted from cell lysate using NucliSense EasyMag automated extraction system (BioMerieux, Craponne, France), eluated in 50 µL, and stored at −80 °C until analysis. Quantification was carried out by real time one-step RT-PCR, using QuantiTect Probe RT-PCR mix, (Qiagen, Dusseldorf, Germany). The reactions were set up in a total volume of 20 µL with 2.0 µL of RNA eluates, QuantiTect Probe RT-PCR enzymes, master mix prepared according to the manufacturer's instructions, enterovirus specific primers (200 nM each) and probe at 300 nM. (Eurogentec S. A.; Seraing, Belgium). Human β-actin (ACTB) (Applied Biosystems, Foster City, CA, USA) was used as endogenous control.

RT-PCR amplification was performed in a Stratagene MX30005P real-time Q-PCR system (Aligent Technologies Inc, Santa Clara, CA, USA) according to the manufacturer's instructions.

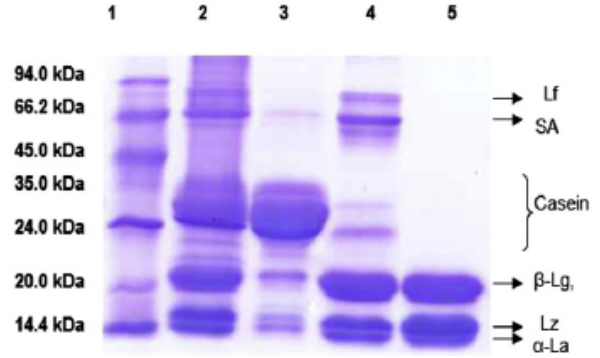

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of donkey milk protein and their fractions: lane 1, marker protein ladder; lane 2, whole donkeys' milk; lane 3, casein fraction; lane 4, whey protein fraction; lane 5, low molecular mass whey protein fraction. The positions of lactoferrin (Lf), serum albumin (SA), caseins, β-lactoglobulin (β-Lg), lysozyme (Lz), and α-lactalbumin (α-La) are indicated.

2.5. Statistical analysis

Effects of the different tested milk protein fractions on enterovirus replication, in comparison to positive controls, were analysed statistically with analysis of variance (ANOVA) (Minitab, 2010) general linear model reference. Statistical significance was defined as $P < 0.05$.

3. Results and discussion

The milk protein fractions CN, WP and LWP were analysed by SDS-PAGE (Fig. 1) and identified on the base of their apparent molecular mass in comparison with the marker protein ladder. The proteins identified in the WP fraction were beta-lactoglobulin (β-Lg), alpha-lactalbumin (α-La), serum albumin (SA), lysozyme (Lz), immunoglobulins (Ig_s), lactoferrin (Lf) and lactoperoxidase (LP). The LWP fraction, containing only β-Lg, α-La and Lz, was free of any high molecular weight protein, whereas traces of whey proteins were found in the casein fraction.

All donkey milk protein fractions showed significant inhibition of virus replication. The WP fraction showed the highest significant inhibition on virus replication at 10 and 5 mg mL^{-1}, when compared with LWP, while there was no significant difference among the WP, LWP and CN at the lowest concentration (Fig. 2). The 1 mg mL^{-1} concentration is the only report for the CN fraction because in our experimental condition ACTB amplification was strongly inhibited when caseins were used at the higher concentrations (5 and 10 mg mL^{-1} – data not shown), suggesting an adverse effect of this fraction, at these concentrations, on the Caco-2 cell line used for growing echovirus.

Donkey milk is particularly rich in two whey proteins, Lf (more than 4% of the whey protein fraction) and Lz (about 21% of the whey protein fraction) having molecular masses of approximately 75 kDa (Salimei et al., 2004) and 14.6 kDa (Vincenzetti et al., 2008), respectively. In our study the LWP fraction, that contains β-Lg, α-La

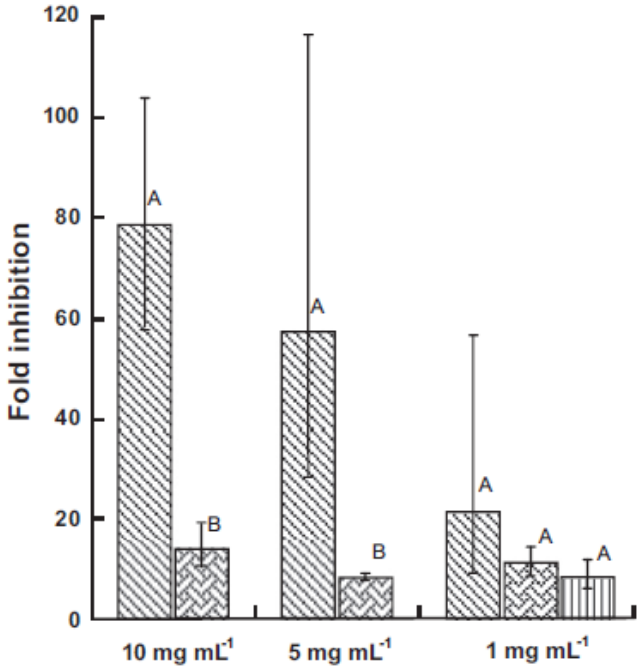

Fig. 2. Fold-inhibition of echovirus type 5 replication. Antiviral effect of the main donkey milk protein fractions: whey proteins (▨), low molecular mass whey proteins (▨) and caseins (▥) at different concentrations on replication of echovirus 5. Error bars represent the standard deviation in fold inhibition from triplicates. For each protein concentration analysed separately (10, 5 and 1 mg mL^{-1}), fractions with significantly different level in viral inhibition are assigned different letters ($P < 0.05$).

and Lz, showed a significantly lower inhibitory effect in comparison with WP at higher concentrations (5 and 10 mg mL^{-1}), suggesting that the main antiviral activity on echovirus type 5 in donkey milk may be attributed to high molecular mass proteins, such as Lf, LP, SA and Ig_s. However, since LWP also showed significant inhibitory effect on echovirus type 5, these high molecular mass proteins are probably not the only proteins responsible for the antiviral activity observed. There might be an additional role of the proteins of the LWP fraction as well, even if only β-Lg has previously been shown to have antiviral activity, inhibiting the replication of rotavirus (Superti, Ammendolia, Valenti, & Seganti, 1997).

Antiviral activity of bovine Lf against gastro-intestinal infectious agents as rotavirus and echovirus has been previously reported (Lin, Chu, & Chiu, 2002; Van der Strate, Beljaars, Molema, Harmsen, & Meijer, 2001). However, synergism with other whey proteins has been also proposed (Joslin et al., 2002). Other antiviral milk proteins are lactadherin, a mucin-associated glycoprotein (46 kDa) and lactoperoxidase, a whey protein (78 kDa) (Seifu, Buys, & Donkin, 2005; Yolken et al., 1992).

Finally, even though some traces of whey proteins could still be found in casein fraction, and these proteins might have been involved in the observed antiviral effect of CN, the fold inhibition difference between CN and WP is less than expected if the traces of Lf only would account for replication inhibition.

4. Conclusion

Donkey milk proteins exhibit antiviral activity on echovirus 5. Maximum inhibitory effect was shown when all whey proteins were present as compared to the fractions containing only low molecular weight whey proteins (β-Lg and α-La). This could, however, be due to a synergic action of the diverse proteins in the whey. The antiviral activity on echovirus 5 exerted by donkey milk proteins might contribute to prevent gastrointestinal virus infection in humans.

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Chapter 5

A preliminary study on potential anti-proliferative effect of donkey milk on SK-N-BE cells line.

This work has been carried out in collaboration with prof. Salvatore Saccone, Claudia Leotta (PhD student), Roberta Picciotto (PhD) and Concetta Federico (PhD), by the “Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Sezione di Biologia Animale", Università degli Studi di Catania, Italy.

Project's outlines

The aim of this work was to investigate the potential anti-proliferative effects of donkey milk on Human Neuroblastoma Cell Lines (SK-N-BE). Skimmed milk was tested on Human neuroblastoma cell line at three different concentrations (5.0, 1.0, 0.2 mg mL^{-1}) and the cell growth was daily monitored for eight days. Subsequently, casein and whey were tested to the same concentration and the cell growth was daily monitored every 24 hours.

Introduction

Malignant neoplasm has a multifactorial etiology with both genetic and environmental factors contributing to risk. The food is a possible factor that could either cause or help prevent cancer (Doll, 1996). Some molecules are able to act (directly or indirectly) on young tumour cells, inducing differentiation and leading to the young tumour cells to maturation (with normal physical functions) (Chen & Chang, 2004; Mao et al., 2009). Among these molecules, there are some proteins with indirect anti-tumour activity.

In the last decades milk has been the focus of interest of several studies because, besides to be rich in healthy compounds with physiological functions, including immuno-regulatory and anti-tumour activity (Ibrahim & Aoki, 2003), milk containing a number of components with anticarcinogenic potential (Parodi, 1997). In particular, its protein fraction has antioxidant activity due to the high level of serum albumin, alfa-lactoalbumin and lactoferrin, three proteins with a relative high content of cysteins. Proteins rich in cystein have antioxidant effect because they increase the glutathione concentration in relevant tissues, which acts to stimulate immunity and detoxify potential carcinogens. Glutathione is a tripeptide made up of cystein, glycine and glutamate, with potent intracellular antioxidant activity. It plays a central role in the body's defence against free radicals and carcinogens (Bounous, 2000).

In addition to this knowledge, several studies have indicated that lactoferrin possesses effective angiogenic inhibition and anti-tumoural activities that inhibit the development and progression of malignant neoplasm (Wakabayashi, 2003; Ye et al.,2008). Mao et al. (2009) working with donkey milk found that it exhibited anti-tumour activities against human adenocarcinoma cells (A 549) in a dose-dependent and time-dependent manner. Moreover, some of donkey whey protein fractions could stimulated.

the production of IL-2, IFN-g, IL-6, TNF-a and IL-1b cytokines (interleukins, lymphokines and cell signal molecules, which are protein molecules secreted by cell of immune system). Their results indicated a potent cytotoxicity which caused cell death by apoptosis (Mao et al., 2009).

Nevertheless donkey milk is scarcely investigated so far. In order to evaluate the potential anti-proliferative effects of donkey milk, we tested the sample on Human Neuroblastoma Cell Line (SK-N-BE). SK-N-BE is a cell line become available in culture since 1972, from a bone marrow biopsy of a child of only 2 years old, affected by neuroblastoma (Biedler et al., 1978). Neuroblastoma is a malignant extra cranial solid tumor in children, and represent the first cause of death by disease in childhood. This cell line, used in studies relating to tumour cell growth and neuronal cell biology, have different morphology, can form aggregates and to replicate in suspension. Moreover, SK-N-BE can be stored at -80°C for long time.

5.1 Materials and methods

Sample preparation

The work was conducted on bulk milk from thirteen Ragusano donkeys, bred in a specialized farm in Sicily. Milk was kept in an ice box during transportation for 40 minutes and processed immediately after arrival. Skimmed donkey milk (SM), was obtained through centrifugation at 3.500 x g, for 15', at 4°C and manual removal of fat layer. Caseins (CN) and whey protein fraction (WP) were obtained from SM by acid precipitation with HCl 1M and, successively, centrifuged at

3.000 x g for 20 minutes to 4°C. Then, the samples were frozen at -80°C and freeze-dried.

Cell culture

Human Neuroblastoma Cell Line (SK-N-BE) was grown at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI Medium supplemented with 10% foetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S), in a 75 cm^2 flask until confluence, then split and seeded in a 24-wells culture plate.

Anti-proliferative assay of skimmed donkey milk (a) and its protein fractions (b)

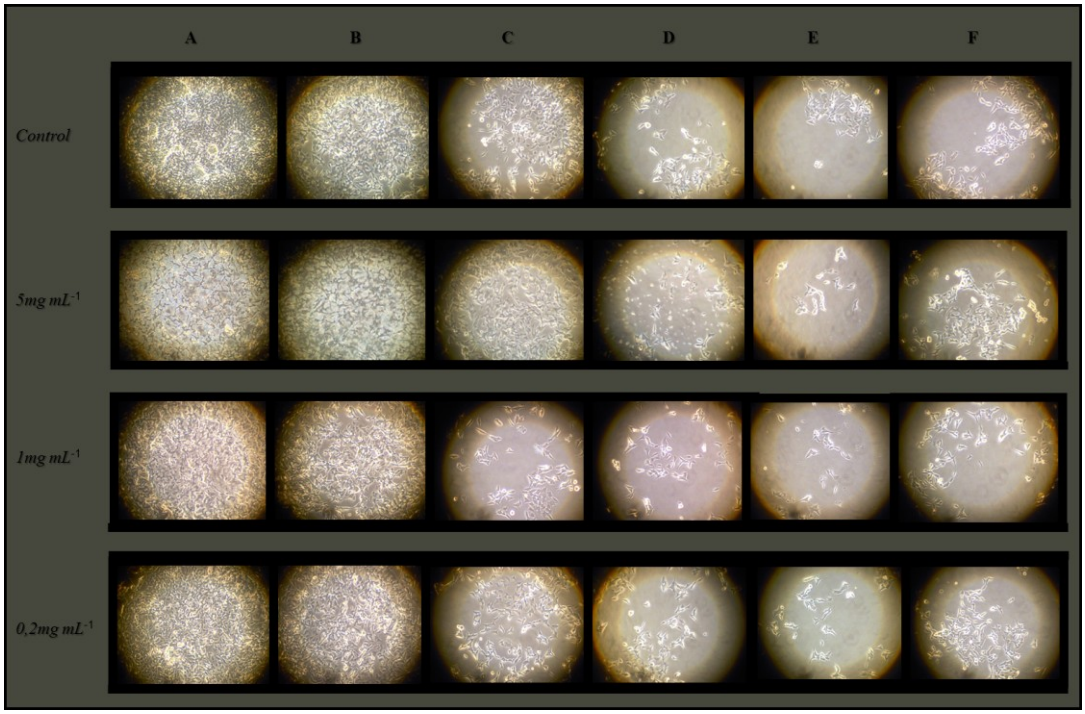
(a) In a first experiment SK-N-BE cells were seeded in 24-wells culture plate at a progressive density ($75x10^3$; $37.5x10^3$; $18.5x10^3$; $9.3x10^3$; $4.52x10^3$; $2.3x10^3$) per well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. SM was dissolved in RPMI Medium, supplemented with 10% FBS and 1% P/S, and added to the cells to obtain a final concentration of 5.0, 1.0, and 0.2 mg mL^{-1}. Negative controls were also included for each cell density. The cells were incubated at 37°C in a 5% CO_2 and the growth was daily monitored for a total of eight days (192h).

(b) In a second experiment SK-N-BE cells were seeded in 24-wells culture plate at a progressive density (*18.5*$x10^3$; $9.3x10^3$; $4.52x10^3$) per well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. CN and WP were dissolved i_n RPMI Medium supplemented with 10% foetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S), and added to the cells to obtain a final concentration of 5.0, 1.0, and 0.2 mg mL^{-1}. Negative controls were also included for each cell density. The cells were incubated at 37°C in a 5% CO_2 and the growth was daily monitored for a total of six days.

5.3 Results *of anti-proliferative assay of skimmed donkey milk (a) and its protein fractions (b)*

(a) After eight days (192 h) no difference was observed in the cell cultures treated with skimmed milk respect to the controls (figure 1). SM did not alter the normal growth of SK-N-BE cells at any different concentrations (5.0, 1.0, and 0.2 mg mL^{-1}).

Figure 1 - Progressive cell density (A, B, C, D, E, F) incubate with skimmed milk at 5.0, 1.0, and 0.2 mg mL^{-1}, after 192 h.



(b) A different result was obtained using DM protein fractions. SK-N-BE cells incubated with the highest WP concentration (5.0 mg mL^{-1}) died within the first 24 h. On the contrary, after six days (144 h) the treated cells with casein and lower whey protein concentration (1.0 and 0.2 mg mL^{-1}) did not show any inhibition on growth.

5.3 Discussion

Donkey milk is known for its peculiar chemical composition and functional properties. It is appreciated and used both in human nutrition and in the industrial preparation of cosmetics. In particular, its high content in lactoferrin, known to have anti-tumoral activities (Wakabayashi et al., 2008) in bovine and caprine milk seems very promising. In a previous study DM and its whey proteins were able to kill the cells by apoptosys (Mao et al., 2009).

In our trial whey protein fraction at the highest tested concentration 5.0 mg mL^{-1}, killed the Human Neuroblastoma Cell Line after 24 h. On the contrary, skimmed donkey milk and casein at serial concentrations did not inhibit SK-N-BE cells cell replication. However it is noteworthy that donkey milk is a good neutral growth medium (pH 7.2), in which the presence of lactose, mineral and vitamins could enhance cells growth. Moreover, skimmed donkey milk contains a low amount of that fraction of whey protein which had showed the anti-proliferative effects in the work carried out by Mao et al., (2009). These reasons could explained the different results obtained from skimmed milk and whey protein fraction.

Although our findings on the effect of the whey protein fraction on Human Neuroblastoma Cell Line need more thorough analysis before we can draw any definite conclusions, this preliminary investigation could represent a starting point for further analysis aimed to identify the role of total whey proteins and of each separate whey donkey milk protein in the observed antiproliferative effect on SK-N-BE cells. A potential strategies could be to use sub-fractions of the whey proteins with high (lactoferrin, immunoglobulin) and low (lysozyme and α - lactoalbumin) molecular weight.

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Chapter 6

Separation of donkey's caseins by cation exchange chromatography: preliminary results

The experimental data shown in this work have been obtained in collaboration with prof. Gerd E. Vegarud, Tove Gulbrandsen (PhD) and Irene Comi (engineer) by the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Science, UMB, Norway (October 2011-March 2012).

Project's outlines

Aim of the work was to obtain protein standards from each of the different donkey's caseins. The whole casein fraction was collected, by isoelectric precipitation, from a single donkey's milk sample. The separation was carried out by means of ion exchange chromatography and two different approaches were followed. The qualitative approach was aimed to find the correct run conditions to separate caseins. Preparative runs were carried out in order to collect the different separated protein peaks. Chromatography collections were then submitted to mass spectrometry analysis in order to identify the separated proteins and measure their purity. The final goal of the project was to produce casein standards to be used in quantification analysis.

Introduction

6.1.Chromatographic analytical method - cation exchange chromatography

Chromatography is a set of general technique with which is possible to separate substances present in a mixtures. It separates the analyte (substance to be separated) by passing the mixture dissolved in a "mobile phase" (eluate) through a stationary phase (immobilized on the support particles or on the inner wall of the column tubing). Therefore, to obtain a good analytical separation is important the appropriate combination between mobile and stationary phase and the optimum physical properties of the column.

Liquid chromatography (LC) is a separation technique in which the mobile phase is liquid. LC can be carried out in a column, with a distribution system (small packing particles) in which one phase is held stationary and another, immiscible phase, allowed to pass through, or by it at a relatively high pressure (high performance liquid chromatography - HPLC).

HPLC can be used for the analysis of organic molecules and ions. It is based on different mechanisms including adsorption, size exclusion and ion exchange.

Depending on the analysis, there are many types of stationary phases: ion-exchange chromatography, in which the separation is based on competition between the ions to be separated and those in the mobile phase, implies resins or polymers with acid or basic groups as stationary phases. The choice of mobile phases is based on the physicochemical properties of the analyte. Ion exchange chromatography (IEC) allow the separation of molecules or groups of molecules such as proteins, which have only slight differences in charge. It relies on charge-charge interactions between the proteinsof the sample and the charges immobilized on the resin present in the column (with opposite charge with respect to the analyte).. Depending on the charge of stationary phase (negative or positive), IEC is named cation exchange chromatography (CEC) or anion exchange chromatography (AEC), respectively. The pH interval in which ion exchange chromatography is carried out is restricted by the pH range in which the protein is stable. Generally, cation exchange chromatography is carried out below the isoelectric point. The isoelectric point of a protein (the pH at which the net charge is zero) depends on the proportions of ionizable amino acid residues in its structure.

Proteins are ampholytes with positive and negative charges. The net surface charge of a protein is highly pH dependent and will change gradually as the pH of the environment changes. Each protein has its own unique net charge versus pH relationship, thus CEC can be repeated at different pH values to separate several proteins which have distinctly different charge properties.

Besides the correct charge of the immobilized phase on the resin present in the column and the best pH for the separation, cation exchange chromatography needs two buffers: the first, starting buffer or sample buffer (buffer A), have a low ionic strength, the latter, elution buffer (buffer B) elutes off the bound molecules by increasing the ionic strength of the eluent solution. Elution can be performed either by a continuous or a stepwise gradient that increases in ionic strength, commonly using NaCl.

6. 2 Mass spectrometry analysis

After separation, generally the peaks are collected and analysed by mass spectrometry (MS), a powerful analytical tool used for measuring the mass-charge ratio of charged particles. The analysis of the whole or digested protein consists of fragmentation, ionisation, extraction and separation of the ions according to their mass-to-charge ratios (m/z). Finally, the separated ions are detected. The structural information is extrapolated through multiple analysers as well as tandem mass spectrometers (MS/MS). The tandem spectrum is converted into peak-list text files. The files are used to browse into database (e.g. NCBI and Swiss-Prot) using tools such as "tool MS/MS ion search" of Mascot (Matrixscience, UK). The score of identification is estimated by Molecular Weight Search (MOWSE) system (inclused in Mascot program).

In figure 1 the different steps of the project are outlined.

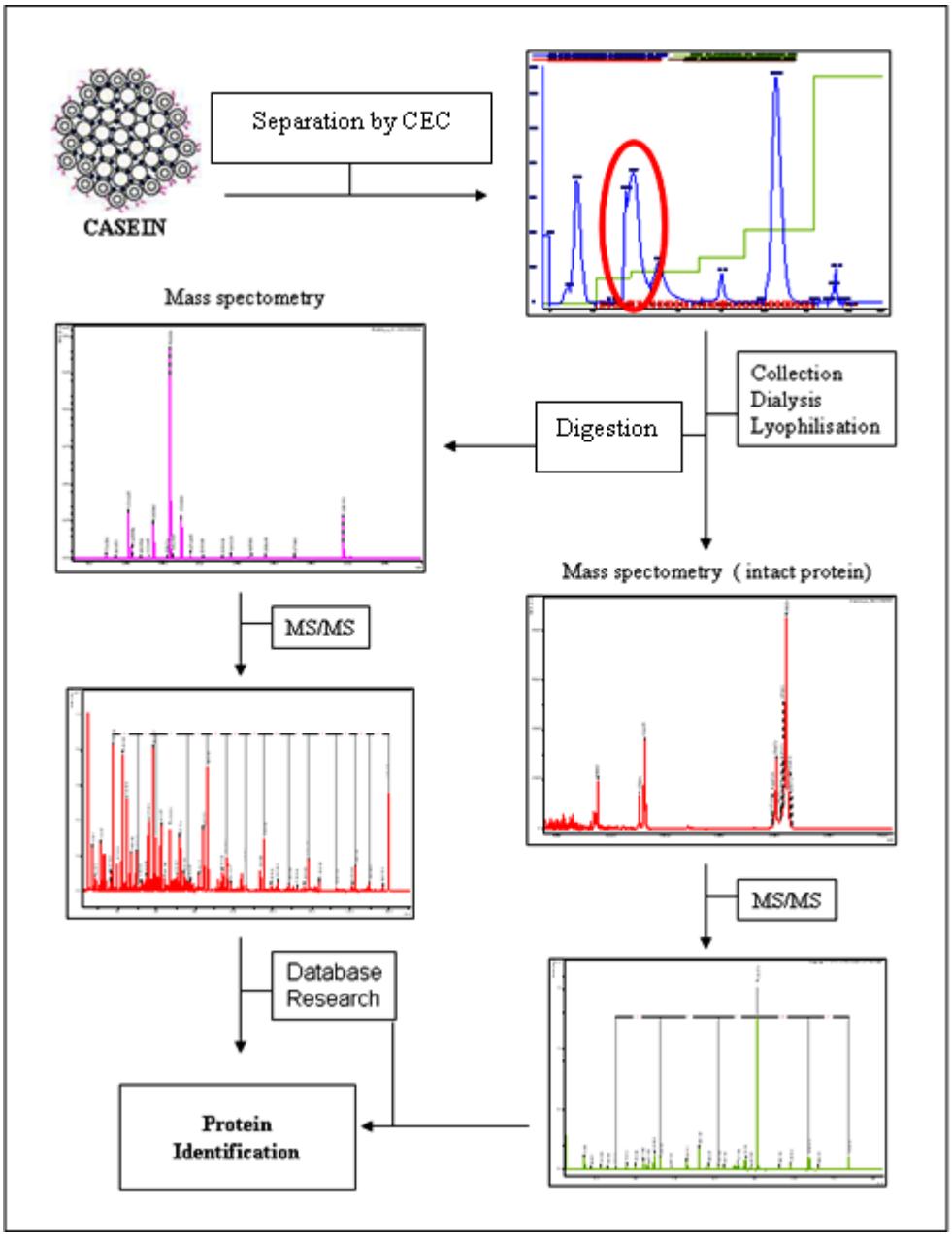

Figure 1 Scheme of the separation survey on the donkey's casein: from whole acid casein to identification by MS and MS/MS, through the separation by cation exchange chromatography (CEC).

6.3 Cation exchange chromatography in relation to casein

Casein (Cn), a family of phosphoproteins, is made up mainly of four proteins (α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein). Although different models have been proposed to describe the shape of the casein, generally it appears spherical, in the form of large colloidal particles, known as casein micelles (Phadungath, 2005).

The main studies available on casein are focused on ruminants (cow, sheep and goat) because of their worldwide distribution and because their milk or derived products are widely addressed to human diet. Recently, studies have been carried out on equidae milk proteins with the aim to characterize their molecular composition (primary structure, disulphide bridges and other post-translational modifications as phosphorylation and glycosylation), which can justify both their functional properties (solubility, clotting aptitude, thermal denaturation) and nutritional quality (amino acid composition, digestibility and bioactivity) (Chianese et al., 2010).

The most effective analytical procedures for structural analysis of proteins are based on chromatographic and electrophoretic techniques. During the analysis is important to consider the high heterogeneity of each single protein fraction that could compromise the purification of substances such as post-translational phenomena (phosphorylation, glycosylation), genetic polymorphism (one or two variants in individual samples, much more in bulk milk), non-allelic deleted forms and proteolysis by action of endogenous proteases (Chianese et al., 2010).

Donkey milk casein represents approximately 47% of the total protein in donkey. To date, four caseins (α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein) have been found in donkey milk by different analytical techniques such as one and two-dimensional electrophoresis followed by N-terminal sequencing, structural MS analysis (Bertino, 2010; Chianese et al., 2012; Vincenzetti et al., 2012; Criscione et al., 2009, Cunsolo et al., 2009) and purification by gel filtration chromatography followed by anionic and cation exchange chromatography (Vincenzetti et al., 2007).

6.4 Materials and methods

Sample collection

Milk was obtained from a single Ragusano donkey at middle lactation stage, bred in a specialized farm in Sicily (Asilat, Milo – CT). The sample was chosen on the base of the protein profile characterized by isoelectric focusing (IEF): according to Criscione et al. (2009), a donkey showing the most common milk protein profile (pattern A) was sampled. The milk sample was kept cool during transportation by an ice box and processed immediately after arrival at the laboratory.

Casein was obtained by acid precipitation with HCl 1M and centrifugation at 3.000xg for 20 minutes at 4°C. Successively, casein was rinse twice with distilled water, frozen at -80°C and freeze-dried.

Buffer composition and Sample preparation

In order to separate the different casein protein fractions (α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein), the whole casein from donkey milk was analysed by cation exchange chromatography. The buffers composition was chosen following the analytic method developed by Gomez-Ruiz et al., (2004) and the subsequent modifications (Criscione, personal communication), adjusting it on the base of the donkey casein properties.

Starting Buffer (Buffer A) and Eluent Buffer (Buffer B) were prepared dissolving in distilled water Urea (6M), Sodium Acetate (0.02M) and DL-dithiothreitol (DTT) (64 micro M). In addition, Buffer B was added with Sodium Chlorate (1M).

The lyophilized casein (10 mg mL^{-1}) was dissolved in Buffer A and 24 uL/mL of mercaptoethanol (used to reduce disulfide) were added. To aid the action of mercaptoethanol, the pH was adjusted to 7.2 while stirring (for 1h) and finally lowered to the working value (pH 3.65, 4.00 or 5.50).

The separation was carried out following two different approaches. Qualitative separations on a small bed-volume column (1ml bed volume) were set up in order to find the run conditions by means of which the analyzed protein showed separation, while, a preparative method (53ml bed volume) was implemented to collect the separated casein fractions for the purpose of produce casein standards to be used in quantification analysis. In the former, sample solution, Buffer A and Buffer B were adjusted at three different pHs: 3.65, 4.00 and 5.50. In the latter, the sample and the same buffers were adjusted pH 5.50.

Cation exchange chromatography - Qualitative approach

A qualitative approach was employed in order to find the best run conditions to separate the casein fractions and apply it to preparative runs (to collect the casein fractions), so limiting the time and the use of sample and chemicals.

Sample casein solution (500uL) was manually injected by syringe, into a HiTrap SP FF-1 ml column (size: 0.7 x 2.5 cm) connected to an HPLC system (Äkta purifier, GE-Healthcare with UNICORN software, see figure 2).

Figure 2. ÄKTA - High Performance Liquid Chromatography System

Initially, the column was equilibrated with Buffer A at different pH values for three corresponding separation runs (pH 3.65, 4.00 and 5.50). For the three different working pH, a linear gradient, from 0% to 40% in 30 Column Volume (CV), was applied. The flow rate was 1 mL min^{-1}. The absorbance was detected at 280 nm, the optical peak due to the effect of aromatic rings in the polypeptide chain from amino acids like tryptophan and tyrosine. Since the resolution was not high, in order to improve the separation and to elute the casein fractions a stepwise gradient at pH 5.5 was used: from 0% to 14% B, 14% B, from 14% B to 18%, 18% B, from 18% to 32% B, 32% B, from 32% to 40%B and 40%B. Each step consisted in 3 Column Volume (1 CV corresponded to 1 mL min^{-1}).

A collection procedure was set up within method, allowing to gather 1ml volume aliquots within and between peaks. The absorbance limit to start the collection was set to 25 mAU.

Aliquots were then pooled together into seven groups (see figure 4), which were dialysed, freeze dried and analyzed by Mass Spectometry (MS) and tandem mass spectrometers (MS/MS).

Cation exchange chromatography - Preparative method

In order to collect enough amount of the separated proteins, the whole casein was analysed by cation exchange chromatography on a HiPrep™ Q Sepharose™ Fast Flow 26/10 column (volumes of 53 ml). Initially, the same method set up during the qualitative analysis (stepwise gradient and pH 5.50) was applied, subsequently, modifications were performed to it in order to improve the separation. Sample casein solution (10 mL) was manually injected, by syringe, into the column connected to an HPLC system (Äkta purifier, GE-Healthcare with UNICORN software).

Initially, the column was equilibrated with Buffer A at pH 5.50 and the caseins were eluted with a stepwise gradient: from 0% to 11% B for 0 CV, 11% B for 2.5 CV, from 11% to 14% B for 0 CV, 14% B for 5% CV, from 14% to 20% B for 3 CV, 20% for 3 CV, from 20% to 32% for 0 CV and 32% B for 5 CV. The flow

rate was 5 mL min^{-1}. The absorbance was detected at 280 nm. Finally, a collection procedure was set up to gather 7 ml volume aliquots within and between peaks. The absorbance limit to start the collection was set to 50 mAU (logarithmic unit used to measure absorbance). Aliquots were then pooled together into four collecting samples, which were dialyzed, freeze dried and analyzed by Mass Spectometry (MS) and tandem mass spectrometry (MS/MS).

Mass Spectometry (MS) and tandem mass spectrometry (MS/MS) analysis

The collections were attended by dialysis (Spectra/Por® dialysis membrane 1-3, MWCO 12,000- 14,000 Da cut off -) and freeze-drying. Analytes powder was then submitted to MS and MS/MS analysis (whole and digested protein). The protein fractions were dissolved in 50mM (NH4)HCO3 and subjected to a rapid trypsin digestion ("digest-in-tip", Poroszyme), mixed with matrix solution (15 mg/ml alpha-cyano-4-OH cinnamic acid) and spotted on a ground steel MALDI target. Intact and trypsin-digested proteins respectively, were then analyzed in the Ultraflex MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) positive ion reflectron mode. The most intense ions from each MS spectrum were selected for MS/MS analysis (LIFT) and these spectra were used to search the NCBInr database, taxonomy Mammals, using Mascot (v2.1). Spectra giving significant ($p<0.05$) hits were reported. A fraction of the good quality spectra, which did not give significant hits, were manually interpreted and the resulting y-ion series were used in Protein BLAST searches against the NCBInr (Mammals) database. Matches to caseins were reported if the hits were among the top two in the search result list.

6.6 Results

First approach - Qualitative analysis

When a linear gradient (0%-40% in 30 CV) was applied to elute proteins from HiTrap Sepharose (SP FF - 1 ml), equilibrated at the different pH values of 3.65, 4.00 and 5.50, two main protein peaks (A and B) where highlighted (see figure 3

a, b and c): the resolution of the separation appeared low, being a wide area of the peaks in common, this indicating a poor separation.

Separation carried out at pH 5.50, showing a considerable anticipation of protein elution, was chosen as starting point to set up a method using a stepwise gradient (previously detailed). The method was set up by monitoring several chromatographic runs in terms of absorbance (280nm) and peaks area. Two distinct main peaks (C and D) were observed (see figure 3d). The method was implemented with a collection sequence which allowed to gather 1ml volume aliquots within and between peaks. Collected aliquots of the chromatographic separation were assembled in seven different fractions (see figure xx).

The MS and MS/MS analysis applied on the seven collected fractions, identified:

- αs_2-casein B precursor (*Equus asinus*) and similar to osteopontin isoform 1 (*Equus caballus*) in the I and II fraction, which corresponded to the unbound;
- β-casein in the III fraction (peak C);
- αs_2-casein (Equus asinus) and k-casein (Equus caballus) in the IV fraction;
- αs_1-casein precursor in the VI fraction (peak D).

The V and VII fraction did not content any casein.

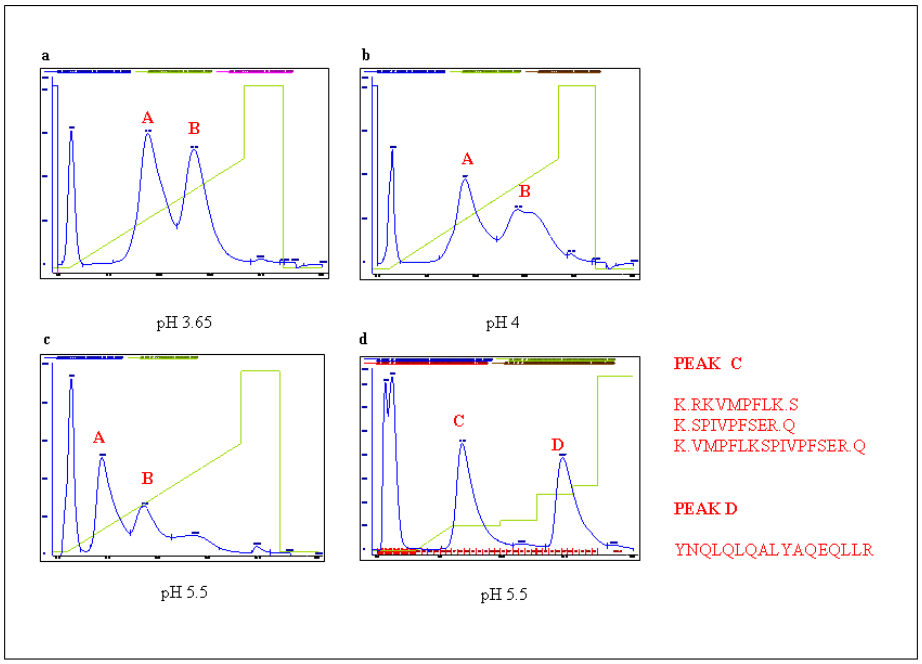

Figure 3 . Cation exchange analysis (qualitative) carried out by means of Äkta purifier equipped with a HiTrap SP FF-1 ml column: a) separation at pH 3.65 and linear gradient; b) separation at pH 4.00 and linear gradient; c) separation at pH 5.50 and linear gradient; d) separation at pH 5.50 and stepwise gradient.

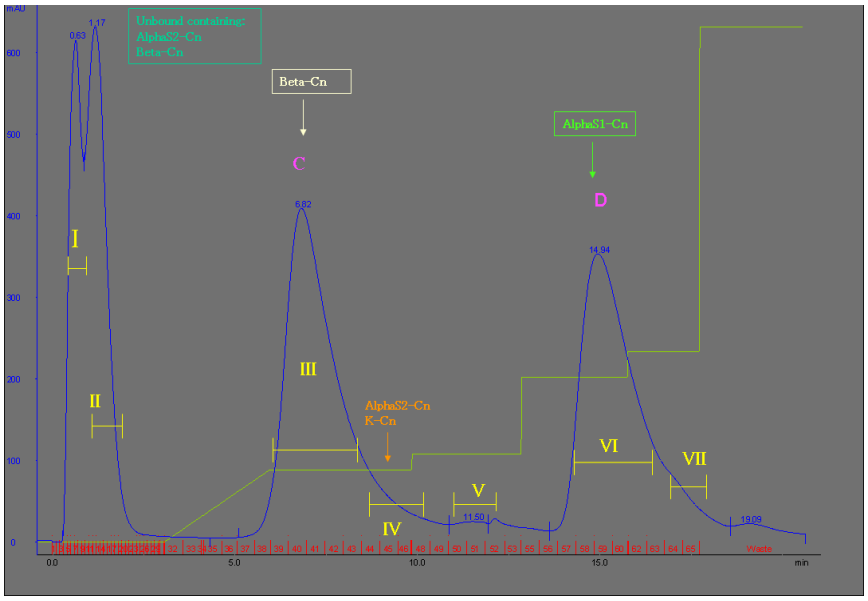

Figure 4. – Cation exchange chromatogram (Äkta purifier equipped with a HiTrap SP FF-1 ml column) of the qualitative separation at pH 5.50 and stepwise gradient. The x-and y-axis represent the elution time (minutes) and the mAU (logarithmic unit used to measure absorbance) respectively, where the area of each peak is proportional to the quantity of

the eluted substance. The green line indicates the trend of the applied stepwise gradient with which the elution was obtained. The red numbers on x-axis indicate the collected aliquots which were pooled in seven fractions (Roman numerals) then submitted to MS and MS/MS analysis.

Second approach - Preparative method

The method implemented in qualitative analysis, using a stepwise gradient (pH 5.50), was subsequently adapted to a preparative column (HiPrep™ Q Sepharose™ Fast Flow 26/10) with the aim of collect enough amount of proteins to use in quantification analysis.

The column was equilibrated at pH 5.50. The method was set up by monitoring several chromatographic runs in terms of absorbance (280nm) and peaks area. The resolution of the separation appeared clear, four main peaks (E, F, G and H) were highlighted, although the first two had an area in common (see figure 5). The method was implemented with a collection sequence which allowed to gather 7 ml volume aliquots within and between peaks. Collected aliquots of the chromatographic separation were assembled in four different fractions (see figure 5).

The MS and MS/MS analysis applied on the collected fractions, identified:

- β-casein (Equus asinus) in the E and F fraction;
- αs1-casein, αs2-casein and k-casein in G fraction;
- αs1-casein and αs2-casein in the H fraction.

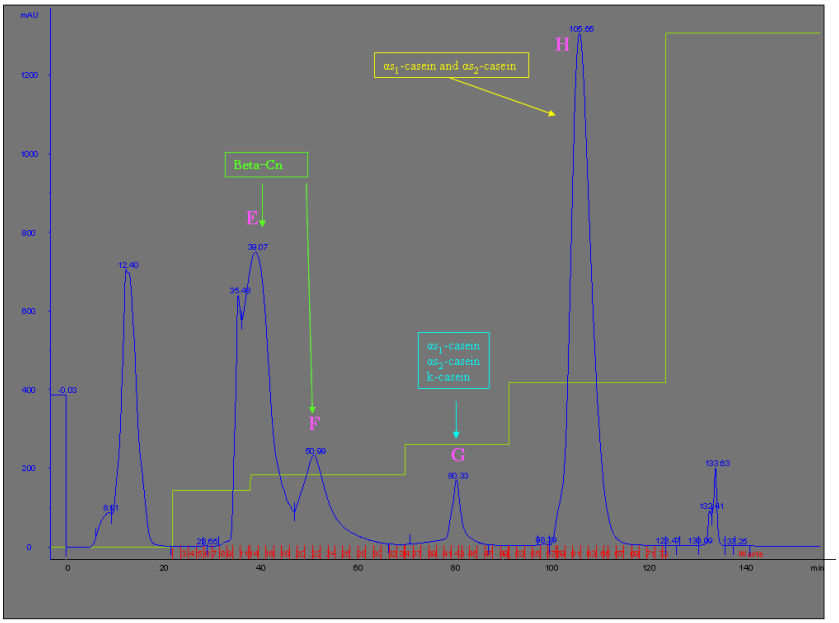

Figure 5. – Cation exchange chromatogram (Äkta purifier equipped with a HiPrep™ Q Sepharose™ Fast Flow 53ml) of the preparative separation at pH 5.50 and stepwise gradient. The x-and y-axis represent the elution time (minutes) and the mAU (logarithmic unit used to measure absorbance) respectively, where the area of each peak is proportional to the quantity of the eluted substance. The green line indicates the trend of the applied stepwise gradient with which the elution was obtained. The red numbers on x-axis indicate the collected aliquots which were pooled in four fractions (E, F, G and H) then submitted to MS and MS/MS analysis.

6.7 Discussion and future prospective

The present outcomes represent a preliminary summary of an ongoing project aimed at identifying and quantify the different protein components of donkey milk. In general, the structure of the project involves the use of chromatographic techniques coupled with spectrometric analysis with the aim of separating and identifying protein components of casein and whey protein fractions. Protein constituents separated and collected using gram scale protocols will be implemented in quantification analysis of individual milk protein profiles by means of capillary electrophoresis technique. The proteomic approach will be accomplished alongside the genomic study of the milk protein genes. Individuals will be characterized at DNA and phenotypic level, in order to identify the

correspondences between protein profiles and the genetic asset with the final goal of setting up a throughput method for rapid qualitative and quantitative analysis.

Ion exchange chromatography is an effective chromatographic technique implemented in the separation rather than the purification of molecules which differ even by small charge. It is widely applied in the research focused on protein separation with analytical as well as preparative purpose (Gomez-Ruiz et al., 2004; Plank et al., 2008). To our knowledge no research surveys dealt with this technique regarding donkey's milk proteins, but for a recent paper by Vincenzetti et al., (2012) who analyzed donkey's milk casein by cation exchange chromatography and identified β- and α_{s1}-casein.

Several runs were carried out on HiTrap Sepharose (SP FF) in order to set up a useful protocol to separate caseins from donkey's milk. The use of a small bed-volume analytical column allowed to vary the separation conditions quickly and limit the use of sample and chemicals. The results of the qualitative approach were not conclusive in terms of clean separation of all caseins, as highlighted by the MS MS/MS tools; notwithstanding, these outcomes represented a starting point to establish a protocol for a gram scale separation of donkey's caseins. Following adaptations of the separation method to the preparative column (HiPrep™ Q Sepharose™ Fast Flow 26/10) also implied several runs to find the best analytical condition, even though a complete separation of all the caseins was not reached.

Although the ion exchange chromatography approach need to be further developed, the actual preliminary results appear promising. Three different fractions were obtained by eluting the whole casein from cation exchanger column. In addition to the separation of the pure β-casein (F1), which clearly eluted at the beginning of the gradient, two additional protein group were obtained, one containing α_s-caseins (F2) and the other showing κ-casein as well as α_s-caseins (F3). Today, in view of establish a preparative protocol aimed at extracting protein standards, we can consider only F1 and F2 useful separations

thanks to their relative amount while F3, though analytically interesting, represents a poor result from a quantitative point of view.

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Abbreviations

AU	absorbance unit
AAF)	amino acid formulas
αs_1-Cn	$Alpha_{s1}$-casein
αs_2-Cn	$Alpha_{s1}$-casein
α-la	Alpha-lactalbumin
ANOVA	Analysis of variance
β-Cn	Beta-casein
β-lg	Beta-lactoglobulin
bLf	Bovine lactoferrin
CaCo-2	Human colon adenocarcinomacell line
CM	Cow milk
CMP	Cow milk protein
CMPA	Cow milk proteins allergy
CN	Casein
CPE	Cytopathic effect
Da	Dalton
DM	Donkey milk

eHF	extensively hydrolysed formulas
FAO	Food and Agriculture Organization
HAMLET	Human Alpha-lactalbumin Made LEthal to Tumor cells
HDJ	Human duodenal juice
HPLC	High performance liquid chromatography
HM	Human milk
kDa	Kilo Dalton
k-Cn	Kappa-casein
IDF	International Dairy Federation
IL	Interleukins
Ig	Immunoglobulin
IS	Immune system
LC	Liquid chromatography
Lf	Lactoferrin
Lfcin	Lactoferricin
LP	lactoperoxidase
Lyz	Lysozime
MALDI	Matrix-assisted laser desorption/ionization
mAU	Mill- absorbance unit

MOI	Multiplicity of infection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
$NaCHO_3$	Sodium bicarbonate
NO	Nitric oxide
PUFA	Poly-Unsaturated Fatty Acid
RT-PCR	Reverse transcription polymerase chain reaction
SA	Serum albumin
SDS- PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	Skimmed milk
SF	Soy formulas
TCID	Tissue culture infective dose
WHO	World Health Organization
WPF	Whey protein fraction