University of Foggia

Department of the Sciences of Agriculture, Food and Environment (SAFE)

PhD Course on Management of Innovation in the Agricultural and Food Systems of the Mediterranean Region (XXXI Cycle)

Milk and human health: relationship between proteic and lipidic compounds of milk from different species and human nutrition

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SESSION 2018-2019

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Abbreviations

AA: aminoacids
ACE: angiotensin converting enzyme
ALA: α-linolenic acid
BAMLET: bovine α-lactalbumin made lethal to tumor cells
BAT: brown adipose tissue
BBB: blood-brain barrier
BM: bovine
BRCA1: breast cancer type 1 susceptibility protein
BSA: bovine serum albumin
BSSL: bile salt simulated lipase
Ca: calcium
CLA: conjugated linoleic acids
CM: caprine milk
CN: caseins
CNS: central nervous system
CPPs: casein phosphopeptides
CSF: blood-cerebrospinal fluid
CVD: cardio vascular diseases
DHA: docosahexaenoic acid
DM: donkey milk
EFA: essential fatty acid
EPA: eicosapentaenoic acid
FA: fatty acid
FABP: fatty acid binding protein
FAME: fatty acids methyl ester
FAS: fatty acid synthase
FBS: fetal bovine serum
FFA: free fatty acids
FM: formula milk
GAPDH: gliceraldeide-3-fosfato deidrogenasi
GF: gastric fluid
GndHCl: guanidine hydrochloride
HAMLET: human α-lactalbumin made lethal to tumor cells
HL-EC: high level-epileptic children
HM: human milk
HMGB1: high mobility group box 1 protein
IF: intestinal fluid
Ig: immunoglobulins
IGF: insulin growth factor
IL: interleukin
k-CN: k casein
K: potassium

LA: linoleic acid
LAB: lactic acid bacteria
LC-FA: long chain fatty acids
LC-FFA: long chain free fatty acids
LCFA: long chain fatty acids
LDL: low-density proteins
LL-EC: low level-epileptic children
LPL: lypoproteinlipase
LPS: lipopolysacarid
MC-FA: medium chain fatty acids
MC-FFA: medium chain free fatty acids
MCFA: medium chain fatty acids
MFGM: milk fat globule mambrane
ML-EC: medium level-epileptic children
MPB: milk basic protein
MUFA: monounsaturated fatty acids
NF-kB p65: nuclear factor kappa-light-chain-enhancer of activated B cells
OM: ovine milk
P: phosphorus
PBMC: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PCR: polymerase Chain Reaction
PHA: phytohemagglutinin
PPARγ: peroxisome proliferator-activated receptors
PUFA: polyunsaturated fatty acids
RNS: reactive nitrogen species
ROS: reactive oxygen species
RP-HPLC: reversed-phase HPLC
SC-FA: short chain fatty acids
SDS-PAGE: sodium dodecil solphate polyacrilamyd gel electrophoresis
SF: simulated salivary fluid
SFA: saturated fatty acids
SPE: solid phase extraction
SREBP-1c: sterol regulatory element-binding proteins
TAG: triacylglycerols
TAME: p-toluene-sulfonyl-L-arginine methyl ester
TCA: tricholoroacetic acid
TFA: trifluoroacetic acid
TNF: tumor necrosis factor
U: units
UCP-1:decoupling protein-1
VA: vaccenic acid
WAT: white adipose tissue
α-LA: α-lactalbumin

α_{s1}-CN: α_{s1}-casein
α_{s2}-CN: α_{s2}-casein
β-CN: β casein
β-LG: β-lactoglobulin
γ-CN: γ casein

Abstract

The Mediterranean Region, even if from a pedoclimatic point of view presents some unfavorable conditions for intensive agro-zootechnical management, it has a potential must to be expressed. It is meaning to focus on the enhancement of animal and vegetable productions of the Mediterranean, both through the increase of knowledge about the production reality of agro-food systems, but above all through a nutritional and nutraceutical characterization of its typical products. In this standpoint, the research attempts to increase the level of innovation and quality of the food chain.

The purpose of the study of this Ph.D. was to bring out the potential positive effect of milk on human health; in detail this was lead, by assessing the existence of a correlation between the proteic and lipidic components of milk from different animal species, paying particular attention to typical species of the Mediterranean Region, and specific *in vitro* cellular systems, able to simulate the biological system *in vivo*. At this regard, different trials have been developed with multiple objectives. Some symptomatic responses that do not seem to have a univocal triggering causes are often related to food intake. The role of bovine, ovine, and caprine milk, caseins, whey proteins and specific milk protein fractions (α_{s1}-CN, α_{s2}- CN, k-CN, β-CN, and a mix of α-LA and β-LG), was studied in an *ex vivo* trial on PBMC cellular system from children with generalized epilepsy, evaluating pro- and anti-inflammatory cytokines and oxidative status. Gastrointestinal digestion profoundly modifies food composition. To better understand the fate of the main nutritional compounds following digestion, a simulation model of gastrointestinal digestion *in vitro* was carried out on human, bovine, ovine, caprine and donkey milk and formula milk, paying particular attention to the study of fatty acids profile modifications.

Finally, to evaluate the correlation between childhood obesity and milk intake, an *in vitro* trial using a cellular system of mature adipocytes 3T3-L1, was conducted. Mature adipocytes are treated with digested milk from different species (human, bovine, ovine, caprine and donkey) and a formula milk, to evaluate the viability,

the apoptotic response, the oxidative and inflammatory response and gene expression levels.

Riassunto

La Regione Mediterranea, anche se da un punto di vista pedoclimatico presenta delle condizioni poco favorevoli ad una management agro-zootecnico di tipo intensivo, possiede delle potenzialità intrinseche tipiche del territorio che necessitano di essere espresse. E' quindi di fondamentale importanza puntare alla valorizzazione dei prodotti di origine animale e vegetale tipici del mediterraneo, sia attraverso l'accrescimento delle competenze nell'ambito della realtà produttiva dei sistemi agro-alimentari, ma soprattutto attraverso una caratterizzazione nutrizionale e nutraceutica di questi prodotti, nell'ottica di aumentare il livello di innovazione e di qualità della filiera e dei prodotti.

Lo scopo di questo percorso di dottorato è stato quello di far emergere le potenzialità salutistiche del latte, quale prodotto di origine animale, attraverso la valutazione dell'esistenza di una correlazione tra la componente proteica e lipidica del latte proveniente da differenti specie animali, con particolare attenzione rivolta alle specie strettamente legate alla Regione Mediterranea, e specifici sistemi cellulari *in vitro* atti a simulare gli analoghi sistemi fisiologici.

A tal fine sono state elaborate differenti prove sperimentali con molteplici obiettivi.

Alcune risposte sintomatiche espresse dal corpo umano che sembrano non essere riconducibili ad un'univoca causa scatenante, sono spesso correlate all'alimentazione. Alla luce di questo, è stato studiato il ruolo del latte, delle caseine, delle sieroproteine e di specifiche frazioni proteiche (α_{s1}-CN, α_{s2}- CN, k-CN, β-CN e un mix di α-LA e β-LG), provenienti da diverse specie animali (bovina, ovina e caprina), in un sistema cellulare *ex vivo* di PBMC derivanti da bambini con epilessia generalizzata; su questo sistema cellulare è stata valutata la risposta immunitaria a seguito del contatto con il latte e le sue frazioni attraverso, la quantificazione della produzione di citochine pro- e antinfiammatorie, e la valutazione dello stato ossidativo.

È noto che il processo di digestione gastrointestinale modifica profondamente la composizione degli alimenti che sono introdotti con la dieta. Per meglio

comprendere il destino dei principali composti nutrizionali contenuti negli alimenti a seguito della loro ingestione e digestione, è stato realizzato un processo di simulazione *in vitro* della digestione gastrointestinale del latte di diversa origine, umano, bovino, ovino, caprino, di asina e un latte in formula, prestando particolare attenzione allo studio delle modificazioni del profilo degli acidi grassi. Infine, per valutare una possibile correlazione tra l'obesità infantile e l'assunzione di latte, è stato condotto uno studio *in vitro* utilizzando adipociti maturi provenienti dalla linea cellulare di fibroblasti 3T3-L1, trattati con il latte digerito proveniente da diverse specie (umano, bovino, ovino, caprino e asino) e con un latte artificiale. A seguito del trattamento è stata valutata la risposta di citotossica, apoptotica, ossidativa, infiammatoria e l'espressione genica di specifiche proteine correlate all'obesità, espressa dalla suddetta linea adipocitaria.

1. GENERAL INTRODUCTION

1.1 Mediterranean Region and diet

Rural landscape of Mediterranean Region is profoundly marked by agro-forestry-pastoral activities, which have contributed to the creation of a mosaic of ecosystems where, small ruminant livestock show a considerable importance. The Mediterrean region is featured by different ecological sub-regions, landscapes and sceneries, ways of life, and traditions, and the single unifying characteristic is the climate, which can be summarised as the perfect union of sun and sea; the poor water availability and the innate characteristics of soil are not suitable for a profitable agriculture. Indeed, the coastal belt shows mostly very sparse grazing, rocky soils, and climatic situations too dry, too hot, or too cold; whereas, the inland is characterised by large continental plateaus with 1000 m of the altitudes. Despite these hard climatic and environmental conditions, some of the first successful agricultural civilisations developed here, and were originally based on the cultivation of cereals. It is generally recognized that, the dairy cow has historically been north-western Europe driving force, the pig that of central Europe and dairy sheep and goats are unquestionably the tipical animals farm of the Mediterranean region (Boyazoglu and Morand-Fehr, 2001).

Physical and human environment of the Mediterranean have conditioned the animal production, characterised by two main property: the close association between the animal factor and cereal production, using grazing in fallow land as a form of fertilization; the livestock management, generally related to natural resources available in different locales of the Mediterrean region, therefore in the northern part of the basin flocks of sheep and goats have traditionally transhumed from summer mountain grazings to the plains in winter. The livestock of small ruminants in the various Mediterranean regions has common characteristics such as low mechanization and therefore the high need for manpower, a close link with the territory and traditions, a positive image of nature, but also an income low when compared with other sectors of production. Furthermore, the link with the territory has led to a great differentiation of products and production systems (de Rancourt et al., 2006). Small ruminants have the ability to transform low quality

forage into products of a high feeding value (Lombardi, 2005) and for that reason these systems have traditionally been related to grazing. Small ruminant grazing systems, offer a number of environmental, sociological or nutritional advantages; they increase the usefulness of farmland unsuitable for cultivation such as the mountainous areas (Mena et al., 2005; Papachristoforou and Markou, 2006) or the semi-desert regions (Degen, 2007); they favour the conservation of the wide variety of vegetation resulting from the different environmental and finally in economically depressed areas, small ruminant farming also plays an important social role by placing the population and maintaining traditions and contributing considerably to multifunctionality (Calatrava and Sayadi, 2003). Furthermore, if they are managed appropriately, goat-grazing systems show a positive level of sustainability, higher than intensive systems (Nahed et al., 2006). Of course, the products obtained from grazing systems present certain features which differentiate them from other feeding systems, such as the high content of polyunsaturated fatty acids, vitamins, and volatile compounds (flavours and terpenes) which favour human nutrition and health (Morand-Fehr et al., 2007).

It is precisely in the Mediterranean region, that the homonymous alimentar regimen is born. The Mediterranean diet, represents the typical dietary pattern consumed by Mediterranean people, has been widely recognised to satisfy the roules of healthy eating and to be a model for a better quality of life and for improve a health status (www.healthierus.gov/dietaryguidelines - Access date: 10-11-2018). In the 1950s the Seven Countries Study, draw attention to which foods were frequently consumed in the Mediterranean area, paying attention about the typical pattern followed mainly by poor rural societies (Trichopoulou, 2004). Of course this mean that the Mediterranean diet is rich in vegetables food such as cereals, fruits, legumes, tree nuts and seeds, and olive oil which rapresents the main source of fat; it provides for a high to moderate intake of fish, a moderate consumption of eggs, poultry, milk and dairy products, a low consumption of red meat. The traditional Mediterrean diet pattern, was popularised by Willett et al. in 1995 by the world famous pyramid representation that graphically highlights the

regularity by which food groups shoud be consumed. Trichopoulou et al. (1995) defined an index to evaluate the grade of adherence to the Mediterrean diet pattern to study of real its associated health effects. Numerous epidemiological studies reportedt the ability of Mediterranean diet pattern to reduce the risk of developing the metabolic syndrome, type 2 diabetes, CVD and some neuro-degenerative diseases and some types of cancers (Serra-Majem et al., 2006; Meydani, 2005) and of human health benefits associated to consumption of products included in this dietary regimen.

In the Mediterrean diet milk and dairy products are well contextualised, and should be consumed moderately two times per day; in fact, although they are rich in Ca and this is important for bones and heart health, dairy products are also a source of saturated fatty acids, so is important to prefere low-fat dairy, such as skimmed milk, yoghurt, cheese and other fermented dairy products. Milk represents the first food for each newborn, able to satisfy by itself the nutritional needs. The various animal species contribute differently in milk world production; cow milk shows about 82.55%, buffalo milk about 13.90%, followed by goat milk with 1.90% and by sheep milk with 1.30%. The other animal species, contribute only for about the 0.3% (FAOSTAT, 2016). However, there is a growing interest for milk of minor species such as goat, sheep and donkey, for the bioactive components that have recently been recognized to such milk, with nutritional, cosmetic, immunological, anti-carcinogenic, antimicrobial and neurological functions (Chiofalo et al., 2004).

1.2 Milk composition

Milk is a complex fluid secreted by the mammary gland of mammals for the nutrition of their newborns. The general composition of milk, primarily depends by the requirements of newborn, and these differ from species to species. For this reason the animal species is the first source of differentiation. Also, milk composition is influenced by several factors grouped mainly in endogenous (genetic and physiological) such as species and breed, animal health status,

milking interval and lactation stage, and in exogenous (zootechnical) such as nutritional factors, feed energy value and composition, climate, breeding system and the type of milking practices. Apart from the nutritional properties, milk provide for different physiological functions such as immunitary one, whit secretion of immunoglobulins and other antibacterial agents, digestive function whit enzymes and binding or carrier protein production and growth function.

Milk gross composition is well characterised for human and for principal dairy species like cow, goat, sheep, buffalo and donkey. A general composition of milk from principal mammals animal species, as average value found in literature, is reported in Table 1 (adapted from Claeys et al., 2014).

Table 1. Gross composition of principal mammals species (adapted from Claeys et al., 2014).

Composition	**Milking species**				
	Human	Donkey	Bovine	Ovine	Caprine
Dry matter (g/L)	107-129	88-117	118-130	181-200	119-173
Protein (g/L)	9-19	14-20	30-39	45-70	30-52
Casein (g/L)	2.4-4.2	6.4-10.3	24.6-28	41.8-46	23.3-46.3
Whey protein (g/L)	6.2-8.3	4.9-8	5.5-7	10.2-11	3.7-7
Casein/whey ratio	0.4-0.5	1.28	4.7	3.1	3.5
Fat (g/L)	21-40	3-18	33-54	50-90	30-72
Lactose (g/L)	63-70	58-74	44-56	41-59	32-50
Ash (g/L)	2-3	3-5	7-8	8-10	7-9
Energy (kJ/L)	2843	1607-1803	2709-2843	4038-4439	2802-2894

Water is the main component in all milks but some differences emerge among the species, indeed dry matter is higher in ruminant milk compared with non-ruminant and human milk. The main carbohydrate is lactose, which provides a ready source

of energy for the neonate and is involved in the intestinal absorption of calcium, magnesium and phosphorus, and in the utilization of vitamin D (Park et al., 2007). Lactose concentration is similary higher in donkey and human milk, and lower in bovine or other ruminant milk (Table 1). In milk there are other carbohydrates that include a small fraction of oligosaccharides, which are free or bounded to lipids, proteins or phosphate and their level is much lower in animal milk compared to human milk (Caleys et al., 2014).

In general, often a distinction is made between "caseinic milk" and "albuminic milk"; the first is typical of ruminant species which produce a milk relatively rich in casein, the second is peculiar of non-ruminant species which make a milk proportionally higher in whey protein content and as a conseguence, with a lower casein/whey ratio (Table 1). Cow milk contains more protein and minerals, especially calcium and phosphorus, than human milk and shows a good balance of all the essential amino acids, including lysine; the protein in this milk is known as high-quality one and is able to supports maximal growth of young calf that grows faster than a child and hence has higher nutritive demands. Although cow milk contains more protein than does human milk, but human milk contains more lactose, resulting in comparable energy contents (FAO, 2013). Caseins comprise nearly 80 percent of the protein in cow milk but less than 40 percent in human milk. The type of caseins that predominate in the two milks also differs; human milk containing more β-casein, which is more susceptible to peptic hydrolysis than αs-casein, particularly α_{s1}-casein, which predominates in cow milk (El-Agamy, 2007).

The proximate composition of goat milk is very similar to cow milk. In contrast with cow milk, the lactose content of goat milk can be increased by supplementing the diet with plant oil (Raynal-Ljutovac et al., 2008). The average contents of protein and fat in sheep milk is higher, only buffalo milk contains more fat on average when compare to the other species.

Donkey milk contains substantially lesser amounts of fat and protein than cow, sheep and goat milk, and this is nearest in composition to human milk becouse of

its high lactose and low protein contents (FAO, 1972). Monogastric species show ash content also lower than cow milk and thus more similar to human milk. Human milk differs in the amounts of various proteins they contain, in particular does not contain β-lactoglobulin, one of the main proteins associated with cow milk allergy (FAO, 2013).

The fat content of donkey milk is remarkably lower than the one of human and ruminant milk, and this which is also reflected by their calorific value (Table 1). Donkey milk fat consists for 80-85% of triglycerides, for 9.5% of free fatty acids and for 5-10% of phospholipids. Bovine, sheep, goat and human milk fat consist for 97-98% of triglycerides, but have only low levels of phospholipids (0.5-1.5%) and free fatty acids (0.7-1.5%) (Claeys et al., 2014). Ruminants and woman milk present similar content of cholesterol much higher than non-ruminant milk. Cholesterol is frequently associated with cardiovascular disease, nevertheless is an essential component of body cell membranes and of the central nervous system (Devle et al., 2014; Claeys et al., 2014).

Donkey milk fat contain a higher percentage of polyunsaturated fatty acids and a lower percentage of saturated fatty acids and monounsaturated fatty acids compared to ruminants. Differences observed in milk fatty acid composition are directly related to type of feed, to mechanisms of fatty acid synthesis and to different gastointestinal apparatus. Therefore, whereas the milk fat composition of non-ruminants reflects closely the composition of dietary lipids, in ruminants, due to biohydrogenation process in rumen, fatty acids in the feed are hydrogenated to SFA by ruminal microbes before absorption. However, during this biohydrogenation process trans fatty acids are formed as well, including vaccenic (C18:1, 11t) and rumenic acid (C18:2 9c,11t), an isomer of the conjugated linoleic acids, and it is known that some positive health effects are attributed to CLA isomers.

The total vitamin content of milk strictly depends on the vitamin status of feeding regime of the mother and is highly variable with the level of water-soluble vitamins being more influenced by the feed than the level of the fat-soluble

vitamins. Generally, the vitamin average content of donkey milk is lower than one of ruminant milk. Additionally sheep, goat and buffalo milk is notable to has a higher vitamin A content than bovine milk, indeed these milks are whiter than cow milk, due to their ability to convert the yellow β-carotene to vitamin A.

The ash content is higher in ruminant milk than non-ruminant milk (Table 1). There are major differences in the mineral content of milk from different species, but generally the mineral concentration is the highest in ruminant milk followed by donkey milk and finally is the lowest in human milk (Fantuz et al., 2012; Uniacke-Lowe, 2011). Calcium and phosphorus are the main minerals of milk with significative effects on bone growth, development, metabolism and maintenance. (Adolphi et al., 2009; Cashman, 2006). Sheep, goat, and buffalo milk contain on average more Ca and P than bovine milk and this last one contains about 50% more Ca and twice as much P and K than donkey milk, but donkey milk contain about 2-3 times more Ca and P than human milk (Salimei and Fantuz, 2012; Abd El-Salam and El-Shibiny, 2011; Park, 2009).

Among the main constituents of milk, fat and proteins represent the fractions that have high nutritional values and exert a potential nutraceutical effect. In recent years, as well as being a food for nutritional purposes, milk assuming a role as a source of bioactive molecules, able to influence some aspects of the human health. It is well known that the diet-health relationship can be a key to preventing diseases and, at the same time, to promoting human health; for this reason recently a considerable growth of the functional food market emerged. Functional foods are defined as food that have a positive effect on human health beyond nutritional value. Drozen and Harrison (1998) defined the functional food as food that contains significant level of biologically active components able to provide specific health benefits beyond the traditional nutrients. These foods may improve the general condition of the body like a pre- and probiotic, decrease the risk of some deseases and could even be used for curing some health disorder. Functional food are similar in appearence to conventional one and should be consumed as part of a normal diet, but have been modified to promote physiological roles

beyond of simple nutrient requirements (Bech-Lanrsen and Grunert, 2003). So functional foods are not pills or capsules but are consumed as part of a normal everyday diet. These foods comprise conventional foods containing naturally occurring bioactive substances (for example dietary fiber) or foods enriched with bioactive substances, such as probiotics or antioxidants, or synthesized food ingredients introduced to traditional foods, such as prebiotics. The most mentioned functional components are, probiotics and prebiotics, soluble fiber, omega-3-polyunsaturated fatty acids, conjugated linoleic acids, plant antioxidants, vitamins and minerals, some proteins, peptides and aminoacids, and frequently phospholipids also (Bath and Bath, 2011).

Dairy products represent a significant part of this market, partly due to the addition during the production process of lactic bacteria, many of them with probiotic activity (Fuller, 1989), and partly due to the natural production of secondary metabolites often associated to positive effects on human health.

1.2.1 Milk protein and human health

Milk protein system is relevant both for nutritional quality and of the implications on the technological characteristics (attitude of milk to cheese transformation).

As reported previously, milk proteins profile changes according to the mammal species evidencing in SDS-PAGE (Figure 1) different protein patterns.

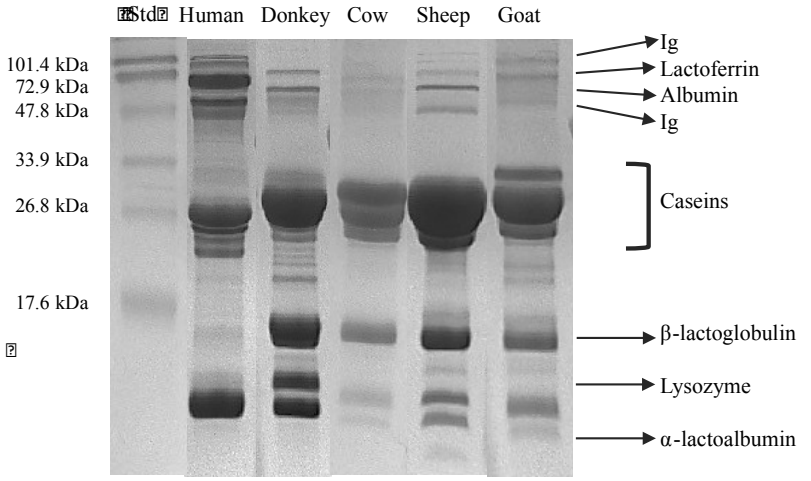

Figure 1. SDS-PAGE of milk from human, donkey, cow, sheep and goat (Std = Broad Range Standard ; Ig = immunoglobulin).

Protein component of milk is divided into two macro-categories, caseins which precipitate by acidification at pH 4.6, and whey proteins, which remain in solution under these conditions. So, over the 95% of the lactoproteins is featured by the

four caseins, α_{s1}-CN, α_{s2}-CN, β-CN, k-CN grouped in casein micelles, and by the two main serum protein, α-lactalbumin and β-lactoglobulin (Martin et al., 2002). The minor proteins include lactoferrin, IGF and the lactoperoxidase system. The ratio casein/whey protein is major in cow milk (about 4.7) intermediate in small ruminants (about 3.3) and low in human and donkey milk (Mølgaard et al., 2011; Claeys et al., 2014). The main proteins of soluble fraction include β-lactoglobulin, α-lactalbumin, Ig, serum albumin, lactoferrin, lactoperoxidase, lysozyme, proteose-peptone, and transferrin (Severin and Wenshui, 2005). The β-lactoglobulin is an important retinol carrier and has shown fatty acid-binding action and antioxidant capacities, and with α-lactoalbumin and lactoferrin have shown suppressing action in tumor development (Parodi et al., 2007). Lactoferrin also shows a crucial role in iron absorption and in exerting antioxidant (Millis et al., 2011). Lactoperoxidase and lysozyme are important antimicrobial agents (Sisecioglu et al., 2010). It is worth to note that immunoglobulins are transferred pass through the placenta to the fetus, for this reason after birth are present in low concentration. The main source of immunoglobulins is colostrum which guarantees immunity defenses after birth, indeed human milk that mainly contains IgA, in colostrum have 100 times more of Ig than milk (Godden, 2008; Politis and Chronopoulou, 2008).

Caseins can be divided in α-, β-, and k-caseins and the main role is mineral binding and the carriage, mainly of calcium and phosphorus forming a coagulum and improving their digestibility in the stomach (Holt et al., 2013). Additionally, caseins are a source of several bioactive peptides with benefits effect on human health. Stated the differences in protein proportions, casein micelles have different characteristics in size but also in hydration and mineralization. Park et al. (2007) reported that sheep and goat casein micelles are similar each other and in comparison with bovine one, are less hydrated, less solvated, and less heat stable and have on average higher mineralization levels. Sheep and goat milk casein micelles are similar and show higher mineralization degrees, less heat stability and idratation compared to cow milk. The size of the casein micelles of goat milk (260

nm) is significantly higher than mare (255 nm), cow (180 nm), sheep (193 nm) and human (64 nm) milk (Park et al., 2007; Raynal-Ljutovac et al., 2008; Potočnik et al., 2011). The structure of casein micells (size, casein distribution, mineralization) varies considerably from species to species, determines the reological properties of milk and affects the milk nutrient digestion. Caseins can form leathery curds in the stomach and be difficult to digest. Cow and sheep milk, which contain hight level of casein, present a coagulum with a spongy and firm structure, while human, goat and donkey milk create a reticular and soft curd (Claeys et al., 2014). This can affects susceptibility to proteic hydrolysis, which, however, depends mainly on the high β-casein micellar content. It is crucial to known the typical casein composition of milk. Caseins distribution in donkey milk was reported by Vincenzetti et al. (2008) and highlighted the presence of α_{s1}-and β-casein but no one other types of caseins, such as α_{s2}-CN, k-CN and γ-CN, were found. Bovine milk has a casein composition rich in α_{s1}-casein and this fraction is probably responsible of the onset of allergic forms in children (Whitelaw et al., 1990) Also sheep milk has a highest content of α_s-casein compared in particular to human that is rich in β-casein but it has a smaller amount of β-casein compared to goat milk.

The nutritional value of milk proteins depends to a great extent on the presence of essential aminoacids. Furthermore, the molecular form and amino acid sequence of the milk proteins are species-specific and affect the protein digestibility, nutritional quality and thermostability (Claeys et al., 2014).

Milk is a dynamic system: the lactoproteins synthesized by the cells of mammary gland are subjected to the action of proteolytic enzymes (proteases), with significant conseguences on the technological (milk processing) and nutritional (production of bioactive peptides) aspects. The milk caseins are also characterized by a species-specifics genetic polymorphism, with important repercussions on the compositional, technological and nutritional characteristics of milk, determining some aspects of great importance that concern: i) the hypoallergenic properties of specific types of milk; ii) the release of peptides with biological actions, from

other lactoproteins (Caroli et al., 2009). Milk protein fraction has been extensively characterized, promoted by the research of non-bovine milk as an alternative protein source for people with hypersensitivity to cow milk proteins, which represents one of the major causes of food allergies (Santos et al., 2010). The genetic polymorphisms of milk proteins play an important role in the allergic reaction, yield, and consistency of curd (El-Agamy, 2007). Some authors reported that goat milk, due to hypoallergenic properties of its proteins, could be considered as a proper alternative to human milk in cow milk allergy cases (Bevilacqua et al., 2001). In goat milk, there are high numbers of alleles at the four casein loci and also the k-CN showed polymorphisms in terms of phosphorylation and glycosylation. The study about goat casein loci, permits to differentiate the goat animal population on the basis of milk utilization: animals which present weak or null casein alleles should be used in breeding programs to producing milk with hypoallergenic properties (Küpper et al., 2010; Albenzio et al., 2009; Sacchi et al., 2005; Moioli et al., 2007) and animals which show strong alleles, associated to a higher cheese yields and a firmer curds, should be used to improve quality and properties of milk used for cheesemaking and dairy products (Albenzio et al., 2009). Less conclusive results are obtained in ovine compared to goats species, where the knowledge of milk protein genetic variants is still limited to α_{s1}-CN and β-LG loci (Amigo et al., 2000; Barillet et al., 2005; Moioli et al., 2007). The casein in goat milk appears to lack an electrophoretic component with the mobility of bovine α_{s1}-casein, and α_{s2}-fraction represents a much smaller proportion in comparison to bovine α_{s2}-casein, making the β-caseins quantitatively the major proteins of goat milk. The very low content or absence of α_{s1}-casein in goat milk makes it possible to detect adulteration of goat milk with cow milk with high precision detecting since of 1% by gel electrophoresis (Aschaffenburg and Dance, 1968). The k-casein isolated and characterized from goat and sheep milk is similar to cow k-casein in many respects, in particular, has the polysaccharide fractions which closely resemble those of cow k-casein one.

The donkey milk protein fraction is particularly rich in whey proteins representing in this milk the 35-50% of the nitrogen fraction, while in cow milk whey proteins represent only 20% (Herrouin et al., 2000). Donkey milk total protein content is very similar to the values for human (Salimei et al., 2004) and mare milk (Malacarne et al., 2002). Indeed, due to the relevant similarity in chemical composition and especially in protein content human milk, both mare, and donkey milk have been widely used to replace human milk. On the basis of the results obtained by Vincenzetti et al. (2008), donkey and mare milk could be considered suitable for feeding young children affected by cow milk allergy. The high lysozyme content found in donkey milk may be responsible for the low bacterial count reported by Salimei et al. (2004) and could be useful to prevent intestine infections in infants. Among other allergenic milk components, it worth to note that the percentage of β-lactoglobulin in donkey milk is much lower than that in bovine milk, where β-lactoglobulin can account for up to 50% of total whey protein (Solaroli et al., 1993). Indeed, β-lactoglobulin is the potential major milk allergen in infants and small children, whereas casein is considered the predominant allergen in adults (Carroccio et al., 1999). These findings, together with the low casein content, are probably related to the hypoallergenic characteristics reported for both donkey milk and mare milk (Carroccio et al., 2000; Iacono et al., 1992). β-lactoglobulin has been purified and characterized in milk from goats and sheep and was reported the existence of two genetic variants in sheep milk, β-LG A and β-LG B (Bell and McKenzie, 1964, 1967; Maubois et al., 1965).

The scientific community has long been focused on the potential therapeutic property of milk, expressed by its protein fraction (Madureira et al., 2007; Teschemacher et al., 1997; Zimecki and Kruzel, 2007). It has been demostrated that some whey proteins such as lactoferrin, β-lactoglobulin, α-lactalbumin and serum albumin have a tendency to slow down the development of tumor cells (Parodi, 2007); α-lactalbumin in the presence of oleic acid forms a complex called HAMLET that in human milk has shown the ability to inhibit a wide range of

tumors cells through a mechanism similar to cell apoptosis (Svanborg et al., 2003). In cow milk the homologue complex, called BAMLET, showed a cytotoxic effect against eight cancer cell lines, through a mechanism of lysosome membrane permeabilization (Rammer et al., 2010). The little milk fat globules are enclosed into a membrane called MFGM; it consists in a lipidic and proteic double layer, and has been shown to have a potential therapeutic effect against some pathological conditions (Spitsberg, 2005). A specific protein isolated from the MFGM named FABP (fatty acid binding protein), has shown the ability to inhibit the cell line that causes the breast cancer (Spitsberg and Gorewit, 1997a, b); as well the BRCA1 protein, an other protein binded to MFGM, acts as a tumor suppressor found in both bovine and human milk. It has been observed that the protein fraction of MFGM is able to inhibiting some Helicobacter Pylori infections (Wang et al., 2001). Sugahara et al., (2005) observed a small protein (about 19kDa), derived from the degradation of a proteose-peptone following the fermentation of fat-free milk by some lactic acid bacteria (LAB), which shows the ability to stimulate the immunoglobulins production from PBMC cell lines of human blood. It has been established that the basic protein fraction of milk (MPB), has a direct effect on bone strengthening in healthy subjects (Toba et al., 2001; Uenishi et al., 2007). In addition to the action of proteins in their native form, it is also interesting to study the action of the degradation products of these proteins, named bioactive peptides. These are identified as latent or encrypted amino acid sequences into the protein in their native form, and which are released after ingestion through proteolysis in the gastrointestinal tract, or during fermentation processes conducted by bacteria, especially lactic bacteria, during the maturation of dairy products. Mellander first described bioactive peptides in 1950; he discovered that the ingestion of peptides deriving from the phosphorylation of caseins, induced an indipendent increase of vitamin D calcification, in children with rickets.

Milk bioactive peptides can be grouped, depending on the health physiological effects, in the following categories: antihypertensives, antithrombotics, opioids,

CPPs, antimicrobials, cytomodulators, and immunomodulators. The antihypertensive activity of some peptides is in the ability to inhibit ACE. The ACE is the key enzyme in the regulation of blood pressure; the ACE-inhibitors substances act with a competitive behavior with this enzyme, so avoiding the conversion of angiotensin I into angiotensin II, which is a powerful vasoconstrictor (Seppo et al., 2003). The main process that induces the release of ACE-inhibitory peptides by milk proteins and in particular by caseins, is the fermentation of milk by LAB. The peptides from dairy origin with ACE-inhibitory activity consist of more than 10 amino acid units (Korhonen and Pihlanto, 2003; Meisel and Bockelmann, 1999; Pihlanto, 2001; Saito, 2008), and among the most studied, there are Val-Pro-Pro peptide (Clare et al., 2003; Hamel et al., 1985; Juillard et al., 1995) and Ile-Pro-pro peptide (Chabance et al., 1995; Drouet et al., 1990), both derived from fermentation of *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, respectively. It has also been demostrated that Ile-pro-pro peptide passes through the intestine intact escaping from intestinal degradation, reaching blood circulation, and that both Ile-Pro-Pro and Val-Pro-Pro have a ACE-inhibitory capacity similar to synthetic molecules.

Thrombosis represents the pathological condition where the abnormal development of clots or thrombus in the veins, arteries and cavities of the heart is observed. From a zootechnical point of view, a correlation was observed between the mechanism that determines the formation of clots in the milk due to the interaction between k-casein and chymosin, and the mechanism that determines the formation of clots in blood, due to the interaction between fibrinogen and thrombin (Jolles, 1975; Jolles and Henschen, 1982; Rutherfurd and Gill, 2000). Some structural similarities (about 80%) have been observed between the milk k-casein and the human gamma-fibrinogen that may have evolved from a common ancestor (Jolles et al., 1978). Casoplatelin is a peptide obtained from k-casein, with antithrombotic activity showing the ability to inhibit the platelet aggregation with a concentration-dependent process, confirming the analogy between fibrinogen and k-casein (Jolles et al., 1986). It is also interesting to know how

these antithrombotic peptids deriving from milk can be absorbed directly into the bloodstream.

Peptides with opioid activity have a pharmacological similarity with opium. Almost all caseins (α_{s1}-, α_{s2}-, β- and k-casein) and whey propteins are potential sources of opioid peptides; however, most of these are produced from β-casein fragments and are known as β-casomorphins (Clare and Swaisgood, 2000; Teschemacher et al., 1997). The β-casomorphine resists to the action of gastrointestinal enzymes (Read et al., 1990) and is associated to the antihypertensive, immunomodulatory, antidepressant, antisecretory and antidiarrheal activities, and it is known to be operative even at very low concentrations (Pihlanto, 2001). Other opioid peptides were found encrypted in the primary sequence of serum proteins such as lactoferrin, β-lactoglobulin and bovine serum albumin (Belem et al., 1999; Rokka et al., 1997). In contrast, k-casein forms the casochine with an opioid-antagonist activity (Séverin and Wenshui, 2005).

CPPs are casein-derived phosphorylated peptides, which contain single or multiple phosphoric residues, and which are released following enzymatic hydrolysis of α_{s1}-, α_{s2}-, β- and k-caseins both *in vivo* and *in vitro* (Adamson and Reynolds, 1995, 1996; Clare and Swaisgood, 2000). These peptides, acting as biocarriers, react by efficiently linking divalent cations such as iron, manganese, copper and selenium; for this characteristic, many researchers have wondered if CPPs could increase human calcium absorption. However, the results are still controversial both in animal and in human models (Scholz-Ahrens and Schrezenmeir, 2000). Some animal model studies report an increase in intestinal calcium absorption with low concentrations of these peptides, while others report that needs to greatly increase the concentrations of CPPs gave to achieve the same results. However, beyond the calcium carrier function, of course these peptides showed an anticanceriogenic property (Mills et al., 2011).

In human and bovine milk there are casein-derived immunopeptides and with ability to stimulate phagocytic activity in human and guinea pig macrophages,

expressing a protective effect against *Klebsiella pneumoniae* (Smacchi and Gobetti, 2000). Immunomodulating peptides can play a key role in the proliferation and maturation of T-lymphocytes and in natural killer cells in newborns, with protective effect against a large number of bacteria, especially of enteric origin (Clare et al., 2003). In detail, the peptide isracin, obtained by the action of chymosin on the α_{s1}-CN, shows an antibacterial activity against *Staphylococcus aureus* and *Candida albicans*; it also has a protective role against mastitis when administered as local injections in sheep and cattle (Lahov and Regelson, 1996).

Milk is also a source of proteins and peptides with antibacterial activity, comparable to an antibiotic (Clare et al., 2003; Lopez-Exposito and Recio, 2008; Séverin and Wenshui, 2005). This is due to the synergistic activity of peptides and immunoglobulins naturally present in milk, such as lactoferrin, lactoperoxidase, lysozyme. It is known that bovine lactoferrin have an anti-viral action against the HIV virus and human cytomegalovirus (Floris et al., 2003). In addition, human and bovine lactofericin have antimicrobial activity against a large number of gram positive and gram negative bacteria, including *Listeria monocytogenes* (Clare et al., 2003; Floris et al., 2003).

1.2.2 Milk fat and human health

Epitelial cells of mammary gland, secrete milk lipids in form of fat globules in emulsion, coated by a biological membrane (MFGM) composed by proteins, glycoproteins, glycerophospholipids, sphingolipids, cholesterol, enzymes and other small compounds (Gantner et al., 2015). Yao et al. (2016) reported that the diameter of the fat globules in caprine milk (3.64 ± 0.33 μm) was smaller than that in bovine milk (4.89 ± 0.17 μm) and in human milk (4.53 ± 0.18 μm). The MFGM has a thickness of about 10-20 nm and constitutes 2-6% of the total globule mass (Heid and Keenan, 2005).

The MFGM has a primary importance for physiochemical properties of fat (Menard et al., 2010); it emulsifies fat globules preventing the aggregation and

coaletion, and acts as a physical barrier avoiding the triacylglicerols hydrolysis by lipolytic enzymes. Milk with high-fat content shows bigger fat globules compare with low-fat milk, so the total specific surface areas of MFGM is higher in milk with small fat globules (Gantener et al., 2015). There is a positive correlation between milk fat content and fat globules size; buffalo milk has the largest diameter of fat globules (about 7 μm) and also produces the highest quantity of fat. In this high-fat milk, just fat globule membrane could represent a limitation in the production of small fat globules. (Menard et al., 2010) The size of milk fat globules is a parameter influencing digestibility and lipidic metabolism efficiency; sheep and goat milk have the smaller size of globules than cow milk, with the 65% of globules <3 μm (Mens, 1985).

Some authors have long studied the interaction between bovine MFGM and human health (Spitsberg, 2005). The MFGM is a source of functional substances and it is considered as a nutraceutical compound. The milk fat globule membrane functionality is conferred by its content in phospholipids, sphingolipids, fatty acids and proteins, which have an antibacterial effect (such as xanthine oxide reductase and mucins) or beneficial health consequence (Dewettinck et al., 2008). Generally, the positive effects have been attributed to the membrane protein fraction with anticancerogenic and antimicrobial activity; nevertheless recently it has been reported that the antibacterial activity of MFGM is also due to the lipidic component.

Cow, buffalo, goat, sheep, and woman milk fat is constituted for about 97-98% of triacylglycerols, 0.5-1.5% of phospholipids and 0.7-1.5% of free fatty acids; while in donkey milk there is a higher content of free fatty acids (9.5%) and phospholipids (5-10%) and a lower content of triacylglycerol (80-85%) than cow milk (Claeys et al 2014; Gantner et al., 2015). Spitsberg et al. (2005) reported that the intake of MFGM through consumption of dairy products or products enriched with MFGM, shows a beneficial effect on human health and that this effect would be attributable to the phospholipid fraction. The three types of MFGM phospholipids with these properties are represented by sphingomyelin,

phosphatidylcholine, and phosphatidylethanolamine. Many cellular processes such as growth, memory development, stress responses and myelination of CNS, seems to be influenced by these phospholipids. In particular, sphingomyelin has shown an anticancerogenic activity and an inhibitory effect on intestinal absorption of cholesterol (Mills et al., 2011). The sphingomyelin is a phospholipid representative of a very important class of lipids exists in nature, known as sphingolipids. These are located mainly in cell membranes, in lipoproteins (especially in the LDL) and in other structures rich in lipids such as skin. It is known that this class of lipids plays an essential role in all cells processes like growth, persistence, and death. They also showed a potential chemotherapeutic and chemopreventive activity (Mills et al., 2011). Sphingolipids show also an important role in the development and regulation of the immune system and in neuronal cell function by regulating rates of neuronal growth, differentiation and death (Cinque et al., 2003; Buccoliero and Futerman, 2003). Cinque et al. (2003), reported that some breakdown products of sphingolipid metabolism work as a lipid messengers controlling the central stages of immune cell development, differentiation, activation, proliferation, and function. In a study conducted by Oshida et al. (2003a) has been demonstrated that feeding the rats with bovine sphingomyelin-supplemented diets the myelination of the central nervous system show beneficial effects.

The fat fraction is also important for the release, upon lipases activity, of the fatty acids. In milk fat, more than 400 fatty acids have been identified, constituted by approximately 1.9 g of SFA/100 g, about 0.8 g/100 g of MUFA and in particular of oleic acid (C18:1 cis-9) and about 0.2 g of PUFA/100 g (Haug et al., 2007).

Milk fat composition mainly varies under the effect of animal species, breed, genotype, and feeding. The feeding factor mostly influences the variation of milk fatty acids profile; as higlighted by some authors, a greater content of CLA, VA and PUFA with consequent improvement of n3/n6 value, is showed in milk from sheep feeded with pasture and fresh forage or supplemented with a vegetables fat source (i.e. flaxseed), (Caroprese et al., 2011; Nudda et al., 2014).

As regard to fatty acid profile, compared to ruminants, donkey milk fat contains a higher percentage PUFA a lower percentage of SFA and MUFA (Claeys et al., 2014). The fatty acids of donkey milk are mainly unsaturated or short-chained, showing higher levels of linoleic acid (n-6 C18: 2) and α-linolenic acid (n-3 C18: 3) than bovine milk (respectively 5 and 224 times more) (Salamon et al., 2009).

Small ruminants milk, compared to cow one, has higher in short- and medium-chain FA from C6:0 to C10:0; these groups of FA represent 8%, 12% and 18% of the total content of fatty acids in cow, sheep and goat milk, respectively, in particular, caprylic acid (C8:0) and, more markedly, capric acid (C10:0) are higher in goat milk. The difference between goat and sheep milk fatty acid profile are the higher presence of some short-chain fatty acids such as caproic, caprylic and capric acids in goat milk than in sheep (Jandal et al., 1996). This is due to the effect of genetic polymorphism at the α_{s1}-casein locus on the fatty acid profile of goat milk, where low α_{s1}-casein genotypes show higher Δ-9 desaturation level and lower medium-chain fatty acids content (Chilliard 2006). Instead, cow milk shows higher levels of butyric (C4:0) and palmitic (C16:0) acids (Glass et al., 1967).

MUFA have a different metabolism from that of long chain fatty acids, indeed be released from triglycerides in the stomach by gastric and pancreatic lipase to be absorbed directly by intestinal cells, without esterification, bypassing the transport via portal vein, to the liver, where they are oxidized (Raynal-Lijutovac et al., 2008). Therefore, MUFA have many positive feedback on human health: for subjects affected by malnutrition or fat malabsorption syndrome, constituting a rapid energetic supply; for pre-term newborns in specific ratio with long chain fatty acids (Telliez et al., 2002); as a molecules able to decrease total circulating cholesterol and especially LDL (Seaton et al., 1986; Kasai et al., 2003); avoiding the fat deposits in adipose tissues, thanks favored absorption pathway (Tsuji et al., 2001); limiting overweight, inducing postprandial thermal expenditure (Raynal-Lijutovac et al., 2008).

Every individual fatty acids, and in partucular SFA, have different influences on blood lipids; lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids are

associated with elevated serum levels of bad cholesterol (LDL) whereas stearic acid (C18:0), which is poorly absorbed in the gut, has no effect on LDL-cholesterol (Shingfield et al., 2008; FAO and WHO, 2010; Gibson, 2011). Butyric acid (C4:0) represents about 10% of total fatty acids in cow milk and exerts a pattern of effect on colonic mucosa. In human, butyric acid is the principal energy source for colonic epithelial cells, and some authors demonstrated its anti-proliferative, anti-inflammatory and apoptotic properties (Mills et al., 2011).

Among milk fat PUFA in particular, oleic, linoleic and linolenic acid, are characterized by one, two and three unsaturations, respectively and are the precursors of the omega-9, omega-6, and omega-3 series. Linoleic and linolenic acids, are also called essential fatty acids (EFA), because the human being is not able to synthesize them on its own and must assume them by food, and they are necessary to guarantee human health, carrying out important functions for the growth, such as the energy production, the maintenance of cellular and mitochondrial membrane health (phospholipid constituents), the hemoglobin synthesis, the sexual and reproductive function, and the proper development of brain and its cognitive functions (Joanne et al., 2015). The EFA determine a reduction of total and bad cholesterol (LDL) and are precursors of eicosanoids, important chemical mediators at the cellular level. In order to perform their nutritional and nutraceutical effect, the omega-6 and omega-3 fatty acids must be taken daily with the diet; the assimilation of these fatty acids is higher when they are naturally present in food and not when they are added through food processing. So, it can be argued the importance of a correct evaluation of the omega-6 and omega-3 content in milk. It is reported that omega-6 and omega-3 fatty acids have showed health beneficial effects reducing the risk of form some diseases such as type-2 diabetes, hypertension (Willett, 2007; Zhao et al., 2007), cancer (Dupertuis et al., 2007), some neurological dysfunctions (Alessandri et al., 2004; Hamilton et al., 2007) and CVD (Siddiqui et al., 2008). In particular omega-3 fatty acids, besides the activity for normal physiological body functioning and the maintenance of health, exhibits a therapeutic potential; in particular omega-3

series are used in the treatment of inflammatory diseases such as rheumatoid arthritis, and in the alleviation of symptoms of mental health such as depression and dementia (Ruxton et al., 2007). EPA and DHA are two significant long-chain n-3 PUFA that are normally involved in cognitive functions and behavioral development, and in addition may contribute to the prevention of CVD, to reduce the risk of other degenerative diseases, type II diabetes, hypertension, cancer and certain disorders in neurological functions (Gogus and Smith, 2010; FAO and WHO, 2010). Moreover, DHA has shown potentiality as a treatment for late-onset Alzheimer's disease (Ma et al., 2007). The recommended ratio omega-6/omega-3 fatty acids ranging from 1/1 to 4/1 and a lower value is preferred for reducing the risk of CVD (Simopoulos, 2002; Balthazar et al., 2017). Although the various studies, the recommended dose of omega 3 and the better proportion between omega 3 and omega 6 in the diet, remains not completely clarify (EFSA, 2010). Humans have shown the capacity to convert with low efficiency ALA to EPA and DHA. Thus, EPA and DHA need to be assumed by the diet, and for this reason, many efforts have been made to increase the content of n-3 PUFA in milk fat using animal feed strategies (Caroprese et al., 2011; Nudda et al., 2014). FAO and WHO (2010) evidenced that replacing SFA with PUFA decreases the risk of CVD and possible and can reduce diabetes risk.

CLA refers to a family of positional and geometric isomers of linoleic acid (an n-6 omega fatty acid), characterized by the presence of conjugated double bonds. CLA is predominantly found in ruminants milk and meat. The isomers with major biological activities are particularly present in milk and dairy products are CLA cis-9, trans-11 (rumenic acid) and CLA trans-10, cis-12. It has been confirmed that CLA can reduce fat mass and increase muscle mass with some probable mechanisms such as the increase in energy consumption, increase in fat oxidation, decrease in adipocyte size and inhibition of the enzymes involved in fatty acid metabolism and lipogenesis (Bhattacharya et al., 2006). Another positive function expressed by the isomers of conjugated linoleic acid is related to the protective effect against atherosclerosis phenomena in the cardiovascular system; CLA

seems to shows an antagonistic effect towards the inflammatory process, which is the main cause of the formation of atherosclerotic plaque. Many *in vitro* studies or based on animal models showed the inhibition effects by CLA towards gastrointestinal cancer, breast carcinogenesis, and prostate cancer; CLA is also able to reduce glucose and insulin levels in plasma, prevent hyperinsulinemia, and improve immune function (Mills et al., 2011). The anti-inflammatory effect of CLA derives from different mechanisms such as the lower production of the eicosaenoic and of the pro-inflammatory cytochine (TNF-α; IL-1, IL-6). In the animal models, CLA induces a negative regulation of the inflammatory genes and an activation of apoptosis in atherosclerotic plaque (Bhattacharya et al., 2006). However, introducing through diet a large number of dairy products may result in an excessive intake of saturated fatty acids and cholesterol with adverse health effects such as increased LDL, the onset of cardiovascular problems and the increase in the concentration of estrogen circulating. In the "Seven Countries" study, a clear relationship between saturated fat intake and coronary mortality was reported. Nevertheless, if saturated fatty acids are grouped according to their length chain, a difference emerges depending on the type of fatty acid: for example stearic acid (C18: 0) has no effect on cholesterol levels if compared to oleic acid (C18: 1); saturated fatty acids such as myristic acid (C14: 0) and palmitic acid (C16: 0) tend to increase cholesterol and LDL levels in plasma.

1.3 Objectives of the Ph.D. study

Milk is a complex and a high nutritional value food able to growth newborn but also used by the adult. The nutritional effect of the lipidic and proteic fractions of milk from different animal species is well studied by the researchers of all countries of the world, nevertheless, recent reports suggest these compounds might have also effects on human health. Milk proteins showed the capacity to stimulate innate immune response causing different physiological reactions such as the hypersensitivity to cow milk proteins, which represents one of the major causes of food allergies, or the activation of pro- and anti-inflammatory cytokines, which cause trigger seizures in epilepsy cases. Nevertheless, milk proteins and lipidis have expressed potential therapeutic properties, and among these cosmetic, immunological, anti-carcinogenic, antimicrobial and neurological functions.

Even if many pieces of information are available about milk composition from different species, little is known on the fate of milk components after digestion. Hydrolytic processes modify milk nutritional compounds during gastrointestinal digestion, to give new molecules often with nutraceutical effect on human health. Lipolysis and proteolysis during gastrointestinal digestion released the fatty acids from the glycerol backbone and the degradation products of native proteins (bioactive peptides), increasing their bioavailability in the gastrointestinal tract. It is worth noting that some fatty acids and some peptides naturally contained in milk have beneficial effects on human health.

In the light of these considerations, the general objective of the Ph.D. study was to evaluate the effect of milk proteic and lipidic compounds before and after simulated digestion process on different biological *in vitro* systems, which simulated the physiological systems in human. For this reason, the Ph.D. work provided for different trials, using various cellular system treated whit digested and not digested milk and its proteic fractions.

In the first trial, have been studied the role of not digested bovine, ovine, and caprine milk, caseins, and whey proteins on the immune status of children with generalized epilepsy; in particular the cultured peripheral blood mononuclear cells

from infants with generalized epilepsy have been used as *ex vivo* cellular system and cytokines, reactive oxygen and nitrogen species was detected as biomarkers to discriminate the effects of milk components on epilepsy in children.

In the second trial, have been evaluated the effects of specific milk protein fractions (α_{s1}-CN, α_{s2}- CN, k-CN, β-CN, and a mix of α-LA and β-LG) from different animal species (bovine, ovine, and caprine), on pro- and anti-inflammatory cytokines and oxidative status, in the same *ex vivo* cellular system (PBMC) from children with generalized epilepsy.

The third trial aimed to understand the effect of gastrointestinal digestion on milk lipidic fraction; in detail the fatty acid profile in milk from different sources (human, formula, donkey, bovine, ovine and caprine) before and after *in vitro* digestion process was studied, in order to assess if the fatty acid profile of ingested milk reflected the one available after digestion. This investigation of the fatty acid profile after *in vitro* digestion could give information on the availability of these compounds for further intestinal absorption.

Finally in the last trial, it has been evaluated the effect of digested milk, with particular regard to its fatty acid composition, from human, donkey, bovine, ovine, caprine and formula milk on mature adipocytes 3T3-L1. In detail cellular viability, apoptosis, oxidative response and gene expression levels of NF-kB p65, HMGB1, SREBP-1c, and FAS were evaluated in this cellular system, to gain information about a correlation between a specific fatty acids profile and childhood obesity.

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2. SECTION I

2.1 Milk digestion

The growing awareness about the relationship between food and human health has led researchers to find a way to understand, the functional value of food. There is the need to evaluate the physicochemical basis of the behavior of food during digestion and consequently its biological fate. Indeed, during the digestion process, food is decomposed in the gastrointestinal tract in macromolecules such as proteins, lipids, carbohydrates, able to satisfy nutritional body requests, but also releases biologically active molecules that could have a beneficial or a damaging effect on human health.

The perfect approach to study food digestion destiny are human clinical trials (Deglaire et al., 2009). However, it is easy to understand how difficult and complex it is to conduct experiment in humana, with invasive approches especially on subjects such as children or young people. Moreover *in vivo* assays lead to technical constraints, high cost, and interindividual variability. To overcome these complications, *in vitro* systems are often used, divided into two principal models, static or dynamic systems. Static *in vitro* systems have been largely used to study food digestion (Hur et al., 2011). The static model is simple and easy to use and consist in a succession of bioreactors reproducing the environmental condition of the various compartments of the digestive apparatus. In each compartment, mouth, stomach, and intestine the pH, enzyme concentrations and ionic strength are determined. However, this model has a limited physiological relevance because it cannot recreate the complexity of the gastrointestinal tract that occurs in the real digestive system (Guerra et al., 2012). *In vitro*, dynamic digestion systems are much more complicated compared with the static ones but there are closer to the physiological conditions thanks to the inclusion in the process of the pH regulation, of the dynamic flows of food and of the concentration of digestive enzymes in every compartment. The most well-known dynamic systems are developed by the Dutch team from TNO, named TIM 1 (Minekus et al., 1995); it is composed of 4 compartments mimic the stomach and the 3 segments, duodenum, jejunum, and ileum of the small intestine. The

various compartments are made in glass with a flexible wall inside and the water circulates into the space between the glass and the flexible wall imitating the peristaltic movements obtained by changing pressure on the water.

Recently, the COST action INFOGEST, an international network constituted by more than 200 scientists from 32 countries in the world that working in the sector of digestion, aimed to consolidate the conditions for a consensual model of simulated digestion of food. In the manuscript, Minekus et al. (2014) tryed to described a set of conditions closer as possible to the physiological situation, and which can be used by different research depending on requests; the process was schematized in Figure 1.

The static digestion method proposed by the COST Infogest network was able to outline a set of parameters in the oral, gastric and small intestinal digestion and its relevance is discussed in relation to available *in vivo* data and enzymes. The protocol obtained from this work gave a detailed guidance, recommendations and justifications but also limitation of the proposed model, that make it easly to use.

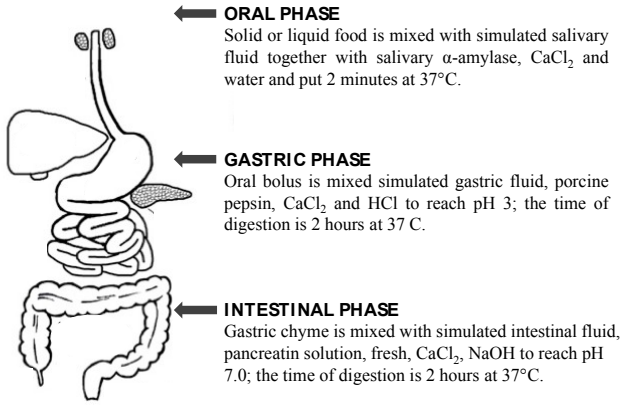

Figure 1. Scheme of a simulated *in vitro* digestion method.

The first step that allows using food is digestion. This process releases nutrients, which can be absorbed and used directly without further hydrolysis. In the mouth, food is chewed and homogenized with the saliva to be then spilled into the stomach; here food comes in contact with mucus, hydrochloric acid (secreted by parietal cells) that decreasing the pH to the 1–5 range (Guerra et al., 2012), pepsinogen (secreted by the main cells), gastrin, gastric lipase and pepsin which initiate the lipolytic and proteolytic reactions, respectively. In the small intestine occurs most of the digestive processes, lipids, and protein hydrolysis are considered complete at the end of the small intestine; pH progressively rises to about 7, pancreas pours the pancreatic juice, very rich in enzymes, able to hydrolyze almost all the food macronutrients. Pancreatic lipase, in association with its colipase, bile acids necessary for the emulsion, digestion, and absorption of fats, and other lipases perform lipolysis (Singh et al, 2009), together with proteases, such as trypsin and chymotrypsin, perform proteolysis (Mackie and Macierzanka, 2010; Damien et al., 2016). Each segment of the intestinal canal is specialized and has different ideal chemical-physical conditions for the correct functioning of the enzymes. The absorption efficiency for the main nutrients such as fat proteins and carbohydrates is as a whole very high, about 95%. The nutrients available for cells are those contained in the aqueous medium surrounds them. The intestinal epithelium is the organ of absorbing for excellence, and the cells that constitute it are enterocytes; these cells are the only one that comes into direct contact with the nutrients coming from the outside, all the other cells receive nutrients through blood (Arienti et al., 2003).

The lipolysis rate depends on physical and chemical properties of water/lipid interface in which lipases act (Favé et al., 2004). The digestive lipases action and the consequently released fatty acid bioavailability are due to some parameters of milk organization such as the milk fat macrostructure (the globule size that is inversely related to surface area of MFGM), the composition and organization of fat-globule surface and the triglycerides molecular structure (Favé et al., 2004).

Milk fat globule size interfered with digestion; the smaller fat globules, the more efficient lipid metabolism, the more milk digestibility (Gantner et al., 2015). Goat milk has on average smaller fat globules (3.05 μm) than sheep (3.4 μm), cow (3.7 μm), human (4 μm) and donkey milk (5 μm); buffalo milk fat globules have generally a larger diameter than those of other species on average 6.1 μm (Claeys et al., 2014). However, also the structure of the fat globule interface constituted by MFGM, plays a role in fat digestibility as gastric lipases must gain access to the triacylglycerols crossing this membraneHuman milk fat globules are coated with three layers made up by, a glycoproteic external layer, a phospholipid membrane as intermediate layer and finally a internal proteic layer; on the surface of glycoproteic layer is branched oligosaccharides structure. In cow milk fat globules are coated with a small addict film comprised by phospholipids and protein without glycoproteins.

Milk with high fat content has a larger fat globules and its membrane is less stable, showing a decreased resistance to deformation under mechanical pressure in comparison with smaller fat globules; moreover the larger fat globules more easly leave aqueus phase to form the creamy layer (Gantner et al., 2015).

The intestinal absorption of fat made up thanks to the action of lipolytic enzymes is related to the triacylglycerol structure. MCFA are hydrolyzed and metabolized more rapidly and completely after absorption by the epithelial barrier in comparison with LCFA (Babayan, 1987; Bach and Babayan, 1992). MCFA are transported directly to the liver via the portal circulation, where follow different catabolic pathways such as beta-oxidation, omega-oxidation, and peroxisomal oxidation. Unlike, the LCFA are preferentially incorporated into chylomicrons and transported via lymph (Mills et al., 2014).

Glycerol backbone shows a different distribution of fatty acids depends on the animal species, and these differences determine the efficacy of lipolysis and the bioavailability of fatty acids (German and Dillar 2006). In human and donkey milk, palmitic acid (C16:0) is often located in sn-2 position (about 74% of the total compared to about 47% in cow milk), and this makes it favorable for the

assimilation by the newborn. The same fatty acid in cow milk is located independently in sn-1 and sn-2 position maintaining equally beneficial health function (Parodi et al., 1982). In cow milk in sn-3 position are mainly bounded fatty acids with a chain length from C4:0 to C10:0 (Gastaldi et al., 2010). Milk butyrate is immediately absorbed as soon as it reaches the small intestine, then processed and released into the bloodstream, and transported to the liver where it is mostly metabolized (Parodi, 1997; Smith et al., 1998). The butyrate seems to show interesting activities oh human health; Yanagi et al. (1993) reported that margarine supplemented with sodium butyrate significantly reduced the incidence of mammary tumors in a rat model; the ingestion of butyrate as a natural component of anhydrous milk fat was able to inhibit mammary tumorigenesis in rats (Belobrajdic and McIntosh, 2000).

Protein digestion is a complex process dependent on the concerted action of proteolytic digestive enzymes e.g., trypsin, chymotrypsin, elastase, carboxy- and aminopeptidases, and their isoenzymes and inhibitors secreted into the gastrointestinal tract on dietary proteins; besides depending on other many factors such as the type of dietary proteins, gastric and intestinal pH, peptic activity, endogenous secretions, and motility (Bouzerzour et al., 2012; Devle et al., 2014). The differences in the composition of milk from different species such as the content and type of protein, lipids, lactose, growth factors, immunoglobulins and enzymes can influence the way proteins are digested. Differences in total protein composition, in detail casein content and casein/whey protein ratio, and in micelle structure, intended like size, casein distribution, mineralization, determine the rheological properties of milk rennet affecting milk nutrient uptake. Indeed, when in the stomach the coagulation and curd formation of milk is higher, the proteins degradation is delayed and this improves their assimilation. Each milk shows different curd formation: in high casein-containing milk, like bovine and sheep milk, is produced a firm and dense coagulum; instead human, donkey and goat milk form soft curds in the stomach, which are easier to digest. A soft curd results physiologically more apt for infant nutrition because is more digestible

(Barlowska et al., 2011; El-Agamy, 2009; Malacarne et al., 2002). Individual milk proteins from different species show a different grade of digestibility; β-LG is more easily digestible in goat and sheep milk than cow milk (Michaelidou, 2008) and horse β-LG are more easy to digest than goat β-LG. About whey proteins, α-lactalbumin of all species seem to be quite hard to digest, in comparison with lactoferrin and serum albumin, that appears to be easily digestible, in human and horse milk as much as in bovine and goat milk (Inglingstad et al., 2010).

During gastrointestinal digestion, the action of digestive enzymes like pepsin, trypsin or chymotrypsin on milk proteins releases bioactive peptides. These peptides derived from milk are initially in an inactive form within the sequence of the precursor proteins; subsequently, in the stomach, the parietal cells secrete hydrochloric acid (HCl), which denatures dietary proteins and activates pepsinogen in its active form, pepsin. Pepsin with other enzymes present in the small intestine such as trypsin or chymotrypsin is responsible for protein hydrolysis acting metabolizing them to amino acids (Korhonen and Pihlanto, 2003).

The activity of peptides is based on their amino acid composition and sequence and generally, the sequences are known to possess multifunctional properties may vary from two to twenty amino acid residues (Meisel and Fitzgerald, 2003).

Various bioactive peptides are released from casein and whey proteins by gastrointestinal digestion (Meisel and Fitzgerald, 2003) with positive or negative effects; for this reasons is very important to study the destiny of milk components after digestion process.

2.2 Focusing on fatty acids profile in milk from different species after *in vitro* digestion

Journal of Dairy Research (2018) **85** 257–262.
doi:10.1017/S0022029918000274

Focusing on fatty acid profile in milk from different species after in vitro digestion

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Received 30 June 2017; accepted for publication 5 April 2018

Abstract

We report the fatty acid profile of raw milk and of the corresponding digested milk from different sources (human milk, formula milk and donkey, bovine, ovine and caprine milk) to gain information on the nutritional quality of different milk sources in infant nutrition.

SC-FA were higher in bovine and caprine milk, intermediate in ovine and donkey and lower in human and formula milk. MC-FA showed the highest values for bovine and caprine milk and the lowest for donkey and formula milk, whereas LC-FA were the highest in donkey and formula milk and intermediate in human milk.

The percentage distribution of fatty acids liberated after *in vitro* digestion did not reflect the patterns found in the corresponding milk sources. In particular, MC-FFA showed the highest and the lowest values in donkey and in formula milk, LC-FFA showed the highest value in human milk. The total FFA was highest in human milk, lowest in formula milk and intermediate in donkey, bovine, ovine, and caprine milk.

Introduction

Human milk is considered the best source of nutrients for the infant, being the first food of the life of each newborn mammal and able to fully satisfy energy and nutritional needs for sugars, minerals, vitamins, proteins and lipids. For women who choose not to breastfeed or have insufficient amount of milk, milk-based formula is recommended (Zou et al. 2013). Although production of an identical product to breast milk is not feasible, every effort has been taken to mimic its nutritional profile for normal infant growth and development. Cow milk is most commonly used as the base for such formula milk (Zou et al. 2017). Less usually formula are soy-based or manufactured from other mammalian milks such as buffalo, donkey, sheep, camel, and goat milk (Zou et al. 2013). However, the benefits of using alternative milk sources to human milk still remain unexplored by the dairy industry.

The fat fraction of milk varies among different species especially in terms of structure and size of fat globule, the amount and structure of TAG, FFA, phospholipid and cholesterol content (Gantner et al. 2015). Milk fat component has been widely investigated in infant nutrition; essential n-3 and n-6 PUFA and their long chain PUFA derivatives such as arachidonic acid and DHA have a central role in cell membrane structure and functions promoting neonatal growth, neurotransmitter metabolism, and visual and nervous system development. Digestion and subsequent bioavailability of lipid and protein components is a complex physico-chemical and enzymatic process; *in vitro* or *ex vivo* model digestion is needed to predict the nutritional quality of food products and their constituents (Devle et al. 2014). The molecular form of fatty acids taken up by the enterocyte modulates its incorporation into either the TAG or the phospholipids fraction of chylomicrons as well as its positional distribution in the TAG of chylomicrons (Martin et al. 1993).

In the light of these considerations the aim of the paper was to study the fatty acid profile in milk from different sources (human, formula, donkey, bovine, ovine and caprine) before and after *in vitro* digestion process, in order to assess if the fatty

acid profile of ingested milk reflects the one available after digestion. The investigation of fatty acid profile after *in vitro* digestion could give information on the availability of these compounds for further intestinal absorption.

Materials and methods

Milk sample collection and analysis

Five lactating women (1–3 months after delivery) were recruited for human milk collection. Breast milk obtained from complete emptying of the gland in the morning and in the afternoon was pooled from each donor. Liquid commercial formula milk reported the following composition according to the nutrition label: fat 3.6%, total protein 1.4%, lactose 5.8%. Commercial bovine milk and caprine milk samples were purchased at a local store, while ovine milk and donkey milk were taken at a dairy farm located in Foggia (Apulian region, Italy) and pasteurised (63 °C for 30 min).

Milk samples were analysed for fat, protein, casein, and lactose content using infrared spectrophotometer (MilkoScan FT 120; Foss Electric A/S, Hillerød, Denmark). Each sample was analysed in duplicate.

For total fatty acids analysis in milk and digested milk, lipids were extracted according to O'Fallon et al. (2007). A calibration curve with a standard mixture of 50 fatty acids (GLC Reference standard 674, Nu-Check Prep, Inc. Elysian MN 56028, USA GLC standard Reference 674, Nu-Check Prep, Inc., Elysian MN 560, USA) and a standard of 12 conjugated linoleic acids (CLA) (C18:2 trans-8, cis-10; C18:2 cis-9, trans-11; C18:2 cis-11, trans-13; C18:2 trans-9, cis-11; C18:2 cis-8, cis-10; C18:2 cis-10, cis-12; C18:2 cis-9, cis-11; C18:2 trans-10, cis-12; C18:2 trans-8, trans-10; C18:2 trans-9, trans-11; C18:2 trans-10, trans-12; C18:2 trans-11, trans-13; GLC Reference standard UC-59M, Nu-Check Prep, Inc. Elysian MN 56028, USA) was used for quantitative determination of FAME. The fatty acids were separated using a capillary column (HP88; 100 m x 0.25mm i.d., 0.20 µm film thickness, Agilent Technologies Inc., Santa Clara, USA) installed on an Agilent Technologies 6890N GC equipped with a flame ionization detector (FID)

and a split injector. The temperature of the injector and FID detector was 250°C, while the temperature ramp of the column was the following (Eulitz et al., 1999): 70°C for 4 min, from 70°C to 175°C (13°C/min), maintained at 175°C for 27 min, from 175°C to 215°C (4°C/min), maintained at 215°C for 45 min. The injection was performed in split mode 1:20 injecting 1μL. The pressure of the carrier gas (helium) was maintained constant at 175 kPa.

Fatty acids were grouped into SFA, MUFA and PUFA according to the level of saturation. Atherogenic and thrombogenic indices were calculated according to Ulbricht & Southgate (1991).

***In vitro* digestion of milk samples**

All reagents used in the analyses were standard analytical grade. Enzymes were purchased from Sigma-Aldrich (S.r.l., Milan, Italy) and enzyme activities for the simulation of digestion fluid were determined according to standard protocols. Porcine Pepsin (EC 3.4.23.1) activity is based on determination of TCA soluble peptides released from haemoglobin detected by spectrophotometric assay; one unit of enzyme produces a ΔA280 of 0.001 at pH 2.0 at 37 °C. Porcine Pancreatine activity is based on Trypsin activity Assay (EC 3.4.21.4); it is based on continous spectrophotometric rate determination of TAME at 247 nm; one unit of enzyme hydrolyses 1 μmole of TAME per minute at pH 8.1 at 25°C. Bile salt concentrations are measured using a commercial kit (Total Bile Acid Assay Kit, STA-631 Cell Biolabs, Inc., San Diego, CA). Simulated salivary fluid at pH 7, gastric fluid at pH 3, and intestinal fluid at pH 7 were prepared whit stock solutions of electrolytes, enzymes, $CaCl_2$, and water according to Minekus et al. (2014) and reported in Table 1.

Table 1. Stock solutions of simulated digestion fluids: Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF). The volumes are calculated for a final volume of 500 mL for each simulated fluid (Adapted from Minekus et al., 2014).

			SSF pH7		SGF pH3		SIF pH7	
Constituent	**Stock conc.**		**Vol. of stock**	**Conc. in SSF**	**Vol. of stock**	**Conc. in SSF**	**Vol. of stock**	**Conc. in SSF**
	$g L^{-1}$	$mol L^{-1}$	mL	$mmol L^{-1}$	mL	$mmol L^{-1}$	mL	$mmol L^{-1}$
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	—	—	11.8	47.2	9.6	38.4
$MgCl_2(H2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	—	—
	$g L^{-1}$	$mol L^{-1}$		$mmol L^{-1}$		$mmol L^{-1}$		$mmol L^{-1}$
$CaCl_2(H_2O)_2$	44.1	0.3		1.5		0.15		0.6

Before *in vitro* digestion, all milk samples were homogenised and divided into aliquots of 5 ml and analysed in duplicate. The procedure was divided into three steps simulating the oral, gastric and intestinal phases according to Minekus et al. (2014). In the oral phase, 5 mL of milk was mixed with 3,5 mL of salivary fluid at pH 7 and 25 µl of 0,3 M $CaCl_2$, and the volume was up to 10 mL with distilled water; finally was added NaOH 1 M increasing pH to 7.0. The mixture was mixed and heated at 37°C for 2 minutes. The oral bolus (10 mL) during the gastric phase, was mixed with 7,5 mL of gastric fluid at pH 3, 1 mL of porcine pepsin stock solution (25000 U/mL^{-1}) made up in GF electrolyte solution, 5 µL of 0.3M $CaCl_2$; the mixture was up to 20 mL with distilled water, and finally was added HCl 1 M reducing pH to 3.0. Gastric phase was conducted in a shaking incubator at 37 °C for 2 h in order to simulate the stomach temperature and peristaltic movement. Finally, 20 mL of gastric chyme during the simulated intestinal phase was mixed with 11,5 mL of intestinal fluid at pH 7, 4 mL of pancreatine stock solution (800

U/mL^{-1}) made up IF electrolyte solution, 2,5 mL 160 mM fresh bile (porcine bile B8631 from Sigma-Aldrich), 40 µL 0,3 M $CaCl_2$ and volume was up to 40 mL with distilled water; was added NaOH 1 M increasing pH to 7.0. Intestinal phase was conducted in a shaking incubator at 37 °C for 2 h in order to simulate the intestine temperature and peristaltic movement. Modifications of the protocol used by Minekus et al. (2014), concerned the oral phase: α-amylase was not added to the salivary fluid due to the absence of starch in milk samples. Gastric and intestinal phases were conducted in a shaking incubator at 37 °C for 2 h. Samples were frozen immediately after analysis in liquid nitrogen to slow down enzymatic reactions, and analysed for fatty acids after 24 h.

Free fatty acid analysis in digested milk sample

Free fatty acids were extracted from digested milk samples according to Devle et al. (2014) and analysed with gas chromatography as previously described. Briefly, 20 mL $CHCl_3$: MeOH (2:1) was added to the lipid samples directly after digestion. Internal standards, dissolved in $CHCl_3$, were also added to the samples. The sample tubes were placed horizontally for 20 minute on an orbital shaker (Biosan Ltd., PSU 10i, Riga, Latvia) set at 350 rpm. NaCl (0.9%, 4 mL) was added to the tubes prior to centrifugation at 20 C for 5 min at 850 g (Beckman Coulter, Allegra 25R Centrifuge, TS-5.1-500 rotor head). The organic phase was transferred to 20 mL tubes (Büchi 20 150 mm, Flawil, Switzerland) and then evaporated by a Büchi Syncore Polyvap at 40 C. The lipids were re-dissolved in 1 mL chloroform and transferred to vials prior to SPE.

The SPE procedure was carried out by a liquid handling robot (Gilson, GX-274 ASPEC, Middleton, WI, USA) with a flow of 1.0 mL min^{-1}. The SPE columns (Chromabond NH2 polypropylene, MachereyeNagel, 500 mg, 3 mL, Dûren, Germany) were conditioned with 7.5 mL hexane. The extracted and re-dissolved lipids (500 mL) were applied to the SPE columns. The neutral lipids were eluted with 5 mL chloroform, the free fatty acids were eluted with 5 mL diethyl ether: acetic acid (98:2) and the phospholipids were eluted with 5 mL methanol. All

fractions were transferred to culture tubes (Duran 12 100 mm, Mainz, Germany) and evaporated under a stream of N_2-gas at 40 C. Neutral lipids fractions were re-dissolved by adding 2.0 mL hexane to the evaporated sample tubes. A sodium methanolate solution was made by dissolving metallic sodium (purum, Merck, Darmstadt, Germany) in methanol, to a concentration of 3.3 mg mL^{-1}. From this solution, 1.5 mL was added to each sample tube. The tubes were placed horizontally for 30 min on an orbital shaker (Biosan Ltd., PSU 10i) set at 350 rpm. The tubes were then placed in vertical position and left to settle for 10 min. The hexane phase was transferred to vials and stored at -20 C° prior to analysis by gas chromatography.

The free fatty acid and phospholipid fractions were each added to 1 mL of boron trifluoride e methanol complex (BF_3MeOH, 14%; SigmaeAldrich). Free fatty acid samples were heated at 70 C° in a water bath for 5 min. The phospholipid samples were heated at 100 C° in a water bath for 90 min. All free fatty acid and phospholipid samples were added to 1 mL of hexane. After mixing, the hexane layer was transferred to vials and stored at -20 C° until analysis by gas chromatography.

Results

Chemical composition of milk

The gross composition of milk from different species and the composition of infant formula are reported in Table 2. Fat content was about 2% in HM and below 1% in DM; regarding ruminant species, fat content was about 4% in bovine and caprine milk and higher than 8% in ovine milk. Casein fraction represented almost 80% of the total protein in ruminant species and about 40% in HM and DM. Lactose content showed levels of about 7% in HM and DM and values about of 4.5% in milk from ruminant species.

Table 2. Gross composition of milk from different species.

Parameter, %	Milk source[1]				
	HM	DM	BM	OM	CM
Fat	2.29 ± 0.12	0.53 ± 0.01	3.63 ± 0.12	8.16 ± 0.32	4.14 ± 0.15
Total Protein	1.21 ± 0.13	1.46 ± 0.04	3.53 ± 0.56	6.01 ± 0.48	3.22 ± 0.35
Casein	0.48 ± 0.21	0.57 ± 0.02	2.72 ± 0.10	4.56 ± 0.13	2.29 ± 0.12
Lactose	7.49 ± 0.13	6.78 ± 0.15	4.69 ± 0.10	4.37 ± 0.11	4.18 ± 0.12

n.d. = not detectable

1 HM=human milk; DM=donkey milk; BM=bovine milk; OM=ovine milk; CM=caprine milk

Fatty acids composition in milk and in digested milk

The effect of milk source on major groups of fatty acids is reported in Table 3. In general, differences emerged in the fatty acid profile of milk from different species and of formula milk. Grouping fatty acids according to the length of the carbon chain, it was evident that SC-FAwere higher in bovine and caprine milk, intermediate in ovine and donkey milk and lower in human and formula milk. MC-FA showed the highest values for bovine and caprine milk, intermediate in human and ovine milk and the lowest for donkey and formula milk. LC-FA were the highest in donkey and formula milk. Among SC-FA, capric acid was the most abundant in milk from ruminant species, particularly caprine, and from donkey milk whereas this fatty acid was about 5 and 13 time lower in human milk and infant formula than in bovine milk. Lauric acid was the most represented among the SC-FA in formula and in human milk (detailed FA composition of milk is reported in Table 4). Myristic acid and palmitic acid were the most represented in MC-FA and their value was about 3 and 2.5 times lower in infant formula and donkey milk compared to bovine milk, respectively. In general regarding LC-FA the most representatives were C18:0, C18:1 cis-9 and C18:2. In particular, donkey

milk was also characterised for a high content of C18:3n3 and C24:1. Furthermore human milk showed EPA and DHA values greater than milk from other species and formula milk.

Higher values of S-FA were found in caprine and bovine and lower for formula milk, maternal and donkey milk, intermediate for ovine milk. Infant formula showed the highest MU-FA, intermediate in human and donkey milk whereas lower values in milk from ruminant species. Lowest PU-FA was found in milk from ruminants, intermediate in formula and human milk and the highest in donkey milk. Atherogenic and Trombogenic were higher in milk from ruminant species than in the other milk sources. In particular, caprine milk showed the highest values whereas ovine milk was under the value of 3 for both indexes.

Table 3. Effect of milk source, on fatty acid composition in raw milk and on free fatty acids composition of digested milk.

	Milk source[1]													
	FM		HM		DM		BM		OM		CM		SEM	*Effect, P*[3]
MILK														
Fatty Acids[2], % FAME														
SC-FA	12.18	d	11.52	d	18.72	c	21.20	b	17.54	c	26.20	a	0.32	***
MC-FA	20.18	d	32.26	c	18.58	d	48.15	a	39.48	b	44.95	a	0.51	***
LC-FA	67.64	a	56.22	b	62.70	ab	30.65	d	42.98	c	28.85	d	0.74	***
S-FA	34.48	d	43.16	c	38.65	cd	73.90	a	65.15	b	77.26	a	0.73	***
MU-FA	41.51	a	37.36	b	32.18	c	22.25	e	27.21	d	19.15	e	0.70	***
PU-FA	24.14	b	19.62	c	29.47	a	3.91	d	7.69	d	3.64	d	0.64	***
	FM		HM		DM		BM		OM		CM		SEM	*Effect, P*[3]
DIGESTED MILK														
Fatty Acids[2], μg/mL of extract														
SC-FFA	31.90	a	36.33	a	14.28	b	32.74	a	43.42	a	24.45	b	4.67	*
MC-FFA	78.40	c	125.88	b	136.68	a	127.46	b	108.82	b	121.04	b	3.75	***
LC-FFA	84.23	b	120.47	a	98.18	b	84.35	b	97.84	b	91.04	b	6.28	*
S-FFA	163.83		231.67		240.50		229.88		218.79		202.90		10.48	NS
MU-FFA	12.32	b	35.00	a	2.60	c	4.64	c	11.73	b	10.43	b	2.51	***
PU-FFA	18.39		16.00		6.03		9.98		19.56		22.92		5.75	NS
Total FFA	194.53	c	282.68	a	249.14	b	244.54	b	250.08	b	236.52	b	13.97	**

[1]FM = formula milk; HM = human milk; DM = donkey milk; BM = bovine milk; OM = ovine milk; CM = caprine milk.

[2]SC-FA = short chain fatty acids; SC-FFA = short chain free fatty acids; MC-FA = medium chain fatty acids; MC-FFA = medium chain free fatty acids; LC-FA = long chain fatty acids; LC-FFA = long chain free fatty acids; S-FA = saturated fatty acids; S-FFA = saturated free fatty acids; MU-FA = monounsaturated fatty acids; MU-FFA = monounsaturated free fatty acids; PU-FA = polyunsaturated fatty acids; PU-FFA = polyunsaturated free fatty acids; Total FFA = total free fatty acids.

[3]NS, $P < 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4. Effect of milk source on fatty acid composition (% of FAME).

	Milk source[1]							
Fatty Acids[2]	FM	HM	DM	BM	OM	CM	SEM	*Effect, P*[3]
C4:0	1.06 bcd	0.76 cd	n.d.	4.25 a	1.37 bc	2.09 b	0.17	***
C6:0	0.15 d	0.34 cd	0.56 c	4.15 a	2.81 b	3.06 b	0.05	***
C8:0	0.79 d	0.33 d	4.64 a	2.36 c	2.46 c	3.30 b	0.05	***
C10:0	0.82 f	2.22 e	7.91 b	5.11 d	6.73 c	11.40 a	0.07	***
C11:0	0.02 b	0.03 b	n.d.	0.11 a	n.d.	n.d.	0.002	***
C12:0	9.34 a	7.84 b	5.62 cd	5.21 d	4.17 e	6.34 c	0.07	***
C14:0	3.61 d	7.24 c	3.68 d	14.02 a	12.29 b	11.93 b	0.14	***
C14:1	n.d.	n.d.	0.01 b	0.71 a	0.64 a	0.01 b	0.01	***
C15:0	n.d.	0.10 b	n.d.	n.d.	n.d.	0.56 a	0.01	***
C15:1	0.01 b	0.02 a	n.d.	n.d.	n.d.	n.d.	0.001	***
C16:0	16.40 cd	21.82 bc	13.35 d	31.60 a	26.00 b	32.07 a	0.55	***
C16:1	0.16 e	3.08 a	1.54 c	1.82 b	0.55 d	0.38 de	0.03	***
C17:0	0.01 e	0.15 d	0.03 e	0.50 b	0.74 a	0.38 c	0.01	***
C17:1	0.01	n.d.	0.01	0.01	n.d.	n.d.	0.001	**
C18:0	1.95 d	2.05 d	0.17 e	6.41 b	8.29 a	5.98 c	0.02	***
C18:1trans-9	0.02 cd	0.05 cd	0.16 bc	0.28 ab	0.02 d	0.31 a	0.02	***
C18:1trans-11	0.18 c	0.17 c	n.d.	1.45 b	2.60 a	0.13 c	0.01	***
C18:1cis-9	40.77 a	33.63 b	15.68 e	17.74 d	23.28 c	17.93 d	0.20	***
C18:2trans-9,trans-12	n.d.	n.d.	0.14 b	0.02 d	0.60 a	0.06 c	0.003	***
C18:2cis-9,cis-12	20.26 a	14.84 b	9.73 c	1.34 d	4.16 d	2.22 d	0.43	***
C20:0	0.11 b	0.06 b	1.58 a	0.09 b	0.22 b	0.09 b	0.03	***
C18:3n-6	0.14 b	0.50 b	1.60 a	0.05 b	0.08 b	0.08 b	0.07	***
C18:3n-3	2.60 b	0.82 c	11.18 a	0.97 bc	1.06 bc	0.41 c	0.16	***
C20:1	0.13 ab	0.13 ab	0.29 a	0.06 b	0.05 b	0.05 b	0.02	**
CLA cis-9, trans-11	0.02 e	0.12 d	n.d.	0.95 b	1.28 a	0.45 c	0.01	***
CLA trans-10,cis-12	n.d.	0.01	n.d.	0.01	0.01	n.d.	0.002	NS
CLA cis-9,cis-11	0.04	0.04	n.d.	0.10	0.03	0.02	0.01	NS
C21:0	0.08 a	0.01 b	n.d.	0.01 b	n.d.	0.01 b	0.004	**
CLA trans-9, trans-11	0.03 b	0.10 b	1.53 a	0.05 b	0.01 b	0.08 b	0.04	***
C20:2n-6	0.25 b	0.14 b	0.85 a	0.04 b	0.05 b	0.05 b	0.04	***
C22:0	0.02 b	0.03 b	0.22 a	0.02 b	0.04 b	0.02 b	0.01	***
C20:3n-6	0.01 b	0.53 a	0.26 b	n.d.	n.d.	0.01 b	0.02	***
C22:1	n.d.	0.10 b	2.08 a	0.11 b	n.d.	0.17 b	0.04	***
C20:4n-6	0.10 b	1.06 a	0.08 b	0.02 b	n.d.	0.01 b	0.03	***
C20:3n-3	0.11 b	0.04 b	0.88 a	0.13 b	0.25 b	0.04 b	0.04	***
C23:0	0.06	0.01	0.11	0.04	n.d.	n.d.	0.02	NS
C22:2n-6	0.16 b	0.12 b	3.22 a	0.05 b	0.05 b	0.09 b	0.08	***
C20:5n-3	0.08 ab	0.22 a	n.d.	0.17 a	0.10 ab	0.11 ab	0.02	*
C24:0	0.06 b	0.18 b	0.79 a	n.d.	0.03 b	0.02 b	0.02	***
C24:1	0.10 b	0.04 b	12.12 a	0.01 b	0.01 b	0.12 b	0.72	*
C22:6n-3	0.34 b	1.08 a	n.d.	0.01 c	0.01 c	0.01 c	0.01	***
Ar.I.	0.61 e	1.03 d	0.55 e	3.55 b	2.27 c	3.78 a	0.02	***
T.I.	0.54 e	0.92 d	0.28 f	3.11 b	2.19 c	3.84 a	0.03	***

[1]FM = formula milk; HM = human milk; DM = donkey milk; BM = bovine milk; OM = ovine milk; CM = caprine milk.

[2] n.d. = not detectable for value < 0.01; Ar.I. = atherogenic index; T.I. = thrombogenic index.

[3] NS, P < 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001

In order to evaluate the impact of *in vitro* digestion on the liberation of free fatty acids from different milk sources, comparison of percentage distribution of free fatty acids in digested milk and total fatty acids distribution of the corresponding milk sources was done and the rate of liberation of fatty acids was calculated as the per cent ratio of free fatty acids and total fatty acids content. The percentage distribution of total S-FA, MU-FA and PU-FA in milk and the percentage distribution of free S-FA, MU-FA and PU-FA in digested milk from different sources are reported in Figure 1. FM, HM, and DM showed a major liberation of S-FA vs. MU-FA and PU-FA. On the contrary milk from ruminant species evidenced a major contribute of S-FA and the liberation of MU-FA after digestion turned out to be limited with an average percentage of 3%.

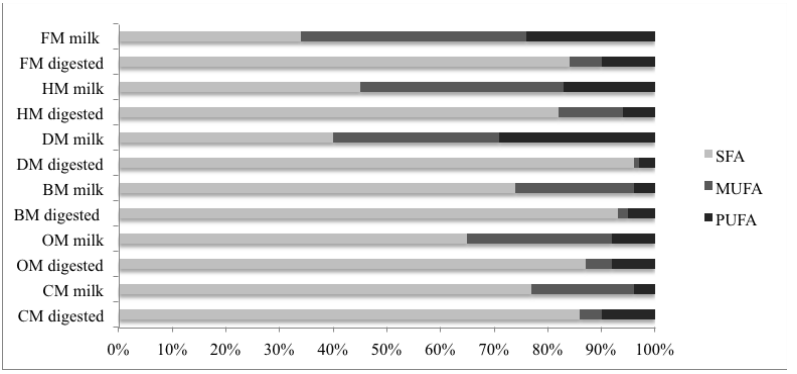

Figure 1. Percentage distribution of total S-FA, MU-FA and PU-FA in milk and percentage distribution of free S-FA, MU-FA and PU-FA in digested milk from different sources.

FM=formula milk; HM=human milk; DM=donkey milk; BM=bovine milk; OM=ovine milk; CM=caprine milk; saturated fatty acids (S-FA); monounsaturated fatty acids (MU-FA); polyunsaturated fatty acids (PU-FA).

The effect of milk source on major groups of FFA of digested milk is reported in Table 3. The FFA grouped according to the length of the carbon chain highlighted significant differences in short-, medium-, and long-chain FFA. Regarding the SC-FFA, donkey and caprine milk showed the lowest content and C6:0 was not detected at all (detailed FFA composition of digested milk is reported in Table 5). Furthermore, the highest level of C12:0 was found in human milk upon digestion whereas intermediate level was ascribed to formula milk.

Table 5. Effect of milk source in free fatty acids of digested milk (µg/mL of extract).

	Milk source[1]							
Free Fatty Acids[2]	FM	HM	DM	BM	OM	CM	SEM	*Effect, P*[3]
C6:0	4.91 ab	0.43 ab	n.d. b	6.99 ab	9.44 a	n.d. b	1.21	*
C8:0	4.85	3.50	4.93	6.69	12.22	5.17	0.91	NS
C10:0	7.11	10.72	5.99	12.03	15.61	11.47	1.79	NS
C12:0	15.02 b	21.68 a	3.36 c	7.03 c	6.15 c	7.82 c	1.40	**
C14:0	4.93 b	11.00 a	5.20 b	9.85 ab	8.88 ab	11.32 a	0.90	**
C16:0	73.47 c	111.74 b	131.46 a	117.14 b	99.93 b	108.96 b	3.07	***
C16:1	n.d.	3.14 a	n.d.	0.42 b	n.d.	0.48 b	0.15	***
C18:0	44.78 c	66.13 b	89.56 a	69.30 b	62.32 b	56.84 bc	2.87	***
C18:1trans-11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	
C18:1cis-9	6.90 b	20.97 a	2.60 b	3.94 b	3.74 b	3.18 b	1.40	***
C18:2cis-9,cis-12	0.43 b	1.04 a	0.16 b	0.14 b	0.29 b	0.14 b	0.08	***
C20:0	5.06	3.26	n.d.	n.d.	3.57	n.d.	0.76	NS
C18:3n-6	0.80	0.79	n.d.	n.d.	n.d.	n.d.	0.17	NS
C18:3n-3	n.d.	1.90	5.87	2.90	1.96	5.90	1.03	NS
C20:1	1.62	1.57	n.d.	n.d.	1.62	n.d.	0.80	NS
C20:2n-6	4.76 b	5.00 ab	n.d.	1.47 b	7.19 ab	9.17 a	1.21	*
C22:0	1.61 a	1.66 a	n.d.	0.86 ab	0.67 b	1.34 ab	0.17	**
C20:3n-6	1.05	1.26	n.d.	1.06	0.75	0.75	0.21	NS
C22:1	3.64	1.40	n.d.	0.29	0.68	1.21	0.94	NS
C20:4n-6	8.79	4.08	n.d.	4.42	4.78	1.95	2.64	NS
C20:3n-3	0.38	0.67	n.d.	n.d.	n.d.	1.91	1.17	NS
C22:2n-6	2.10	0.00	n.d.	n.d.	3.93	2.82	0.57	NS
C20:5n-3	0.00	0.89	n.d.	n.d.	0.55	n.d.	0.17	NS
C24:0	2.08	1.55	n.d.	n.d.	n.d.	n.d.	0.65	NS
C24:1	0.16 b	7.93 a	n.d.	n.d.	5.69 a	5.56 a	0.69	*
C22:6n-3	0.08	0.36	n.d.	n.d.	0.12	0.30	0.09	NS

[1]FM = formula milk; HM = human milk; DM = donkey milk; BM = bovine milk; OM = ovine milk; CM = caprine milk.

[2]n.d. = not detectable for value < 0.01.

[3]NS, P < 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001

Medium-chain FFA showed the highest and the lowest values in donkey and in formula milk, respectively; accordingly C16:0 showed the same behaviour, representing the most abundant among MC-FFA. Furthermore, free C14:0 had the highest values in human and caprine milk, whereas C16:1 was detected only in human, bovine, and caprine milk with the highest level found in the former milk. The total LC-FFA showed the highest value in human milk which also showed the highest content for C18:1 cis-9, C18:2 cis-9, cis-12, C22:0, C24:1. Apart from the milk source analysed, C18:0 represented more than 50% of the whole class of LC-FFA. In particular, in donkey milk almost the total LC-FFA was represented by stearic acid. Monounsaturated FFA were significantly different in the digested milks, with the highest value found in human milk with the major contribute of oleic acid. This FFA was six times higher in human than in digested milk from animal species, and about three fold higher than in formula milk. Furthermore, it is worth noting that, although the difference was not significant, EPA and DHA were always found in human digested milk and were absent from bovine and donkey milk. Finally the total FFA were highest in human milk, lowest in formula milk and intermediate in donkey, bovine, ovine, and caprine milk.

Discussion

Chemical composition of milk

In general, data for chemical composition of milk from different species were in accordance with reference literature (Gantner et al. 2015; Claeys et al. 2014); however, fat content of human milk from the present study showed lower values than previously reported for the same milk. It is known that in human milk fat varies much more than any other major constituents due to stage of lactation, prematurity, changes during a feed and during the day, age and parity and especially due to maternal diet and weight gain during pregnancy. In general human and donkey milk showed a closer similarity, whereas milk from ruminant species showed mean values of fat and protein double those found in human and donkey milk; in particular, ovine milk was confirmed to be the richest in both

nutritional components. The lower casein content in human milk is in accordance with its peculiar protein distribution. Non-protein nitrogen is a remarkable fraction in human milk compared to other milk source, representing over 20–25% (Armaforte et al. 2010) and consists mainly of free amino acids and urea, peptides and ammonium, polyamines and nucleotides (Claeys et al. 2014). Finally lactose is the most abundant carbohydrate, whatever milk source, providing energy, and usually it is added into formula to standardise the lactose concentration into the human milk range (Zou et al. 2017).

Fatty acids composition in milk

The fatty acid pattern in human milk derives from endogenous synthesis in the mammary gland and uptake from maternal plasma and both sources are influenced by maternal nutrition (Innis, 2014). Fatty acid composition of milk fat in non-ruminant species tends to only reflect the lipid composition of diet, whereas diet has a minor impact on fatty acid profile in milk from ruminant species due to the ruminal biohydrogenation processes that modify the fatty acid profile of base food. It is worth noting that among ruminant species, PU-FA in ovine milk showed a mean value double that in caprine and bovine milk likely due to the feeding system being based on pasture and fresh forage (Nudda et al. 2014) and to the specific activity of Δ9 desaturase.

Human milk contains naturally long chain PU-FA n-3 and n-6 series and formula milk is generally enriched with essential nutrients such as EPA and DHA, with beneficial effects on proper brain and visual development in the fetus and maintenance of neural and retinal photoreceptors throughout life (Zou et al. 2017). Apart from the milk source analysed, the most represented LC-FA C18:1cis-9 and C18:2 are of interest in human nutrition. The former is an important source of energy for the baby, and the latter an essential fatty acid precursor of other long chain fatty acids (Lopez-Huertas, 2010). As a consequence of fatty acid profile atherogenic and thrombogenic indexes were more favourable to human nutrition in formula, human and donkey milk.

Fatty acids composition in digested milk

Lipid digestion is characterised by different steps which occur firstly in the stomach and secondly in the small intestine where the bile salts emulsify intermediate products, making them available to pancreatic lipases that hydrolyse the bonds in the sn-1 and sn-3 position by releasing a 2- monoacylglycerol (Gallier et al. 2013a; Devle et al. 2014). In all the milk sources subjected to the study, the percentage distribution of fatty acids liberated upon gastrointestinal digestion did not reflect the patterns found in the corresponding milk sources. In particular the most important changes were observed in FM, HM, and DM with a major liberation of S-FA, whereas comparable fatty acids profiles were observed in milk and in the corresponding digested milk from ruminant species, where the predominant group is always represented by S-FA.

Differences in the FFA of digested milk sources could be attributed to multiple factors concerning the interaction between milk substrate and digestion process, such as the presence of endogenous enzymes to milk, the different structure and organisation of the milk fat globules, and the position of bounds in TAG. However, similarities between acidic profile of human milk and milk from other animal sources after digestion are relevant for the industrial exploitation of the latter milk sources for their use as basic components in infant formula more respondent to human milk profile.

Milk, and particularly human milk, contains lipolytic enzymes including LPL and BSSL. LPL is an enzyme similar to pancreatic lipase, which is stimulated by bile salts (de Oliveira et al. 2016). Both LPL and BSSL are considered to be crucial for the digestion of lipids by human babies, as they secrete low levels of both pancreatic lipase and bile salts (Fox & Kelly, 2006). In contrast to human milk, infant formula is devoid of any BSSL activity and a consequent reduction in hydrolysis ester bonds of TAG may be expected (Martin et al. 1993). The size and structure of the fat globules affect digestion; the smaller the fat globules the more efficient are the process (Claeys et al. 2014). On the other hand, the size and dispersion of the fat globules confer greater consistency to goat and sheep milk

favoring freezing without phase separation (Balthazar et al. 2017), with positive implications on their industrial exploitation. In particular, goat milk has smaller globules (diameter of about 3 μm) than sheep (about 3.5 μm) and bovine (about 4.8 μm) (Gantner et al., 2015); human milk has similar dimensions to donkey milk (about 4 μm) while infant formula is characterised by a very small dimension of milk fat globules (about 0.4 μm) (Nguyen et al. 2015). The structural characteristics of MFGM are another notable factor that influences the different digestibility of fat. The MFGM in human and donkey milk consists of three layers, the internal protein layer, intermediate phospholipids layer and the external layer of glycoproteins on which are bound branched oligosaccharides (Ye et al. 2011). In bovine milk the external layer is further coated with phospholipids, and pancreatic lipase has more difficulty reaching the triglyceride core (Gallier et al. 2013a, b). Recent reports highlight the negative consequences on infant's health of formula supplemented with vegetable oil as a major source of fat, since these formulas specifically lack MFGM (Zou et al. 2017).

The S-FA in human milk are located mainly in sn-2 position which is unfavourable for the action of lipolytic enzymes. Accordingly, the rate of liberation of C12:0, C14:0, C16:0, and C18:0 was lower than that reported for the corresponding fatty acids in milk from ruminant species. However, the highest level of C12:0 in human milk upon gastro-intestinal digestion and the intermediate level in formula milk were in accordance with the higher content of lauric acid in the corresponding milk sources. The amount of free S-FA in digested human milk may be a balance between the preferential position occupied by those fatty acids and the size of the fat globules. It is reported that in human milk C16:0 is located mainly in the sn-2 position whereas in bovine, ovine, and caprine milk it is primarily found in sn-1 position (Claeys et al. 2014); however the 2-monoglycerides with palmitic acid at the sn-2 position are easier to absorb by infant than free fatty acids (Sidnell & Greenstreet, 2011). The highest level C16:1 found in human milk may be ascribed to the sn-1 and sn-3 position in TAG. Overall, the physiological significance of MC-FA relys on their rapid hydrolysis

and, absorption by the epithelial barrier and their transport through the bloodstream where they are rapidly catabolised (Mills et al. 2011). The higher level of LC-FFA found in human milk maybe ascribed to the binding site at sn-3 position whereas it is reported that mainly SC-FA and MC-FA are bound at the sn-3 position in bovine milk (Nguyen et al. 2015).

Conclusion

The analysis of the fatty acid profile in human milk, formula milk and different animal species showed that SC-FA were higher in bovine and caprine milk, intermediate in ovine and donkey milk, and lower in human and formula milk. The group of MC-FA showed the highest values for bovine and caprine milk and the lowest for donkey milk and formula milk, whereas LC-FA were the highest in donkey and formula milk and intermediate in human milk. Infant formula and human milk showed higher percentage of MU-FA and PU-FA than other milk sources.

The percentage distribution of fatty acids liberated upon gastrointestinal digestion did not reflect the patterns found in the corresponding milk sources likely due to the different susceptibility of milk sources to the action of the gastrointestinal enzymes used in the *in vitro* digestion process. The amount of total FFA was highest in human milk, lowest in formula milk and intermediate in donkey, bovine, ovine, and caprine milk. In particular, the total LC-FFA and MU-FFA were highest in human milk due to the major contribute of oleic acid.

The study of fatty acid pattern liberated upon milk digestion is useful in the exploitation of alternative milk sources for design and optimisation of innovative substitutes in infant feeding and may be further associated to the study of fatty acids bioavailability upon intestinal absorption.

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3. SECTION II

3.1 Childhood epilepsy and nutrition

Epilepsy is one of the most common chronic childhood diseases and is the fourth most common neurological problem, only migraine, stroke and Alzheimer's disease occurs more frequently. It affects about 65 million people throughout the world and 10.5 million of who are children under 15 years of age. The World Health Organization found the average incidence of epilepsy to be 6/1000 in developed countries and 18.5/1000 in developing countries (www.epilepsy.com/learn/about-epilepsy-basics - Access date: 04.01.2019; İşler et al., 2014). The epilepsies represent a spectrum of brain disorders ranging from severe, life-threatening and disabling, to ones that are much more light. During epilepsy seizures, the normal pattern of neuronal activity becomes disturbed, causing strange sensations, emotions, and behavior or sometimes convulsions, muscle spasms, and loss of consciousness. The causes of epilepsies are many and get involved in several types of seizures; may due by an abnormality in brain wiring, an imbalance of nerve signaling chemicals called neurotransmitters, or by changes in important features of brain cells called channels, or by some combination of these and other factors. That the causes of the disturb of the normal pattern of neuron activity is illness, brain damage or abnormal brain development, this can lead in any way to seizures (www.ninds.nih.gov/Disorders/All-Disorders/Epilepsy-Information-Page -Access date: 04.01.2019). When a person has a single seizure as the result of a high fever or head injury does not necessarily mean that has epilepsy; it is necessary that the seizures repeat separated by at least 24 hours.

The basic classification according to International League Against Epilepsy (ILAE) criteria is based on 3 key features: i) where seizures begin in the brain; ii) the level of awareness during a seizure; iii) the features of seizures. Seizures are separate for the where they begin in, focal (seizures which start in an area or network of cells on one side of the brain), generalized seizures (that involve networks on both sides of the brain at the onset), unknown onset seizures (the onset of a seizure is unknown), focal to bilateral seizure (that starts in one side or

part of the brain and spreads to both sides). For the describe awareness the groups are focal aware (the awareness remains intact during a seizure), focal impaired awareness (the awareness is affected at any time during a seizure), awareness unknown (sometimes it's not possible to know if a person is aware or not), generalized seizures (thus no special terms are needed to describe awareness in generalized seizures). Other symptoms may occur during a seizure and on basis of these, seizure behaviors are separated into groups that involve movement in focal motor seizure (some type of movement occurs during the event such as twitching or jerking), focal non-motor seizure (other symptoms occur first, such as changes in sensation, emotions, thinking, or experiences), auras (describes symptoms a person may feel at the beginning of a seizure).

About generalized onset seizures can be motor or non-motor. Generalized motor seizure uses the generalized tonic-clonic seizure term to describe seizures with stiffening (tonic) and jerking (clonic). Tonic seizures cause stiffening of muscles of the back, legs, and arms while clonic seizures cause repeated jerking movements of muscles on both sides of the body and the movements cannot be stopped by restraining the limbs. Tonic-clonic seizures cause a combination of symptoms, including stiffening of the body and repeated jerks of the arms and legs as well as loss of consciousness. Myoclonic seizures cause brief shock-like jerks of a muscle or group of muscles. This group of symptoms typical of generalized motor seizure corresponds to "grand mal". Generalized non-motor seizures are primarily absence seizures, and the term corresponds to the old term "petit mal." Finally, atonic seizures cause a loss of normal muscle tone, which leads to fall down or drop the head involuntarily.

Secondarily generalized seizures, they only become generalized (spread to both sides of the brain) after the initial event (a partial seizure) has already begun (www.epilepsy.org - Access date: 04.01.2019). Berg et al. (2010) reported a classification in a table shown below (Table 1).

Table 1. The list of recognized seizure types, reported by Berg et al. (2010).

Table 1. Classification of seizures[a]

- Generalized seizures
  - Tonic–clonic (in any combination)
  - Absence
    - Typical
    - Atypical
    - Absence with special features
      - Myoclonic absence
      - Eyelid myoclonia
  - Myoclonic
    - Myoclonic
    - Myoclonic atonic
    - Myoclonic tonic
  - Clonic
  - Tonic
  - Atonic
- Focal seizures
- Unknown
  - Epileptic spasms

[a]Seizure that cannot be clearly diagnosed into one of the preceding categories should be considered unclassified until further information allows their accurate diagnosis. This is not considered a classification category, however.

The debilitating CNS disorders like epilepsy, brain tumors, HIV encephalopathy, neurodegenerative disorders, distress a high number of people, higher then those are affected by systemic cancer or cardiovascular diseases (Nasreen et al., 2015).

For these disorders there are not a specific therapy, only medication or drugs to alleviate the symptoms can use, but no one of these can stop definitively disease progression.

However, our body implements defensive systems to protect the most delicate organs such as the brain; a series of structute was generated by central nervous sistem to preserve itself by neurotoxic molecules and circulating blood cells or pathogens. These barriers are rapresented by CSF barrier, the BBB, the blood-retinal barrier and the blood-spinal cord barrier, and show various degrees of permeability (Saraiva et al., 2016)

The BBB is a complex multicellular structure morphologically is constitute by of nonfenestrated endothelial cells, basement membrane, glial cells such as astrocytes and pericytes, and inter-endothelial tight junctions that have the task to separate the CNS from the systemic circulation.

Under normal physiologic conditions, the BBB is the most extensive and exclusive barrier among those of CNS able to protect it by pathogens and neurotoxic molecules and to regulate the entry of plasma-born substances and immune cells into the brain and into the nervous tissue.

The CNS injuries like seizures, infections, traumatic and ischemic events, cause transient changes in the physiologic and structural features of the BBB (Zucker et al., 1983) impairing the BBB integrity and inducing an inflammatory state that is a common features of several neurologic conditions (Krizanac-Bengez et al., 2004) associated with the late onset of epilepsy. During inflammation a series of events like enhanced production of cytokines by the endothelial cells of the BBB, the upregulation of adhesion molecules of the BBB, the activation of metalloproteinases, and the catabolism of arachidonic acid at the level of the brain, cause the increase of the permeability of the BBB, but the mechanism of how it happens, is not yet fully clarified (Lossinsky et al., 2004). The phenomena of neuroinflammation and neurodegeneration may develop as a consequence of failure in maintaining intact the BBB (Vezzani et al., 2008).

Numerous study in animal model evidenced a complex linkage between epilepsy and the immune system; indeed were observed an anomaly in the expression of cytokines and immune cells in patients that has epilepsy especially in proinflammatory conditions.

During epileptic seizures, the immunity system and its association with inflammatory reactions seem to play an important role, highlighting the production of cytokines as mediators of spontaneous seizures (Li et al., 2011). Cytokines more studied that mainly are involved in epilepsy are IL-1β, IL-6, and TNFα. After seizures, either generalized tonic-clonic or complex partial the level of some cytokines increase quickly to return to baseline in fluctuate time intervals.

However, during epilepsy events the expression of cytokines grows in the brain but also change the peripheral cytokine levels.

Available studies highlight the ambiguous role of IL-1β, IL-6 and TNFα in epileptogenesis because these molecules have demonstrated pro- and anticonvulsive properties in different animal studies. Remain a certain difficulty to extrapolate the results of cytokines studies in human epilepsies because in epileptic patients the cytokine detection is often limited in blood (Li et al., 2011).

In many cases epilepsy seizures are provoked by equivocal external or internal stimuli such as sleep deprivation, excess alcohol intake, premature awakening, menstruation, psychological stress, and photic stimulation, in some cases also simple action like reading, thinking, writing, calculating, and playing musical instruments can trigger seizures.

It is well known that much physical illness is often associated with dietary risk factors however a growing interest is born for the relationship between diet and risk of mental illness.

Many studies reported some healthy dietary patterns are associated with mental health and reduced risk of cognitive damage (Parletta et al., 2013). Epilepsy is one of those mental disorders that is often associated with diet. Several reports suggesting that some foods trigger seizures, for example, the consumption of large amounts of Gingko nuts can trigger convulsions like also monosodium glutamate can induce convulsions in rats (Miwa et al., 2001; Bhagavan et al., 1971), while in rats feed with an excess of dietary amino acids was highlighted a reduction of seizures (Asadi-Pooya et al., 2008).

It is well known that milk proteins play an important role in the ability to stimulate innate immune response through the activation of pro- and anti-inflammatory cytokines (Albenzio et al., 2012) and these molecules rapidly increase in the peripheral blood. Milk is the principal food for human newborns, and fulfills nutritional needs and ensures safe development and growth during the first stages of life. More studies are necessary to understand if milk proteins can express a

response pro- or anti-inflammatory of the immune system and if this can trigger seizures in the child.

3.2 Milk from different species: Relationship between protein fractions and inflammatory response in infants affected by generalized epilepsy

J. Dairy Sci. 99:5032–5038
http://dx.doi.org/10.3168/jds.2015-10704

Milk from different species: Relationship between protein fractions and inflammatory response in infants affected by generalized epilepsy

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Abstract

The present study was undertaken to evaluate the effect of protein fractions from bovine, caprine, and ovine milk on production of cytokines and ROS and RNS by cultured PMBC from infants with generalized epilepsy. Bovine, caprine, and ovine bulk milks were pasteurized and analyzed for chemical composition. Then, PBMC were isolated from 10 patients with generalized epilepsy (5 males; mean age 33.6 ± 5.4 mo). Production of TNF-α, IL-10, IL-6, and IL-1β was studied in cultured PBMC (from infants with epilepsy and controls) stimulated by bovine, caprine, and ovine milk and casein and whey protein fractions, and levels of ROS and RNS were measured in the culture supernatant. The ability of PBMC to secrete cytokines in response to milk and protein fraction stimulation may predict the secretion of soluble factor TNF-α in the bloodstream of challenged patients. Bovine, caprine, and ovine bulk milks induced low-level production of IL-10 by cultured PBMC in at least 50% of cases; the same behavior was observed in both casein and whey protein fractions for all species studied. Bovine and ovine milk and their casein fractions induced production of lower levels of IL-1β in 80% of patients, whereas caprine milk and its casein fraction induced the highest levels in 80% of patients. The amount of IL-6 detected after stimulation of PBMC by milk

and its fractions for all species was lower than that of other proinflammatory cytokines. In the bovine, total free radicals were higher in bulk milk and lower in the casein fraction, whereas the whey protein fraction showed an intermediate level; in caprine, ROS/RNS levels were not different among milk fractions, whereas ovine had higher levels for bulk milk and casein than the whey protein fraction. Lower levels of ROS/RNS detected in PBMC cultured with caprine milk fraction could be responsible for the lower levels of TNF-α cytokine in the corresponding fraction. Cytokines might be useful biomarkers to discriminate the effects of foods on the inflammatory response; dietary strategies could help in alleviating the negative effects of epilepsy in infants.

Introduction

Milk composition of the principal dairy species varies widely in terms of genetic, physiological, and nutritional factors, and environmental conditions. Milk protein is a very heterogeneous group of molecules mainly influenced by genetic variants (Ng-Kwai-Hang and Grosclaude, 2003). Genetic polymorphism varies among ruminant species and is associated with different level of protein synthesis in milk, different rates of phosphorylation and glycosylation of the peptide chain, and different AA sequences of the protein (Michaelidou, 2008).

Epilepsy comprises a group of neurological disorders characterized by the periodic occurrence of spontaneous seizures (Vezzani et al., 2008); the World Health Organization estimates that it affects 0.8% of the world's population (Li et al., 2011). Several reports suggest that certain foods might trigger seizures; studies using animal models reported a reduction in seizure threshold in rats administered excess dietary AA and the induction of convulsions by monosodium glutamate (Asadi-Pooya et al., 2008). Furthermore, in recent study, at least 30% of children with intractable epilepsy had intakes below the recommended dietary allowance for vitamins D, E, and K, folate, calcium, and linoleic acid (Volpe et al., 2007). Gordon and Dooley (2015) conducted a cross-sectional survey on food insecurity

and health status and concluded that the experience of food insecurity appears to be more frequent among persons living with epilepsy.

In human newborns, milk fulfils nutritional needs and ensures safe development and growth during the first stages of life. Recently, the role of animal food products on diet has been widely recognized, with particular regard on the effects of protein fractions and human health. It is well known that milk proteins play an important role in the ability to stimulate innate immune response through the activation of pro- and antiinflammatory cytokines (Albenzio et al., 2012); these molecules rapidly increase in the peripheral blood. In recent years, an increasing body of evidence has indicated a complex relationship between epilepsy and the immune system (Li et al., 2011). Abnormalities in expression of cytokines and immune cells have been observed in patients with epilepsy and in animal models (Plata-Salamán et al., 2000; Ravizza and Vezzani, 2006). Oxidative stress is known to occur in the pathogenesis of the most prevalent form of epilepsy and contributes to acute injury-induced neuronal damage (Pearson et al., 2015).

To the best of our knowledge, no studies have been reported on the role of milk protein fractions from different ruminant species on immune status of infants with epilepsy. Therefore, the present study was undertaken to evaluate the effect of milk protein fractions from different animal species bovine, ovine, and caprine on production of pro- and antiinflammatory cytokines and reactive oxygen species by cultured peripheral blood mononuclear cells from infants with generalized epilepsy.

Materials and Methods

Experimental Design and Milk Sampling

Pasteurized bovine, caprine, and ovine bulk milks were lyophilized (lyophilization cycle −50°C for 24 h; Lio5P, 5 Pascal s.r.l., Trezzano, Milan, Italy) and the resulting lyophilized powders stored at 4°C. Bovine, caprine, and ovine milk samples were analyzed for chemical composition using an infrared

spectrophotometer (Milko Scan 133B; Foss Electric, Hillerød, Denmark). Casein and whey protein fractions were obtained according to IDF (1993).

Patients

Ten patients with generalized epilepsy (5 males; mean age 33.6 ± 5.4 mo) and 10 control (5 males; mean age 35.6 ± 6.8 mo) were recruited at the Complex Structure of Neuropsychiatry Childhood– Adolescence of Ospedali Riuniti (Foggia, Italy) and were included in the study.

Inclusion criteria were diagnosis of active and unambiguous focal or generalized epilepsy according to International League Against Epilepsy (ILAE) criteria (Berg et al., 2010) with no family history of epilepsy without any other concomitant systemic, neurological, or psychiatric diseases and without any abnormalities on neuroradiological investigations.

Exclusion criteria were occurrence of clinical seizures within the last 3 d before blood drawing, malignant tumor, concomitant inflammatory disease, severe neurological or neuroimmunological disease (i.e., stroke, cerebral hemorrhage, encephalitis, meningitis), immunosuppressive or immunomodulatory treatment during the last 6 mo, surgery or significant trauma within the last 2 wk, hepatic or renal insufficiency, or severe psychiatric disease.

Approval was obtained from the Ospedali Riuniti (Foggia, Italy) Institutional Review Board for these studies. Written informed consent was obtained from the parents in accordance with the Declaration of Helsinki on the Ethical Principles for Medical Research Involving Human Sub jects (http://www.wma.net/ en/30publications/10policies/b3/17c.pdf).

Production of TNF-α, IL- 10, IL-6, and IL-1β was studied in PBMC stimulated with bovine, caprine, and ovine milk and casein and whey protein fractions. The PBMC were obtained from infants with generalized epilepsy and from control infants. The PBMC were isolated from heparinized blood (3–5 mL) by gradient centrifugation in Ficoll-Histopaque (Sigma Aldrich, Milan, Italy). The viability of PBMC was checked by measuring Trypan blue dye exclusion and was >90%. The

PBMC were resuspended at a final concentration of 1.5 × 105 cells/mL in RPMI 1640 (Sigma Aldrich) supplemented with L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum (Sigma Aldrich). The PBMC were distributed in 0.2 mL of calcium medium into flat-bottomed 96-well microtiter plates, cultivated for 5 d at 37°C in a 5% CO_2 incubator in the presence of bulk milk, casein, or whey protein obtained from bovine, caprine, and ovine species. After the 5-d incubation, PBMC viability was >90%. Concentration of the tested proteins used for stimulation was 100 µg/mL. The PBMC were stimulated to determine production of anti- and proinflammatory cytokines with 50 µL of PHA (final concentration 10 µg/mL, Sigma Aldrich). The mitogen PHA (10 µg/mL) was used as positive control stimulus to ensure normal immune reactivity to control stimuli in the subjects, whereas cells not activated with mitogen represented negative controls. At the end of the incubation period, quadruplicate culture supernatants were harvested and stored at −20°C until used in cytokine assays. Levels of TNF-α, IL-10, IL-6, and IL-1β in the culture supernatants were determined in duplicate using commercial Luminex Multiplex Assays (Labospace, Milan, Italy); results were expressed in picograms per milliliter. Briefly, the Bio-Plex Calibration kit (#171203060, Labospace) and Bio-Plex Validation kit (#171203001, Labospace) were used to prepare a standard curve for each cytokine. The analysis was performed using a BioRad Bio-Plex 100 with the magnetic luminex screening assay (BioRad, Milan, Italy).

The level ROS and RNS were measured in the culture supernatants using an OxiSelect *in vitro* ROS/ RNS Assay Kit (Cell Biolabs Inc., San Diego, CA) according to the manufacturer's instructions. Data were normalized to protein level (measured by BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). The ROS/ RNS were expressed as 2,7-dichlorodihydrofluorescein (DCF) in millimoles per liter (mM).

Cytokines and ROS/RNS levels in control patients did not show differences for milk species and protein fractions; mean values of TNF-α, IL-10, IL-1β, and IL-6 in bovine, ovine, and caprine milks were 150 ± 55, 25 ± 3, 45 ± 18, and 140 ± 52

mg/mL of protein and values of ROS/RNS were 0.7 ± 0.02, 0.5 ± 0.01, and 0.4 ± 0.03 mM in the 3 milks, respectively.

Statistical Analysis

All variables were tested for normal distribution using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Data on cytokines and ROS/RNS were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 2011). The tested parameters were always lower ($P < 0.001$) in controls than in infants with epilepsy; data on control infants were used as a baseline to evaluate the response of infants with epilepsy. The effects of milk source, milk protein fractions, and their interaction were tested within infants with epilepsy. For each cytokine, the levels in controls were used as a threshold level to group the response of infants with epilepsy into 3 levels (low, medium, and high), where low, medium, and high groups had increases <30%, from 30 to 70%, and >70% relative to the threshold level, respectively. The percentage of patients ascribed to each level is reported. When significant effects were found ($P < 0.05$), Student's t-test was used to identify significant differences between means.

Results and Discussion

Composition of Bovine, Caprine, and Ovine Milks

Chemical composition of pasteurized bovine, caprine, and ovine milks is reported in Table 1. Milk composition from more represented dairy species is characterized by a great complexity of milk nutrients (Fox, 2003). In this study, percentage composition of bovine and caprine milks was comparable for protein content and for casein and whey protein fractions. As expected, ovine milk exhibited a higher percentage of principal chemical components reported in Table 1; protein fractions in ovine milk were twice as high as those found in bovine and caprine milks. The percentage of fat content ranged from 3.63 to 8.16% from bovine to ovine milk; in caprine milk, fat content was 4.14%. Among milk components, proteins are species-specific mainly due to genetic polymorphisms; in particular,

the genetic polymorphisms of milk proteins from small ruminant species are of importance as they are associated with quantitative and qualitative parameters in milk (Albenzio and Santillo, 2011).

Table 1. Chemical composition of bovine, caprine, and ovine pasteurized milk (means ± SEM).

	Milk species		
Component	Bovine	Caprine	Ovine
Protein, %	3.53 ± 0.56	3.22 ± 0.35	6.01 ± 0.48
Casein, %	2.72 ± 0.10	2.29 ± 0.12	4.56 ± 0.13
Whey protein, %	0.85 ± 0.02	0.96 ± 0.01	1.42 ± 0.02
Fat, %	3.63 ± 0.12	4.14 ± 0.15	8.16 ± 0.32
Lactose, %	4.69 ± 0.10	4.18 ± 0.12	4.37 ± 0.11

Cytokines and ROS/RSN in Cultured PBMC

Effects of milk protein fractions of bovine, caprine, and ovine milks on cytokine levels in cultured PBMC are presented in Table 2. Cytokines are generally synthesized and secreted in response to immunological stimuli; they are soluble, potent glycoproteins involved in the regulation of growth, immune cell activation, and inflammatory and immune responses able to travel to distant cells in other organs via the peripheral circulation (Youn, 2013). Tumor necrosis factor-α produced by PBMC cultured with bulk milk was lower ($P < 0.05$) for bovine and ovine milk in 80 and 70% of the infants respectively, whereas caprine milk induced higher levels of this cytokine in 80% of infants. However, the levels of TNF-α detected in PBMC stimulated with caprine milk reached lower levels ($P < 0.05$) than those against bovine and ovine milks. For the casein fraction, a higher percentage of patients ascribed to a lower production of TNF-α regardless of species. The whey protein fraction regardless of species was able to induce higher levels of TNF-α in 10% of infants; in particular, this value was 4-fold and 11-fold

higher ($P < 0.01$) in bovine and ovine than the level of TNF-α produced by PBMC cultured with the caprine whey protein fraction, respectively. Furthermore, TNF-α produced by PBMC cultured with bovine and ovine whey proteins was lowest in 70% of patients, whereas TNF-α produced by PBMC cultured with the caprine whey protein fraction showed intermediate levels in 80% of cases. The ability of PBMC to secrete cytokines in response to milk and protein fraction stimulation may be a predictor of the secretion of soluble factor TNF-α in the bloodstream of challenged patients. Several studies have reported that TNF-α might acts in a concentration-dependent manner; TNF-α has been shown to play a proconvulsive role in Shigella-mediated seizures at low concentration but exert an anticonvulsive effect at higher concentration (Yuhas et al., 2003). In the central nervous system, TNF-α can activate its 2 receptors, p55 and p75, and may modulate cell-signaling pathways: a low concentration of TNF-α may predominantly activate proconvulsive effects via p55, whereas a high concentration of TNF-α can play anticonvulsive role through the p75 pathway (Li et al., 2011).

Table 2. Effects of bulk milk and milk protein fractions of bovine, caprine, and ovine milk on cytokine levels (means ± SEM; pg/mL) in cultured peripheral blood mononuclear cells of infants with generalized epilepsy.

Cytokine	Species and level [1]	Patients %	Bulk milk			Patients %	Casein			Patients %	Whey protein		
Tumor necrosis factor-α	**Bovine**												
	Low	80	1,023.5	±	105.4[c]	70	718.6	±	95.2[c]	70	52.5	±	8.5[c]
	Medium	10	1,507.7	±	128.8[b]	20	1,254.5	±	110.8[b]	20	3,414.2	±	320.5[b]
	High	10	2,257.7	±	253.8[a]	10	3,909.1	±	301.7[a]	10	11,740.7	±	1,165.1[a]
	Effect, P-value			**				***				*	
	Caprine												
	Low	10	161.67	±	74,3	80	965.6	±	93.2	10	686.44	±	92.5
	Medium	10	653.3	±	103.2	10	1,444.9	±	108.5	80	1,088.5	±	107.1
	High	80	1,398.3	±	150.2	10	3,112.07	±	322.7	10	2,788.22	±	998.2
	Effect, P-value			***				***				***	
	Ovine												
	Low	70	560.10	±	85.6	50	656.6	±	95.3	70	50.82	±	9.5
	Medium	20	1,581.5	±	125.6	40	1,253.5	±	98.5	20	8,620.5	±	1,430.5
	High	10	3,530.2	±	320.5	10	3,526.5	±	320.5	10	31,442.4	±	3,195.1
	Effect, P-value			*				**				***	
IL-10	**Bovine**												
	Low	50	3.67	±	1.05	50	0.58	±	0.1	50	1.72	±	0.8
	Medium	40	22.79	±	2.3	40	17.91	±	1.6	40	20.87	±	1.8
	High	10	74.05	±	8.8	10	84.4	±	5.5	10	63.78	±	6.0
	Effect, P-value			**				***				***	
	Caprine												
	Low	70	1.6	±	0.1	70	1.4	±	0.2	70	0.83	±	0.1
	Medium	20	33.9	±	1.6	20	26.36	±	2.1	20	27.5	±	1.2
	High	10	90.84	±	3.4	10	118.52	±	5.6	10	83.06	±	4.2
	Effect, P-value			***				***				***	
	Ovine												
	Low	50	7.91	±	1.2	70	2.3	±	0.8	50	1.4	±	0.1
	Medium	40	22.4	±	1.6	20	25.3	±	2.5	10	12.88	±	1.5
	High	10	53.6	±	5.22	10	81.83	±	8.5	40	32.5	±	1.4
	Effect, P-value			**				**				***	
IL-1β	**Bovine**												
	Low	80	825.5	±	25.5	80	240.8	±	28.1	10	0.8	±	0.1
	Medium	10	1,150.4	±	90.2	10	1,928.4	±	77.9	30	746.5	±	98.2
	High	10	1,958.4	±	110.5	10	5,898.5	±	427.2	60	1,586.2	±	80.2
	Effect, P-value			***				***				***	
	Caprine												
	Low	10	0.5	±	0.1	10	0.5	±	0.1	0		-	
	Medium	10	4,689.7	±	150.2	10	1,367.87	±	89.9	80	7,578.5	±	502.5
	High	80	14,095.3	±	1,022.2	80	14,619.5	±	1,101.2	20	11,828.5	±	898.5
	Effect, P-value			***				***				***	
	Ovine												
	Low	0		-		80	5,475.6	±	175.5	80	0.5	±	0.1
	Medium	60	5,159.6	±	189.2	10	26,073.6	±	1,825.2	10	72,806.4	±	3,911.5
	High	40	13,405.6	±	952.3	10	72,093.5	±	5,215.6	10	228,878.8	±	9,526.5
	Effect, P-value			***				***				**	
IL-6	**Bovine**												
	Low	10	0.6	±	0.1	10	0.8	±	0.2	60	4,405.5	±	150.2
	Medium	50	8,645.5	±	450.2	10	2,486.5	±	150.2	30	13,072.5	±	859.5
	High	40	11,668.5	±	852.3	80	15,283.5	±	1,223.5	10	18,268.2	±	2,225.3
	Effect, P-value			***				***				***	
	Caprine												
	Low	10	0.5	±	0.1	10	0.7	±	0.2	0		-	
	Medium	10	4,690.5	±	186.5	10	1,368.5	±	952.2	70	7,532.1	±	395.5
	High	80	14,098.1	±	896.8	80	14,618.8	±	989.5	30	12,859.2	±	898.5
	Effect, P-value			***				***				***	
	Ovine												
	Low	0		-		80	5,475.6	±	259.6	80	0.8	±	0.1
	Medium	60	5,159.6	±	156.7	10	26,074.5	±	2,159.5	10	72,806.5	±	4,896.2
	High	40	13,405.6	±	895.6	10	72,094.5	±	5,528.6	10	228,879.5	±	6,548.6
	Effect, P-value			***				***				***	

[a–c]Means with different superscripts differ ($P < 0.05$).

[1]The percentage of patients ascribed to each level is reported. The low, medium, and high levels grouped values that showed an increase <30%, between 30 and 70%, and >70% in respect to the threshold level, respectively.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Bovine, caprine, and ovine bulk milks induced low level production of IL-10 by cultured PBMC in at least 50% of cases; the same behavior was observed in both casein and whey protein fractions for all milk species studied. In general, the total amount of IL-10 detected was lower than that of other cytokines involved in this study. Interleukin-10 has broad anti-inflammatory properties by its inhibition of antigen-presenting cell function and suppression of production of proinflammatory cytokines (O'Garra et al., 2008). Indeed, IL-10 deactivates macrophages, which in turn decreases the production of cytokines by T cells (Youn, 2013). Several animal studies and clinical observations suggest an anticonvulsant effect of IL-10; one report showed protective effects of IL-10 against the development of epileptiform activity evoked by transient episodes of hypoxia in rat hippocampal slices (Levin and Godukhin, 2007). Production of IL-10 in blood is controversial; some authors reported no differences in plasma IL-10 between patients with focal seizure and control (Virta et al., 2002), whereas IL-10 production was significantly lower in patients suffering from focal seizure than in healthy control (Li et al., 2011). Youn (2013) reported that IL-10 was significantly elevated in plasma 48 to 72 h after seizure onset, leading to the hypothesis that IL-10 may have an anticonvulsive effect in neonatal seizure patients by suppressing proinflammatory cytokines production. In a previous study, Albenzio et al. (2012) found higher levels of IL-10 in PBMC cultured against bovine and caprine milk frac- tions in both healthy infants and in infants with cow milk allergy; in the current study, the lower level of IL-10 could be ascribed to the impaired immunological status of infants affected by generalized epilepsy.

Bovine milk and casein fraction induced the lowest levels ($P < 0.01$) of IL-1β from cultured PBMC in 80% of patients, whereas whey protein induced the highest level in 60% of cases. Caprine milk and casein fraction induced the highest levels ($P < 0.01$) of the same cytokine in 80% of cases and whey protein induced intermediate level in 80% of cases. Ovine milk induced medium levels of IL-1β in 60% of cases and low levels in 80% of cases for casein and whey protein fractions. The IL-1 family comprises 3 ligands, IL-1α, IL-1β, and IL-1Ra; IL-1β is

mostly secreted, whereas IL-1α is predominantly membrane-bound. Interleukin-1β plays a role in promoting excite-toxicity and perhaps in seizure generation (Vezzani and Baram, 2007). A notable example of a dual role of cytokines on neuronal survival in diseased tissue exists; in particular, neuroprotective actions of IL-1β have been reported, likely mediated by its ability to induce the synthesis of the growth factors of astrocytes, promoting cell repair mechanisms. Other mechanisms of neuroprotection induced by cytokines include stimulation of antioxidant pathways; in this respect, IL-1β and TNF-α can either reduce or exacerbate glutamate receptor-mediated excite-toxicity depending on their extracellular concentrations, the length of time the tissue is exposed to these cytokines during injury, and the receptor time activated by this cytokines (Bernardino et al., 2005). Chronic expression of IL-1β during epileptogenesis highlights the possibility that this cytokine might be involved in the mechanisms underlying the onset of spontaneous seizures (Vezzani et al., 2008).

Caprine bulk milk showed the highest level ($P < 0.01$) of IL-6 in 80% of patients, bovine milk showed interme- diate levels in 50% of patients, and ovine milk showed the lowest level in 60% of patients. Bovine and caprine casein fractions stimulated higher levels of IL-6 in 80% of cases, whereas ovine casein stimulated a lower level in 80% of cases. Apart from animal species, the whey protein fraction stimulated a lower IL-6 level in most of the studied patients. The complexity of the PBMC response against stimulation by milk protein fractions relies on the ambiguous nature of IL-6, which is necessary for the normal development of the nervous system but has neurotoxic and proconvulsive effects when increased levels are detected in the brain (Samland et al., 2003). The increase in IL-6 in the central nervous system after generalized seizure was more pronounced than the increase in plasma (Li et al., 2011). In general, the amount of IL-6 detected after stimulation of PBMC with milk and its fractions for all species was lower than that of other proinflammatory cytokines detected in this study, probably because this cytokine is not a reliable marker of epilepsy in the

bloodstream. Indeed, Li et al. (2011) reported that the IL-6 level in plasma does not reliably reflect its level in brain.

Effects of stimulation of cultured PMBC with milk and protein fractions from different species on ROS/ RNS levels are presented in Figure 1. Levels of ROS/ RNS were higher in bovine and ovine milks than in caprine milk; within bovine milk, total free radicals were higher in bulk milk and lower in casein fraction whereas whey protein showed an intermediate level. In caprine milk, the ROS/RNS levels were not different among milk fractions; finally, ovine milk displayed higher levels for bulk milk and casein than for whey protein fraction.

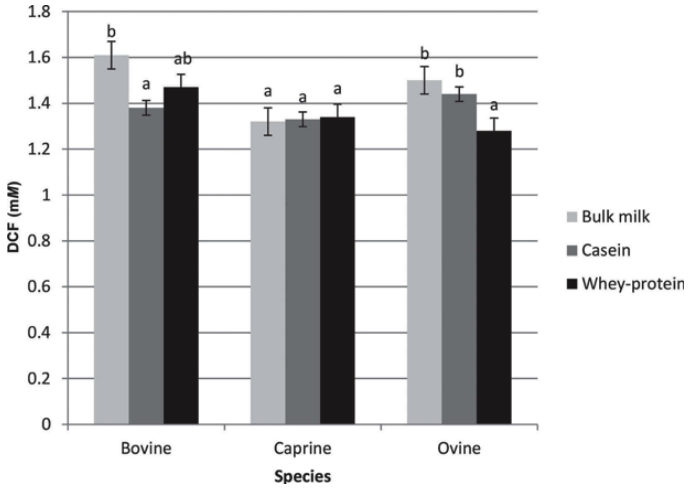

Figure 1. Effects of bulk milk and protein fractions from different species on reactive oxygen species and reactive nitrogen species (ROS/ RNS) levels in cultured peripheral blood mononuclear cells. Mean values with different letters (a, b) differ ($P < 0.05$); error bars indicate SEM. DCF = 2',7'-dichlorodihydrofluorescein.

The increased levels of ROS are a direct indicator of elevated oxidative stress in biological systems; excessive production contributes to cell dysfunction and cell death (Hensley et al., 1995). The production of ROS is part of the killing strategy of effector cells within the T helper (Th) 1-type immune response, and it is also involved to further amplify the release of proinflammatory cytokines. An oxidizing milieu is also a trigger of the redox-sensitive signal transduction pathway in cells, including the induction of proinflammatory cytokines such as TNF-α (Murr et al., 2005). Lower levels of ROS/RNS detected in PBMC cultured with caprine milk fraction could be responsible for the lower levels of TNF-α in the corresponding fraction. It was reported recently that oxidative damage occurring during epileptogenesis contributes to acute injury-induced neuronal damage leading to detrimental effects on areas of the brain associated with learning and memory function (Pearson et al., 2015). Those authors also suggested that the possibility of pairing antioxidants with anti-seizure drugs, as a combination therapy, might help to reduce the cognitive impairment as comorbidities occurring in epileptic patients.

Conclusions

Production of cytokines and ROS/RNS by cultured PBMC from infants with generalized epilepsy was influenced by protein fractions of milk from bovine, caprine, and ovine species. The ability of PBMC to secrete cytokines in response to stimulation by milk and protein fractions may be a predictor of the secretion of pro- and antiinflammatory cytokines in the bloodstream of challenged patients. Detection of TNF-α and ROS/RNS *in vitro* may provide information on inflammatory status and oxidative damage occurring in infants with generalized epilepsy. Cytokines might be useful biomarkers to discriminate the effects of foods on the inflammatory response; dietary strategies could help in alleviating the negative effect of epilepsy in infants. Further investigations on milk components are needed to better clarify the mechanisms affecting immunological status in infant with generalized epilepsy.

3.3 Milk Nutrition and childhood epilepsy: An *ex vivo* study on cytokines and oxidative stress in response to milk protein fractions

J. Dairy Sci. 101:4842–4852
https://doi.org/10.3168/jds.2017-13104

Milk nutrition and childhood epilepsy: An ex vivo study on cytokines and oxidative stress in response to milk protein fractions

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Abstract

We present a pilot study on the effects of milk protein fractions (α_{s1}-CN, α_{s2}-CN, k-CN, β-CN, and a mix of α-LA and β-LG) from different animal species (bovine, ovine, and caprine) on pro- and anti-inflammatory cytokines and oxidative status in cultured peripheral blood mononuclear cells from children with generalized epilepsy. PBMC were obtained by density gradient from blood of 10 children with generalized epilepsy (5 males; mean age 33.6 ± 5.4 mo) and 10 controls (5 males; mean age 35.6 ± 6.8 mo). Children with epilepsy were grouped according to cytokine levels as follows: children with epilepsy having low levels of cytokines not different from those of control children; children with epilepsy having cytokine levels at least 5-fold higher (medium levels) than those of control children; and children with epilepsy having cytokine levels at least 10-fold higher (high levels) than those of control children. The production of tumor necrosis factor-α (TNF-α), IL-10, IL-6, and IL-1β was studied in cultured PBMC incubated with α_{s1}-CN, α_{s2}-CN, k-CN, β-CN, and a mix of α-LA and β-LG from bovine, caprine, and ovine milks. The levels of reactive oxygen and nitrogen species and catalase activity were assessed in cultured supernatant. In the HL-EC group, β-CN from small ruminant species (ovine and caprine) induced the highest levels of TNF-α, whereas PBMC incubated with α_{s2}-CN from ovine milk and the mix of β-LG and α-LA from all tested milk species had the lowest levels of TNF-α. Within

the HL-EC group, production of IL-1β was higher for bovine and ovine α_{s2}-CN fractions and lower for caprine and ovine β-CN and k-CN. In the HL-EC group, IL-6 was higher in cultured PBMC incubated with α_{s2}-CN from bovine and ovine milk than from caprine milk. The cytokine IL-10 did not differ among milking species. The highest levels of ROS/RNS were found after incubation of PBMC with the β-CN fraction in bovine milk. Catalase activity was higher in PBMC cultured with β-CN isolated from bovine and caprine milk and with α_{s1}-CN from ovine milk.

Introduction

Epilepsy is the third most common brain disorder, and it is characterized by a persistent predisposition to seizures and by emotional and cognitive dysfunction (Duncan et al., 2006). Overwhelming evidence indicates that epilepsy is associated with inflammation and elevated levels of cytokines (Galic et al., 2012). Cytokines are immunological molecules involved in the progression of many diseases (Damsgaard et al., 2009); different cytokine patterns can be used to characterize immune responses such as immune maturation, allergic tendencies in infancy, and later development of autoimmune disease (Gutcher and Becher, 2007).

Inflammation of the central nervous system is characterized by microglial activation with an increase in pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 (Riazi et al., 2010). Seizures not only induce the expression of cytokines in the brain but also alter peripheral cytokine levels (Li et al., 2011); indeed, peripheral inflammation reflects a similar inflammatory state in the brain (Riazi et al., 2010). The involvement of the blood–brain barrier and its dysfunction in epileptic syndromes suggest that blood–brain barrier failures precede and are required for the initiation of status epilepticus (Marchi et al., 2009).

A role of diet in triggering epilepsy disorders has been claimed, and research has focused on the relationship between epilepsy and modulation of the gut–brain

axis, brain inflammatory reactions, and dietary allergic disorders (Albenzio et al., 2016a). Autoimmune processes and associated pathogenic autoantibodies have received increasing attention for their role in epilepsy disorders (Palace and Lang, 2000; Pollak et al., 2014).

A population-level epidemiological study documented that epilepsy and some autoimmune diseases frequently co-occur, and the potential role of autoimmunity in epilepsy must be considered (Ong et al., 2014). Of particular interest are the many species of intestinal microbes that reside in great numbers in the digestive tract. These gut microbiota may play a vital role not only in the maintenance of microbiota homeostasis and food digestion, but also in the progression of autoimmune diseases through modulation of immune responses (Albenzio et al., 2016b; Wu et al., 2016).

In a prospective study based on pediatric experience, a link between cow milk allergy and epileptic events was reported (Falsaperla et al., 2014). In childhood, milk nutrition is particularly relevant because human newborn milk fulfils nutritional needs and ensures safe development and growth during the first stages of life (Albenzio et al., 2016a).

In a previous study, we investigated the role of bovine, ovine, and caprine milk, caseins, and whey proteins on the immune status of children with generalized epilepsy; cytokines and reactive oxygen and nitrogen species detected *ex vivo* were useful biomarkers to discriminate the effects of milk components on epilepsy in children (Albenzio et al., 2016b).

The present study was undertaken to evaluate the effects of milk protein fractions (α_{s1}-CN, α_{s2}-CN, k-CN, β-CN, and a mix of α-LA and β-LG) from different animal species (bovine, ovine, and caprine), on pro- and anti-inflammatory cytokines and oxidative status in cultured PBMC from children with generalized epilepsy.

Materials and methods

Patients

Ten children with generalized epilepsy (5 male, 5 female; mean age 34.6 ± 4.4 mo) and 10 controls (5 male, 5 female; mean age 36.5 ± 5.8 mo) were recruited at the Complex Structure of Neuropsychiatry Childhood-Adolescence of Ospedali Riuniti (Foggia, Italy) and included in the study. Inclusion and exclusion criteria were in accordance with those reported in Albenzio et al. (2016b). Approval was obtained from the Ospedali Riuniti (Foggia, Italy) Institutional Review Board for these studies. Written informed consent was obtained from the parents in accordance with the Declaration of Helsinki on the Ethical Principles for Medical Research Involving Human Subjects.

Separation of Milk Proteins by Reversed-Phase HPLC

Samples from pasteurized bovine, ovine, and caprine milks were prepared following the method proposed by Bobe et al. (1998). Briefly, aliquots of 500 µL of milk were frozen at −20°C. A solution containing 0.1 M Bis-Tris buffer (pH 6.8), 6 M GndHCl, 5.37 mM sodium citrate, and 19.5 mM dithiothreitol (pH 7) was added to frozen aliquots in a 1:1 ratio (vol: vol). Samples were incubated for 1 h at room temperature, and centrifuged for 5 min at 16,000 × g in a microcentrifuge. The fat layer was then removed with a spatula, and the remaining solution was diluted in a 1:3 ratio (vol: vol) with a solution containing 4.5 M GndHCl prepared in acetonitrile, water, and TFA in a ratio 100:900:1 (vol: vol: vol, pH 2). For the identification of milk proteins, a standard was prepared containing purified bovine milk proteins (α_s-CN, β-CN, k-CN, α-LA, and β-LG, purchased from Sigma-Aldrich, St. Louis, MO). Separation of milk proteins was achieved by RP-HPLC. The HPLC system consisted of an Agilent 1260 Infinity Series chromatograph (Agilent Technologies, Santa Clara, CA), equipped with a binary pump (Agilent 1260 Infinity series, G1312B), a diode-array detector (Agilent 1260 Infinity series, 1315C), and a fraction collector (Agilent 1260 Infinity series, G1364C). The Agilent Chem-Station for LC Systems software

controlled the equipment. Separations were performed on an RP semi-preparative column C8 (Zorbax-300SB-C8 RP, Agilent Technologies), with a silica-based packing (5 µm, 300 Å, 250 mm × 9.4 mm internal diameter). Gradient elution was carried out with a mixture of 2 solvents. Solvent A consisted of 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. Separations were performed with the following gradient: linear gradient from 33 to 35% B in 5 min, from 35 to 37% B in 4 min, from 37 to 40% Bin 9 min, from 40 to 41% Bin 4 min, followed by an isocratic elution at 41% B during 5.5 min, followed by a linear gradient from 41 to 43% B in 0.5 min, returning to the starting condition in 1 min, and re-equilibrating the column for 8 min. The flow rate was 2 mL/min, and column temperature was kept at 45°C, and detection was made at a wavelength of 214 nm. The injection volume was 80 µL. Separated protein fractions were collected through the fraction collector, frozen at −80°C, and finally freeze-dried. Figure 1 shows the RP-HPLC profile of ovine milk displaying peaks attributed to k-CN, α_{s2}-CN, β-CN, α_{s1}-CN, and the mix of α-LA and β-LG according to elution time.

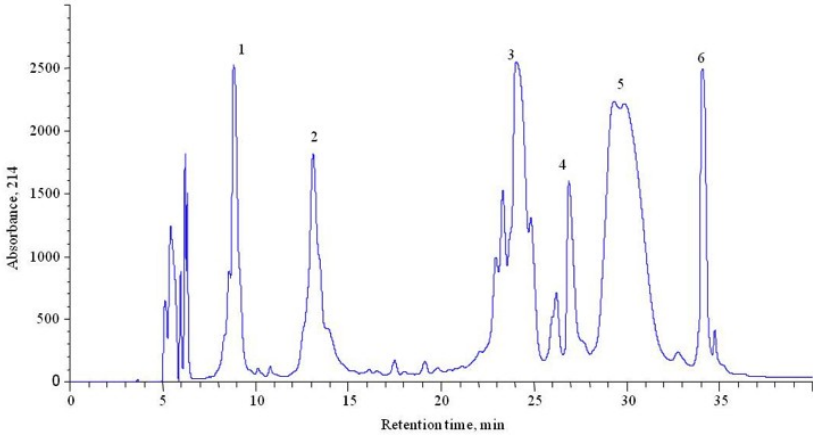

Figure 1. RP-HPLC profile of ovine milk. Identity of peaks: 1, k-CN; 2, α_{s2}-CN; 3, α_{s1}-CN; 4, the mix of α-LA and β-LG; 5, 6, β-CN.

SDS-PAGE of Milk Protein Fractions

The milk protein fractions collected by RP-HPLC were subjected to electrophoretic separation to confirm the presence and purification of the isolated protein. Sodium dodecyl sulfate PAGE was performed according to Laemmli (1970). The same standards used in RP-HPLC analysis were loaded onto the SDS gels to allow comparison with a reference protein. The gels were stained with Coomassie Brillant Blue G250 (Bio-Rad, Watford, UK), and destained in an aqueous solution of acetic acid and methanol (20% and 7%, vol/vol, respectively). The destained gels were acquired using a Gel Doc EQ system (Bio-Rad). Figure 2 shows the SDS-PAGE electrophoretogram of the protein fraction collected by RP-HPLC analysis from ovine milk. The electrophoretogram is given as an example to show the successful separation of the main protein fractions in milk from different species.

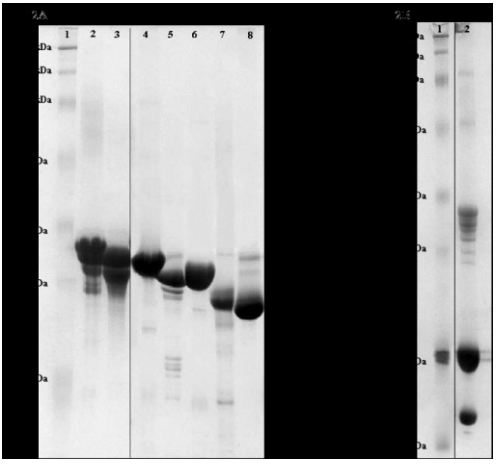

Figure 2. SDS-PAGE electrophoretogram of the milk protein fractions, collected by RP-HPLC. 2A: SDS-PAGE 12%, line 1, molecular weight standard; lines 2, 5, 7, bovine milk proteins (α_s-CN, β-CN, k-CN, respectively) 3, 4, 6, 8, ovine milk proteins (α_{s2}-CN, α_{s1}-CN, β-CN, k-CN, respectively. 2B: SDS-PAGE 15% line 1, molecular weight standard; line 2 ovine milk proteins (β-LG and α-LA).

Analysis of Cytokines in Cultured PBMC

Production of TNF-α, IL-10, IL-6, and IL-1β was studied in PBMC from blood of patients and control children, incubated with 100 μg/mL of k-CN, α_{s1}-CN, α_{s2}-CN, β-CN, and a mix of α-LA and β-LG obtained from bovine, caprine, and ovine milks according to the procedure previously described in Albenzio et al. (2016b). Briefly, PBMC were obtained by Ficoll-Histopaque (Sigma Aldrich, Milan, Italy) density gradient from heparinized venous blood of 10 children with generalized epilepsy and 10 children without clinical signs of disease as controls. The trypan blue dye exclusion test was applied to check PBMC viability, which was >90%. Finally, 1.5 × 105 PBMC/mL were resuspended in RPMI-1640 medium (Sigma Aldrich) supplemented with L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum (Sigma Aldrich), distributed into 96- well microtiter plates, and cultured for 5 d in 5% CO_2 in a humidified incubator. The PBMC were incubated with 100 μg/mL (final concentration) of k-CN, α_{s1}-CN, α_{s2}-CN, β-CN, or a mix of α-LA and β-LG obtained from bovine, caprine, and ovine milks. At the end of the incubation time, supernatants were harvested and stored at −20°C until cytokine assays were performed using Luminex Multiplex Assays (Thermo Fisher Scientific, Waltham, MA) at Labospace (Milan, Italy). An independent laboratory performed assays.

Determination of ROS/RNS and Catalase Activity

The levels of ROS and RNS were detected in accordance with Albenzio et al. (2016b) using an OxiSelect *in vitro* ROS/RNS Assay Kit with Green Fluorescence (Cell Biolabs Inc., San Diego, CA). The amount of catalase was measured using Human CAT ELISA Kit (Wuhan Fine Biotechnology Co. Ltd., Hubei, China) according to the manufacturer's instructions. Data were expressed in pictograms per milliliter and normalized to protein level (measured by BCA Protein Assay Kit, Thermo Scientific, Rockford, IL).

Statistical Analysis

All variables were tested for normal distribution using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Data on cytokines, ROS/RNS, and catalase were analyzed by ANOVA using the GLM procedure of SAS Institute (2011). The effects on patients of experimental factors (species: bovine, caprine, and ovine; and milk protein fraction: k-CN, α_{s1}-CN, α_{s2}-CN, β-CN, and α-LA and β-LG mix) and their interaction were tested for cytokine levels, catalase, and ROS/RNS according to the following model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk},$$

where yijk is the dependent variable; μ is the overall mean; α is the effect of animal species (i = 1–3); β is the effect of milk protein fraction (j = 1–5); αβ is the interaction of the animal species × milk protein fraction; and ε is the error.

For cytokine response, patients were grouped as follows: LL-EC (n = 4) = group of children with epilepsy having low levels of cytokines not different from those of control children; ML-EC (n = 3) = group of children with epilepsy having cytokine levels at least 5-fold higher (medium levels) than those of control children; HL-EC (n = 3) = group of children with epilepsy having cytokine levels at least 10-fold higher (high levels) than those of control children. When significant effects were found (at $P < 0.05$), Student's t-test was used to locate significant differences between means.

Results and discussion

Cytokine Pattern in Cultured PBMC

Monocytic cytokine production can be measured *ex vivo* in stimulated monocytes isolated from peripheral blood, which is thought to reflect their potential to produce cytokines (Albers et al., 2005; Damsgaard et al., 2009).

Levels of different cytokines in cultured PBMC incubated with α_{s1}-CN, α_{s2}-CN, k-CN, β-CN, and the α-LA and β-LG mix of obtained from bovine, ovine, and caprine milks were measured in children with epilepsy. The percentage distribution of children grouped according to level of cytokines is reported in

Table 1. For TNF-α, 50% of children were in LL-EC, 30% in ML-EC, and 20% in HL-EC. For IL-6, 50% of children were in LL- EC, 20% were in ML-EC, and 30% were in HL-EC. For IL-1β, 40% of children were in LL-EC, 30% were in ML-EC, and 30% were in HL-EC. Finally, for IL-10, all children were in LL-EC; that is, levels were comparable with those of controls. It is worth noting that half of the children with generalized epilepsy showed TNF-α and IL-6 patterns comparable with those of control children.

Cytokine responses in children are characterized by variable behavior due to the dynamism in the development of the immune system and immune maturation. Furthermore, during weaning, children are challenged to food antigens able to influence expression of cytokines. Albenzio et al. (2016a) reviewed the implications of diet on the modulation of gut–brain axis, brain inflammatory reactions, and dietary allergic disorders in children with epilepsy.

Table 1. Percentage distribution of epileptic children grouped according to the level of cytokines.

	Groups[1]		
Cytokines	LL-EC	ML-EC	HL-EC
TNF-α	50	30	20
IL-6	50	20	30
IL-1β	40	30	30
IL-10	100	-	-

[1] Low Level-Epileptic Children (LL-EC) - group of epileptic children that showed cytokine levels not different from the control children; Medium Level-Epileptic Children (ML-EC) - group of epileptic children that showed an increase of cytokine levels of at least five time higher than control children; High Level-Epileptic Children (HL-EC) - group of epileptic children that showed an increase of cytokine levels of at least ten time higher than control children.

TNF-α Produced by Cultured PBMC of Children with Generalized Epilepsy

The production of TNF-α by PBMC after incubation with milk protein fractions from different species is re- ported in Figure 3. In general, concentration of TNF-α was affected by species ($P < 0.01$), protein fraction ($P < 0.01$), and their interaction ($P < 0.001$). The HL-EC group had a lower concentration of TNF-α in PBMC incubated with bovine milk than with ovine or caprine milk (284.72, 336.40, and 329.78 ± 10.37 pg/mL; mean ± SEM from the 3 children with epilepsy enrolled in the HL-EC group, respectively); the results were the same for all of the milk protein fraction tested. The interaction between species and milk protein fractions showed no differences in the concentration of TNF-α in the LL-EC group. In contrast, children in the ML-EC group had the highest TNF-α concentrations in response to the ovine β-CN fraction and the lowest in response to the mix of β-LG and α-LA from ovine and caprine milks. In the HL-EC group, β-CN from ovine and caprine milks induced the highest levels of TNF-α, whereas α_{s2}-CN from ovine milk and the α-LA and β-LG mix from all tested milking species resulted in the lowest levels of TNF-α. Riazi et al. (2010) reported that TNF-α might be considered a link between peripheral and central inflammation, suggesting that the production of TNF-α within the brain during peripheral inflammation increases seizure susceptibility. Indeed, blocking production of TNF-α in the central nervous system was effective as an anticonvulsant mechanism. In a previous *ex vivo* study, Albenzio et al. (2016b) reported no differences according to species in TNF-α produced by cultured PBMC isolated from children with generalized epilepsy and incubated with the whole casein fraction of bovine, ovine, and caprine milks. In the present study, when casein fractions were isolated and tested on PBMC from children with epilepsy in groups ML-EC and HL-EC, we observed a major effect of β-CN from ovine milk on TNF-α production. Sheep milk is the richest of bovine and caprine in casein with a content of approximately 4.18 g/100 g (Dario et al., 2008); about half as much is present in bovine (2.80 g/100 g; Guo et al., 2007) and caprine (2.81 g/100 g; Leitner et al., 2004) milks. Furthermore, the main individual casein fractions (α_{S1}-,

α_{S2}-, k-, and β-CN) are characterized by different levels of synthesis in milk, and their proportions vary according to polymorphic expression of the encoding genes. Whey proteins exhibit genetic polymorphism that influences milk composition; ovine milk has been shown to be the richest in whey proteins (1.02 g/100 g) compared with other milking species (Dario et al., 2008). In the present study, the differences in TNF-α response after stimulation with different protein fractions may be associated with genetic polymorphism related to differences in polypeptide chains and AA sequences.

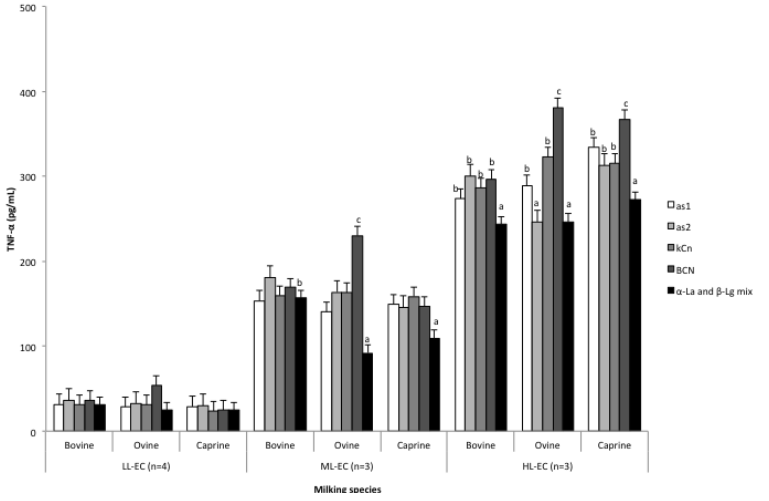

Figure 3. Concentration (μg/mL) of TNF-α produced by PBMC after incubation with milk protein fractions (k-CN, α_{s2}-CN, α_{s1}-CN, β-CN and a mix of α-LA and β-LG) from different species (bovine, ovine, and caprine). Low Level-Epileptic Children (LL-EC, n=5) - group of epileptic children that showed cytokine levels not different from the control children; Medium Level-Epileptic Children (ML-EC, n=3) - group of epileptic children that showed an increase of cytokine levels of at least five time higher than control children; High Level-Epileptic Children (HL-EC, n=2) - group of epileptic children that showed an increase of cytokine levels of at least ten time higher than control children. Error bars represent standard error of the means (SEM). a, d Mean values with different letters differ for $P < 0.05$ (Student t-test).

IL-1β Produced by Cultured PBMC of Children with Generalized Epilepsy

The production of IL-1β by PBMC after incubation with milk protein fractions from different species is reported in Figure 4. Interleukin-1β contributes to the development of epilepsy through rapid effects on neuronal survival ad transcription pathways and long-lasting effects on expression of selective gene families involved in brain structural and functional changes (Vezzani and Baram, 2007). The concentration of IL-1β was affected by species ($P < 0.001$), protein fractions ($P < 0.001$), and their interaction ($P < 0.01$). In the LL-EC group, mean values (± SEM) of IL-1β were 14.99, 14.21, and 10.82 ± 1.11 pg/mL in response to bovine, ovine, and caprine milks, respectively; in the ML-EC group, values were 28.49, 23.47, and 22.05 ± 1.21 pg/mL, respectively; and in the HL-EC group, values were 58.01, 53.44, and 50.44 ± 1.11 pg/mL, respectively. The concentration in LL-EC was higher in PBMC incubated with the α-LA and β-LG mix from bovine milk compared with that from ovine and caprine milks. In ML-EC, IL-1β concentration was higher in PBMC incubated with α_{s1}-CN and α_{s2}-CN and with the α-LA and β-LG mix isolated from bovine milk. In the HL-EC group, production of IL-1β was higher in bovine and ovine α_{s2}-CN fractions, whereas lower levels were detected for caprine and ovine β-CN and k-CN. Finally, the lowest concentrations of IL-1β were detected in PBMC incubated with the α-LA and β-LG mix in ovine milk and the highest in caprine and bovine milk. Vezzani and Baram (2007) reported an implied role of pro-inflammatory cytokines in seizure activity and epilepsy, with a predominant role of IL-1β as a pro-convulsive cytokine. In the present study, the lower IL-1β response after incubation with caprine α_{s2}-CN fraction could be ascribed to a more complex polymorphism of this casein fraction in goats. Albenzio et al. (2012, 2016c) found high numbers of alleles at the 4 casein loci in goat milk. Genetic polymorphisms of milk proteins play an important role in eliciting different degrees of allergic reaction (El-Agamy, 2007); caseins, especially α-CN, are among the most important milk allergens (Restani et al., 1999; Ballabio et al., 2011). The PBMC of children with cow milk allergy stimulated with casein and β-LG from bovine milk showed

higher TNF-α levels than the same cells stimulated with proteins from caprine milk (Albenzio et al., 2012), indicating a role of pro-inflammatory cytokines such as TNF-α in the immunological reaction to milk proteins. Evaluating the production of cytokines by PBMC after exposure with milk protein fraction is of great interest to identify potential antigens in milk. It is worth noting that α_{s2}-CN and β-LG are absent in human milk and that the proportion of caseins and whey protein fractions is different in milk during different phases of lactation (Armaforte et al., 2010).

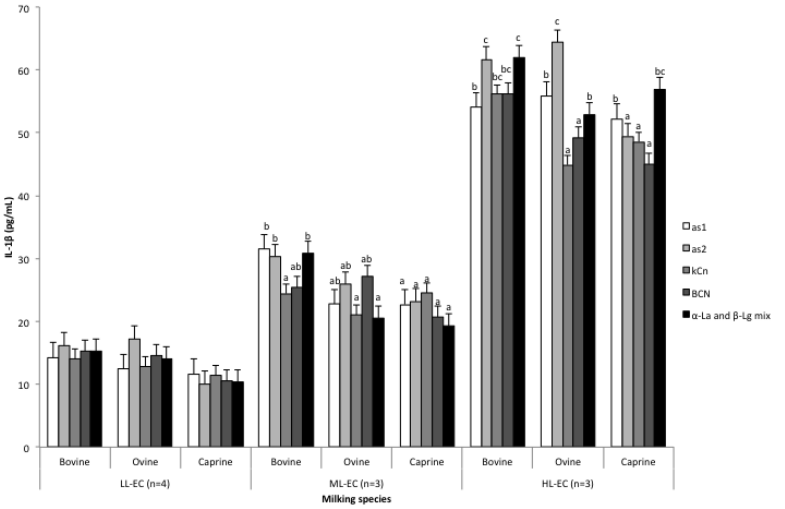

Figure 4. Concentration (μg/mL) of IL-1β produced by PBMC after incubation with milk protein fractions (k-CN, α_{s2}-CN, α_{s1}-CN, β-CN and a mix of α-LA and β-LG) from different species (bovine, ovine, and caprine). Low Level-Epileptic Children (LL-EC, n=4) - group of epileptic children that showed cytokine levels not different from the control children; Medium Level-Epileptic Children (ML-EC, n=3) - group of epileptic children that showed an increase of cytokine levels of at least five time higher than control children; High Level-Epileptic Children (HL-EC, n=3) - group of epileptic children that showed an increase of cytokine levels of at least ten time higher than control children. Error bars represent standard error of the means (SEM). a, d Mean values with different letters differ for $P < 0.05$ (Student t-test).

IL-6 Produced by Cultured PBMC of Children with Generalized Epilepsy

The production of IL-6 by PBMC after incubation with milk protein fractions from different species is reported in Figure 5. The concentration of IL-6 was affected by species ($P < 0.01$) and by an interaction between species and milk protein fraction ($P < 0.05$). There have been conflicting reports on the influence of IL-6 in seizures. Indeed, it remains unclear whether elevated blood levels of IL-6 allow conclusions to be drawn regarding the expression and function of IL-6 in epileptogenic brain areas (Li et al., 2011), probably because this cytokine penetrates the blood–brain barrier poorly (Kalueff et al., 2004). Other research reported that elevated levels of IL-6 occurred in the plasma and cerebrospinal fluid of patients with recent epileptic seizures, in which the levels of IL-6 generally correlate with the severity of seizure (Kalueff et al., 2004).

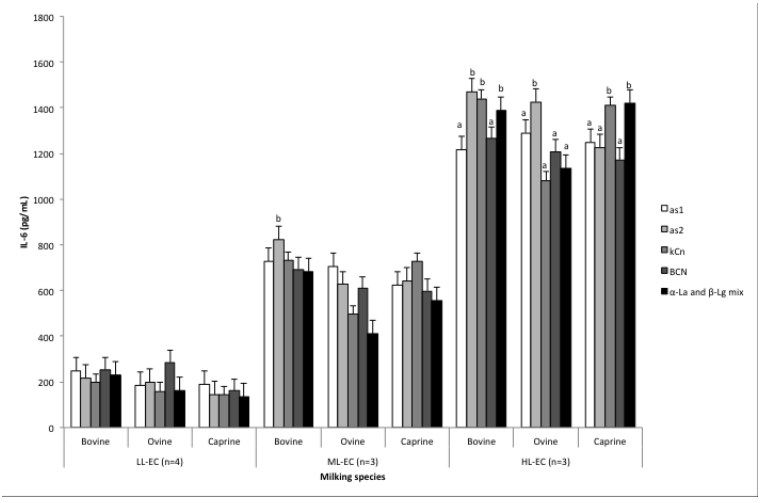

Figure 5. Concentration (µg/mL) of IL-6 produced by PBMC after incubation with milk protein fractions (k-CN, α_{s2}-CN, α_{s1}-CN, β-CN and a mix of α-LA and β-LG) from different species (bovine, ovine, and caprine). Low Level-Epileptic Children (LL-EC, n=5) - group of epileptic children that showed cytokine levels not different from the control children; Medium Level-Epileptic Children (ML-EC, n=2) - group of epileptic children that showed an increase of cytokine levels of at least five time higher than control children; High Level-Epileptic Children (HL-EC, n=3) - group of epileptic children that showed an increase of cytokine levels of at least ten time higher than control children. Error bars represent standard error of the means (SEM). a, d Mean values with different letters differ for $P < 0.05$ (Student t-test).

We detected differences in IL-6 production among milking species ($P < 0.01$); IL-6 concentration was lower for caprine, intermediate for ovine, and higher for bovine milk (160.40, 211.14, and 234.31 ± 22.97 pg/mL; mean ± SEM) in the LL-EC group, whereas IL-6 concentration was lower in ovine and caprine milks than in bovine milk (1,266.38, 1,245.12, and 1,347.86 ± 29.81 pg/mL; mean ± SEM) in the HL-EC group. No differences by milking species were found in the ML-EC group, where IL-6 reached a mean level of 662.6 ± 44 pg/mL in response to bovine, ovine, or caprine milk.

In the ML-EC group, the α_{s2}-CN fraction from bovine milk resulted in the highest concentration of IL-6 ($P < 0.05$). No differences in IL-6 concentration were observed in the HL-EC group after incubation with α_{s1}-CN and β-CN. Concentrations of IL-6 were higher in cultured PBMC incubated with α_{s2}-CN from bovine and ovine milks than in that from caprine milk. The concentration of IL-6 was higher after incubation with k-CN and with the α-LA and β-LG mix from bovine and caprine milks than from ovine milk. In a previous study, Albenzio et al. (2016b) found lower levels of IL-6 in cultured PBMC, from children with epilepsy, incubated with whole casein fractions from caprine milk than bovine and ovine milk. The ability of caprine milk to induce lower expression of IL-6 than bovine and ovine milk may be influenced by the polymorphic nature of α_{s2}-CN fraction.

IL-10 Produced by Cultured PBMC of Children with Generalized Epilepsy

We observed no differences in production of IL-10 among milking species (1.59 ± 0.19, 1.57 ± 0.21, and 1.55 ± 0.18 pg/mL; mean ± SEM) in cultured PBMC incubated with bovine, ovine, and caprine milks, respectively. Interleukin-10 is one of the major cytokines produced by regulatory T cells, and it exerts inhibitory actions on monocytes and T cells, partly suppressing the formation of pro-inflammatory cytokines (Reuss et al., 2002). In this study, all patients with generalized epilepsy produced levels of IL-10 comparable with those of controls, demonstrating the ability of PBMC to exert an anti-inflammatory response when

exposed to different protein fractions from bovine, ovine, and caprine milks. In agreement, no differences in plasma IL-10 were found between patients with febrile seizures and controls (Virta et al., 2002). Tiemessen et al. (2004) investigated the role of IL-10 in T-cell reactivity of children with cow milk allergy, and suggested that activated allergen-specific T cells might contribute to an active form of immune suppression *in vivo* through the production of IL-10 and thereby prevent aberrant reactions toward antigens such as cow milk proteins.

Oxidative Status in Cultured PBMC of Children with Generalized Epilepsy

The effects of milk protein fractions from different species on ROS/RNS levels in cultured PBMC from children with epilepsy are reported in Figure 6. The level of ROS/RNS was affected by species ($P < 0.001$) and the interaction between species and milk protein fractions ($P < 0.001$). The lowest level of ROS/RNS was found in PBMC incubated with the α-LA and β-LG mix isolated from bovine milk, whereas the highest level was found after incubation with β-CN isolated from bovine milk.

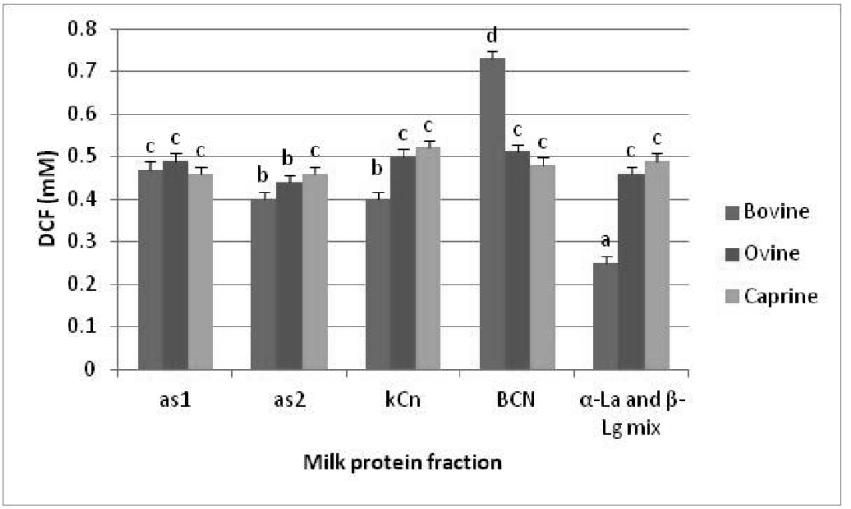

Figure 6. Effects of milk protein fractions from different species on ROS/RNS levels (mM/m) in cultured PBMC from epileptic children. Mean values with different letters (a-d) differ ($P < 0.05$); error bars indicate SEM. DCF = 2', 7'-dichlorodihydrofluorescein.

Reactive oxygen species include the superoxide anion, hydrogen peroxide, and hydroxyl radical, which are formed by excitation of the solitary electrons of oxygen; these oxygen species exert detrimental effects on unsaturated fats and proteins in cell membranes (Valko et al., 2007; Keskin Guler et al., 2016). The effects on catalase activity of cultured PBMC after incubation with milk protein fractions from different species are reported in Table 2. Catalase activity was affected by the interaction between species and milk protein fraction ($P < 0.05$), with higher levels being found in PBMC incubated with the β-CN fraction isolated from bovine and caprine milks and with α_{s1}-CN from ovine milk. In contrast,

lower levels of catalase activity were found after incubation with k-CN from all milking species tested and with β-CN and the α-LA and β-LG mix isolated from ovine milk.

Catalase is part of the antioxidant system that functions to neutralize ROS produced in the body; specifically, catalase is involved in the breakdown of H_2O_2. Recently, Keskin Guler et al. (2016) investigated the oxidant and antioxidant status of patients with epilepsy in relation to its balance in antiepileptic therapy. The higher catalase activity after incubating PBMC with β-CN and α_{s1}-CN in the current study might be related to the need to neutralize the highest accumulation of ROS/RNS in the corresponding fraction.

Conclusions

In the human newborn, nutrition from milk ensures growth and development during the first stages of life; however, protein components of milk may be related to immune disorders observed in children with generalized epilepsy. Pro- and anti-inflammatory cytokines produced *ex vivo* from PBMC isolated from children with generalized epilepsy were influenced by stimulation with milk protein fractions from different milking species. The differences in cytokine responses may be associated with genetic polymorphisms of the milk proteins. Evaluating the production of cytokines by PBMC after exposure to different milk protein fraction is of interest to identify potential antigens in milk. In particular, the β-CN fraction induced the highest levels of TNF-α in ovine and caprine milk, whereas the α_{s2}-CN fraction from bovine milk stimulated the highest level of IL-6 and played a major role in production of IL-1β from cultured PBMC. The higher levels of ROS/ RNS found after stimulation of PBMC with β-CN and α_{s1}-CN, together with higher catalase activity, may be related to an oxidative stress response in children with generalized epilepsy. Further *in vivo* studies should be performed to evaluate the influence of milk protein fractions on gut microbiota in the pathophysiology of epilepsy.

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4. SECTION III

4.1 Childhood obesity and nutrition

According to data provided by the World Health Organization (WHO), the number of obese people in the world has tripled since 1975. In 2016 over 1.9 billion adults were overweight, and 650 million of these were obese; the 39% of adults aged 18 years and over (39% of men and 40% of women) were overweight and about 13% of the world adult population (11% of men and 15% of women) were obese.

Obesity and overweight, before considered as problems only for high-income countries, are also increasing in low- and middle- income countries, especially in urban settlements, and are now recognized as real public health problems. Indeed, in Africa, the number of overweight or obese children under 5 has increased by nearly 50 percent since 2000. Over 340 million children and adolescents aged 5-19 were overweight or obese in 2016 and nearly half of the children under 5 who were overweight or obese lived in Asia (www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight - Access date: 07.01.2019)

The basic cause of obesity and overweight is an energy imbalance between calories consumed and calories expended. This is due to an increased intake of energy-dense foods that are high in fat and to an increase in physical inactivity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization. Changes in dietary and physical activity patterns are often the result of environmental and societal changes associated with the development and lack of supportive policies in sectors such as health, agriculture, transport, urban planning, environment, food processing, distribution, marketing, and education. Overweight and obesity show consequences on human health also on insurgence of other diseases. One most important of these are represented by the cardiovascular diseases (mainly heart disease and stroke), which were the major cause of death in 2012; but also diabetes, the musculoskeletal disorders (especially osteoarthritis - a highly disabling degenerative disease of the joints) and some types of cancers (including

endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon) are disease closely related to obesity.

In late decades, research in the pediatric field deals the issue of childhood obesity looking for a relationship between this phenomenon and the feeding of the newborn in the first six months of life (Han et al., 2010). In fact, it is in the period between the last stages of fetus growth and the first months of newborn life that the preadipocyte cells develop in mature adipose tissue under hormonal and nutritional stimuli. Childhood obesity is associated with a higher possibility of obesity, premature death, and disability in adulthood. But in addition to increased future risks, obese children show breathing difficulties, an increased risk of fractures, hypertension, early markers of cardiovascular disease, insulin resistance, and psychological effects. It seems that the nutritional factors of the infants diet influence the development of adipose tissue in adults (Fuijsawa et al., 2013) so, making the choice of healthier foods, is possible to preventing overweight and obesity.

Until a few years ago, adipose tissue was considered just an organ for energy reserve but the recent epidemic diffusion of obesity and its clinical complications in the last twenty years has attracted researchers' attention (Bray and Bellanger, 2006), in particular after the discovery that adipose tissue is the site of production and secretion of leptin, a hormone able to influence the instinct of intakes food (Friedman 2004). Actually, the adipose tissue is considered not only as a simple energy reserve but as an important endocrine organ that has numerous targets, including some areas of the brain like the hypothalamus. The adipose tissue is involved in the regulation of many processes: in the homeostasis of fat mass and nutrient, in the immune response, in the control of blood pressure, of thyroid functions and of the reproductive system (Trayhurn 2005; Grant and Dixit 2015).

The adipose organ is composed of two types of tissue cells, white adipose tissue and brown adipose tissue. WAT consists mainly by unilocular cells of considerable size (70-80 μm), with a flattened nucleus located peripherally and characterized by the presence of a single large cytoplasmic lipid vacuole

composed by triglycerides, which employs 90% of the total cell volume. White adipocytes have the physiological role to accumulate FFA for replenishing the organism of this substratum in the intervals between one meal and another; this is an essential relevance role especially when the interval is prolonged for weeks, becoming the tissue of survival. A crucial feature of white adipocytes is their ability to expand (Cinti, 2001; Friedman, 2009). In a condition like as in genetic obesity and obesity induced by high-fat diet, the adipocytes can increase their volume of about 6-7 times.

The BAT is made up by polyhedral cells (about 30-40 μm in diameter) that are characterized by a large, round and central nucleus and an abundant cytoplasm full of characteristic mitochondria and small lipid vacuoles. The presence of more lipid vacuoles is the reason why brown adipocytes are also called multilocular cells. Brown adipocytes are distinguished from white ones for the expression of the UCP-1 that is responsible to disperse the proton gradient generated in the electronic transport chain inside the mitochondria at the level of internal membrane, determining the production of heat instead of ATP (Cannon and Nedergaard, 2004; Richard and Picard, 2011). In fact, the main function of brown adipocytes is to dissipate the energy of fatty acids contained in lipid vacuoles to produce heat.

Overall the adipose tissue consists of two distinct main components: one represented by completely differentiated cells, the adipocytes, the other is called vascular-stromal fraction and includes preadipocytes, endothelial cells, macrophages, and fibroblasts.

The formation of adipose tissue is namely adipogenesis, and during this process, preadipocyte cells are transformed into mature adipocytes under the guidance of specific hormonal stimuli and particular genetic transcription factors (Ali et al., 2013). The adipogenesis is a highly controlled process, whose cellular and molecular events have been extensively studied in recent years, thanks to generation of some cell lines, such as the murine preadipocytic line 3T3-L1 (Green and Kehinde, 1975), which has permitted to understanding of both the

differentiation mechanism of preadipocytes in mature adipocytes, and the mechanisms underlying the main metabolic functions of the cell, such as lipolysis, incorporation of insulin-mediated glucose and lipogenesis.

Obesity is a metabolic disorder that is expressed by two phenomena, the increase of the adipocytes number that makes up the adipose tissue, due to hypertrophic phenomena, and an abnormal lipid filling of existing adipocytes due to hyperplastic phenomena (Berry et al., 2014). The hyperplasia occurs mainly in the developmental age, while hypertrophy is more characteristic in adulthood obesity, and it also represents the most common and most studied form.

The trigger reason for this disorder there is an excessive energetic intake, due to the ingestion of fats and carbohydrates, which induces mature adipocytes to store such excess energy in form of triglycerides; within the adipocytes cells the triglycerides thus accumulated, activate the process of adipogenesis with consequent growth of the cells in size and number. This unexpected cellular transformation causes a change in the adipokines secretion, the activation of pro-inflammatory processes and the increase of oxidative stress phenomena (Baret et al., 2013; Kusunoki et al., 2013).

As previously advanced there is a close link between childhood obesity and the feeding of the newborn in the first six months of life; during the last stages of fetus growth and the first months of life of the newborn, preadipocyte cells transforms into mature adipocytes and for this reason infant diet to influence the development of adipose tissue in adults.

Just because milk is the only food taken by the newborn in these phases, many researchers are interested to study the nutritional profile of this food, and the effects that milk fat component may have on the adipose tissue. In particular be worth considering the effect of milk fatty acids on human health and on adipose tissue. Flachs et al., (2009) observed that some n-3 series LC-PUFA such as EPA and DHA have a metabolic action on adipose tissue to prevent obesity. Some authors have tested the effect of MUFA and CLA as herbal extracts for obesity (Chang et al., 2015). Guo et al., (2006) have shown that caprylic acid and MCFA

have the ability to inhibit the synthesis of triglycerides by adipocytes. Oleic acid also shows an effect of reducing the viability of mature adipocytes by inducing cell apoptosis (Rohana et al., 2011). Other authors have also confirmed the existence of a relationship between fatty acid composition of foods taken with diet and childhood obesity highlighting some synergistic effects among fatty acids; more precisely the association of n-6 series PUFA with high concentrations of LA and of ALA support a continuous growth of adipose tissue under nutritional stimuli (Ailhaud and Guesnet 2003). However, the biochemical processes influence both the quantity and quality of fatty acids that reach the adipose tissues during digestion (Devle et al., 2014). It is therefore interesting to understand how the digestive process affects the bioavailability of fatty acids contained in milk, and whether this may influence adipose tissue.

3.2 Effect of lipid fraction of digested milk from different sources in mature 3T3-L1 adipocyte

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Effect of lipid fraction of digested milk from different sources in mature 3T3-L1 adipocyte

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Abstract

We evaluated the effect of *in vitro* digested milk on mature adipocytes 3T3-L1, paying particular to its fatty acid composition, and comparing human, donkey, bovine, ovine, caprine and formula milk. Cellular viability, apoptosis, oxidative response and gene expression levels of NF-kB p65, HMGB1, SREBP-1c and FAS were evaluated. Digested milk treatments significantly reduced 3T3-L1 mature adipocytes viability and caspase activity compared with control group, but no significant differences were observed among different sources of digested milk. In all digested milk samples, ROS level was higher than the control, however, the digested human and formula milk showed lower levels of ROS than DM, BM, OM and CM samples. Lower capacity of HM and FM to induce oxidative stress in mature adipocytes was ascribed to the peculiar free fatty acids profile of digested milk samples. All milk treatments elicited a significant over-expression of NF-kB p65 in 3T3-L1 adipocytes compared to the control; the lowest gene expression was found in HM, BM, OM and CM, the highest in FM and an intermediate behavior was shown in DM. All digested milk treatments influenced the gene

expression of SRBP-1c with FM and HM showing the highest levels. For FAS expression, BM showed the highest level, OM and CM intermediate and FM, HM and DM the lowest levels, however HM and DM had comparable levels to the control.

Introduction

Obesity is a metabolic disease caused by the increment of adipose tissue mass due to hyperplastic and hypertrophic growth of adipocytes. Obesity is closely associated with others diseases, such as insulin resistance, type 2 diabetes, hypertension, atherosclerosis, cardiovascular disorders, metabolic syndrome and some type of cancers. The main cause of obesity is the excessive intake of dietary carbohydrate and fat that stimulate the adipogenic process, which in turn causes changes in adipokine secretion, activation of pro-inflammatory pathways and increase of oxidative stress (Baret et al., 2013; Kusunoki et al., 2013). Childhood obesity is increasingly an important concern in pediatric research. The relationship between breast- feeding in the first six months and childhood obesity (Han et al., 2010), as well as milk nutritional factors that influence newborn health, have been studied (Fujisawa et al., 2013; Romacho et al., 2015). Preadipocyte cells are transformed under hormonal and nutritional stimulation into adipose tissue during the late-fetal and postnatal period, therefore, the nutritional factors in the diet of newborn babies influence the future development of adipose tissue (Spalding et al., 2008; Tang et al., 2008). Milk is undoubtedly a complete food for the newborn with high nutritional value and several bioactive compounds. The main high-energy value compounds in human milk are lactose and fat, and in the latter case the fatty acid profile can exert a complex effect on adipose tissue both promoting and controlling inflammation (Guenther Boden, 2011; Romacho et al., 2015).

During gastrointestinal digestion low pH and enzymes alter food composition to a very considerable extent, and in particular this process impacts both the quality and the quantity of fatty acids through physical and biochemical mechanisms. Milk of various animal species is different for structure and size of fat globules,

composition in triglycerides and also enzymatic activity and these features can influence the milk fat digestion (Devle et al., 2014). Recently Santillo et al. (2018) submitted milk from different sources (human, formula, donkey, bovine, ovine and caprine) to *in vitro* digestion to gain information on the fatty acids pattern liberated upon simulated enzymatic hydrolysis. The present study aimed to evaluate the effect of digested milk, with particular regard to its fatty acid composition, from human, donkey, bovine, ovine, caprine and formula milk on mature adipocytes 3T3-L1. In particular cellular viability, apoptosis, oxidative response and gene expression levels of NF-kB p65, HMGB1, SREBP-1c and FAS were evaluated.

Materials and methods

Milk treatments

Commercial brands of bovine milk, caprine milk and liquid formula milk were purchased at a local store. Ovine milk and donkey milk were taken at a dairy farm located in Foggia (Apulian region, Italy) and pasteurized (63 °C for 30 min). Five lactating women (1–3 months after delivery) were recruited for human milk samples collection. Gross composition of the milk samples is reported in Table 1. Milk sources were previously subjected to *in vitro* digestion according to Minekus et al. (2014). Free fatty acids in digested milk sources were analyzed as described in Santillo et al. (2018) and are reported in Table 2.

Table 1. Gross composition of milk source, (adapted from Santillo et al., 2018).

Parameter, %	Milk source[1]					
	HM	FM	DM	BM	OM	CM
Fat	2.29 ± 0.12	3.6 ± 0.01	0.53 ± 0.01	3.63 ± 0.12	8.16 ± 0.32	4.14 ± 0.15
Protein	1.21 ± 0.13	1.4 ± 0.01	1.46 ± 0.04	3.53 ± 0.56	6.01 ± 0.48	3.22 ± 0.35
Lactose	7.49 ± 0.13	6.03 ± 0.02	6.78 ± 0.15	4.69 ± 0.10	4.37 ± 0.11	4.18 ± 0.12

[1] HM = human milk; FM = formula milk; DM = donkey milk; BM = bovine milk; OM = ovine milk; CM = caprine milk.

Table 2. The effect of milk source in free fatty acids of digested milks (μg/mL of extract), (adapted from Santillo et al., 2018).

	Digested milk source[1]							
Free Fatty Acids[2]	FM	HM	DM	BM	OM	CM	SEM	Effect, P[3]
C8:0	4.85	3.50	4.93	6.69	12.22	5.17	0.91	NS
C16:0	73.47 c	111.74 b	131.46 a	117.14 ab	99.93 b	108.96 b	3.07	***
C18:1c9	6.90 b	20.97 a	2.60 b	3.94 b	3.74 b	3.18 b	1.40	***
C18:2c9c12	0.43 b	1.04 a	0.16 c	0.14 c	0.29 bc	0.14 c	0.08	***
C20:5n3	0.00	0.89	n.d.	n.d.	0.55	n.d.	0.17	NS
C22:6n3	0.08	0.36	n.d.	n.d.	0.12	0.30	0.09	NS
SC-FFA	31.90 a	36.33 a	14.28 b	32.74 a	43.42 a	24.45 b	4.67	*
MC-FFA	78.40 c	125.88 ab	136.68 a	127.46 ab	108.82 b	121.04 ab	3.75	***
LC-FFA	84.23 b	120.47 a	98.18 b	84.35 b	97.84 b	91.04 b	6.28	*
Total FFA	194.53 c	282.68 a	249.14 b	244.54 b	250.08 b	236.52 b	13.97	**
S-FFA	163.83	231.67	240.50	229.88	218.79	202.90	10.48	NS
MU-FFA	12.32 b	35.00 a	2.60 c	4.64 c	11.73 b	10.43 b	2.51	***
PU-FFA	18.39	16.00	6.03	9.98	19.56	22.92	5.75	NS

[1]FM = formula milk; HM = human milk; DM = donkey milk; BM = bovine milk; OM = ovine milk; CM = caprine milk.

[2]SC-FFA = short chain free fatty acids; MC-FFA = medium chain free fatty acids; LC-FFA = long chain free fatty acids; S-FFA = saturated free fatty acids; MU-FFA = monounsaturated free fatty acids; PU-FFA = polyunsaturated free fatty acids; Total FFA = total free fatty acids.

[3] NS, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Cell culture conditions

Murine 3T3-L1 fibroblasts were propagated and differentiated as described by Lin et al. (2005). Briefly, the cells were propagated in DM0 (DMEM containing 5 mM glucose, 10% FBS, and penicillin/streptomycin [100 units/ml each]) and allowed to reach confluence. After 2 d (day 0), the medium was changed to DM1 (containing 10% FBS and 160nM insulin, 250 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine). Two days later (day 2), the medium was switched to

DM2 (DMEM containing FBS 10% and 160 nM insulin). After another 2 d, the cells were switched backed to DM0 for 2 d. Finally 3T3-L1 adipocytes were treated with BSA medium (DMEM medium add BSA 500 μM) conjugated with 2% of different sources digested milk, previously filtered through 0.2 mm filter. Control group (CTR) was treated only with BSA medium. Time of contact was 24 h for XTT and ROS assay and 48 h for gene expression analysis.

Cell viability

Cell viability was determined with XTT Cell Proliferation Assay Kit (ATCC, Manassas, VA). Briefly, the cell were maintained in BSA medium added with digested milk from different sources for 24 h, then washed with PBS and incubated for 4 h with activated-XTT solution. The absorbance was read using a spectrophotometer at 630 nm and subtracted from the 450 nm values to eliminate non-specific readings. The viability was expressed as the percentage relative to absorbance of BSA.

Caspase 3–7 assay

Apoptotic assay was performed using CellEventTM Caspase-3–7 Green Detection Reagent (Molecular Probes-Life Technology) a fluorogenic substrate for activated caspase-3–7. Briefly, 3T3-L1 adipocytes were incubated with 5 μM of CellEventTM Caspase-3– 7 Green Detection Reagent at 37 °C for 30 min. The excitation/ emission was 502/530nm, measured using a Wallac 1420 Fluorescent Plate Reader.

Measurement of ROS generation

Treated cells seeded in a 96-well plate were incubated with 10 μmol/l CM-H2DCFDA (Molecular Probes-Life Technology, Brooklyn, NY) for 45 min at 37 °C, and the intracellular formation of ROS was measured at excitation/emission wavelengths of 485/530 nm using a Wallac 1420 Fluorescent Plate Reader (D'Apolito et al., 2017).

RT reaction and real-time quantitative PCR

Total RNA from treated cells was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Total RNA was then isolated following the manufacturer's instructions. The mRNA was reverse transcribed by SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, USA). Experiments were performed in quadruplicate in optical 96-well reaction plates on a CFX96 Touch Real-Time PCR detection System using iQ SYBR green supermix (Bio-Rad Laboratories Inc., California, USA). Experiment setup and data analysis were performed using CFX manager software. Expression levels of NF-kB p65, HMGB1, SREBP-1c and FAS were normalized to β-actin and GAPDH levels in the same sample. Melting curves were analyzed to ensure that fluorescence signals solely reflected specific amplicons. PCR conditions were as follows: 7 min at 95 °C and 45 cycles of 30s at 95°C and 30s at 60°C (D'Apolito et al., 2010).

Statistical analysis

Data on the effect of different digested milk treatments on mature adipocytes 3T3-L1 cellular viability, apoptosis, oxidative response and gene expression levels of NF-kB p65, HMGB1, SREBP-1c and FAS were analyzed using ANOVA for repeated measures (SAS Institute, 2011). Where significant effects were found ($P < 0.05$), the Student's t-test was used to locate significant differences between means.

Results and discussion

3T3-L1 adipocytes viability and apoptosis

The cytotoxic effect of digested human, bovine, caprine, ovine and donkey milk and of liquid commercial formula on mature 3T3-L1 adipocytes, detected by using XTT assay, is reported in Table 3 and graphically in Figure 1.

Table 3. Effect of digested milk treatments on viability, caspase 3-7 activities and ROS concentration activity in 3T3-L1 mature adipocytes.

	Treatments[1]								
Cellular parameters	**CTR**	**FM**	**HM**	**DM**	**BM**	**OM**	**CM**	**SEM**	***Effect, P*[4]**
Cell viability, % control [2]	100	41	38	40	39	40	43		NS
Caspase 3-7, RFU [3]	67,077.0	27,318.0	29,746.0	26,552.0	26,503.0	27,242.0	24,108.0	2,714.0	NS
ROS, RFU [3]	165,467.0	786,027.0[b]	630,477.0[b]	1,068,204.0[a]	1,243,015.0[a]	1,140,366.0[a]	1,203,505.0[a]	13,250.0	***

[1]CTR=control treatment, FM= digested formula milk, HM= digested human milk, DM= digested donkey milk, BM= digested bovine milk, OM= digested ovine milk, CM= digested caprine milk.

[2]Cell viability was expressed as % of control (BSA).

[3]Caspase 3-7 activity and ROS concentration were expressed as relative fluorescence units (RFU)

[4]NS, *** $P < 0.001$

Treatments with digested milk samples were compared to each other as percentages, keeping the control group as 100% of viability. Digested milk samples reduced cell viability compared with the control group; however, no significant differences were observed among different sources of digested milk.

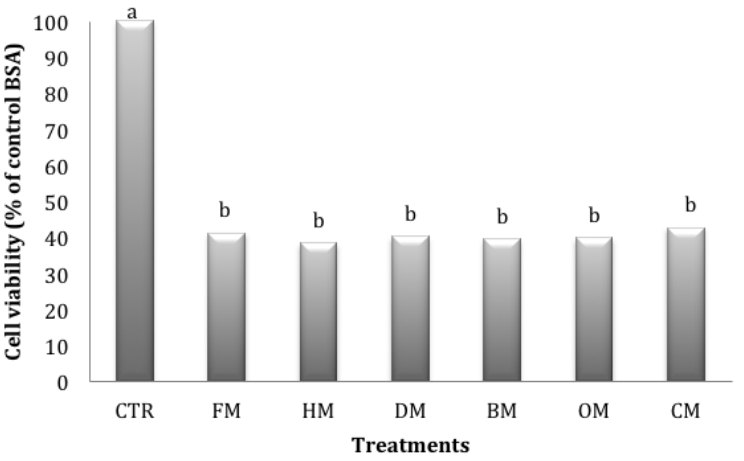

Figure 1. Effect of digested milk treatments on viability of 3T3-L1 mature adipocytes.

CTR=control treatment, FM= digested formula milk, HM= digested human milk, DM= digested donkey milk, BM= digested bovine milk, OM= digested ovine milk, CM= digested caprine milk.

Treatments not sharing a common letter differ significantly from one another ($P < 0.05$).

Cell death may be ascribed to different mechanisms such as necrosis or apoptosis. Apoptosis is an important biological process by which the body removes aged cells during physiological or pathological conditions (Sergeev, 2009) and it is described as an active, programed process of autonomous cellular dismantling that avoids eliciting inflammation. Necrosis has been characterized as passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents.

In order to evaluate the mechanism by which digested milk reduced cell viability the caspase 3–7 activity was evaluated. Caspase-3–7 are effector proteins that,

activated by caspase initiators, induce apoptosis in cells (Fink and Cookson, 2005). Table 3 shows the effect of digested milk treatments on caspase 3–7 activity in 3T3-L1 mature adipocytes; graphically is reported in Figure 2.

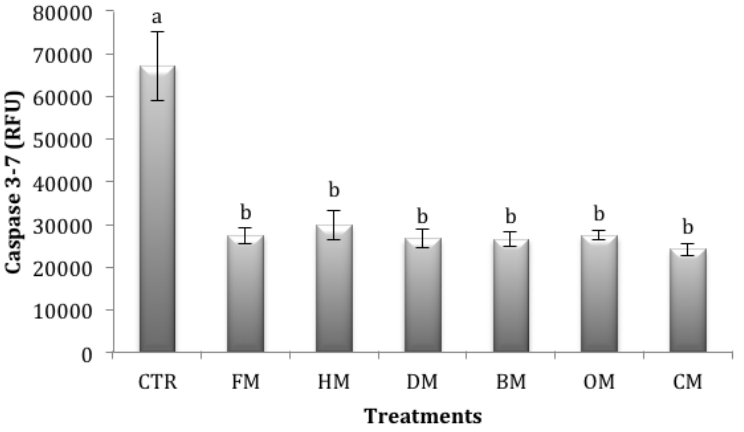

Figure 2. Effect of digested milk treatments on caspase 3-7 activity in 3T3-L1 mature adipocytes. CTR=control treatment, FM= digested formula milk, HM= digested human milk, DM= digested donkey milk, BM= digested bovine milk, OM= digested ovine milk, CM= digested caprine milk. Treatments not sharing a common letter differ significantly from one another ($P < 0.05$).

All of the treatments significantly reduced the caspase 3–7 activity compared to that found in the control. However, no significant differences in caspase activity were found among digested milk treatments. Results on caspase activity were in accordance with cell viability and indicate a late stage of apoptotic events in the control group where the major presence of live cells was able to produce caspase.

Level of ROS concentration

Since cell death is induced by oxidative stress (Navarro-Yepes et al., 2014), we next investigated the effect of digested milk samples on ROS production in 3T3-L1 mature adipocytes, as shown in Table 3 and graphically in Figure 3. In all digested milk treatments ROS level was higher than the control, however, the digested human and formula milk showed lower levels of ROS than DM, BM, OM and CM digested samples. Mitochondria are the main source of ROS due to cellular respiration; the respiratory process converts metabolic compounds such as carbohydrate, fat and protein to CO_2 and H_2O releasing various types of ROS at the same time.

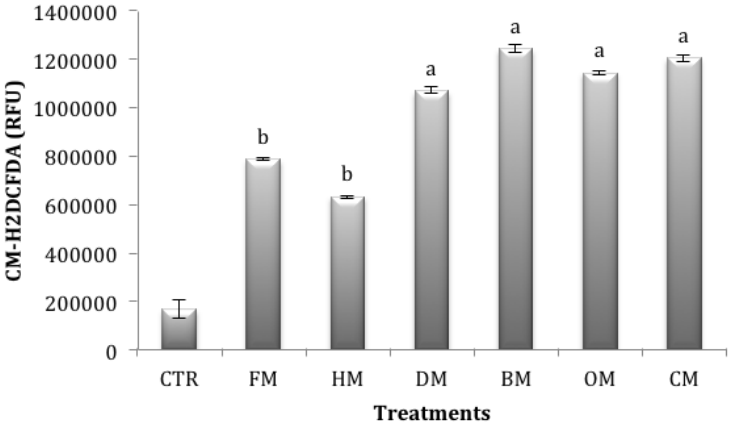

Figure 3. Effect of digested milk treatments on ROS concentration in 3T3-L1 mature adipocytes.

CTR=control treatment, FM= digested formula milk, HM= digested human milk, DM= digested donkey milk, BM= digested bovine milk, OM= digested ovine milk, CM= digested caprine milk. Treatments not sharing a common letter differ significantly from one another ($P < 0.05$).

This event is opposed by endogenous cellular antioxidant mechanisms such as superoxide dismutase, catalase and glutathione peroxidase but when ROS output exceeds antioxidant defenses, the cell enters a state of oxidative stress (Rigoulet et al., 2011). The role of ROS in adipose tissue is complex. In preadipocytes the accumulation of mitochondrial ROS could inhibit cell proliferation (Carrière et al., 2003, 2004; Wang and Hai, 2015), whereas in mature adipocytes from obese rats high level of ROS were observed (Furukawa, 2004) and protection of adipocytes from oxidative stress is recognized as a potential clinical strategy in obesity treatment (Kusunoki et al., 2013). In this study the lower capacity of HM and FM to induce oxidative stress in mature adipocyte could be ascribed to the peculiar free fatty acid profile of digested milk. The activity of PPARγ, involved in adipogenic pathways, can be influenced by fatty acids transported into the adipocytes (Fernyhough et al., 2007). Caprilic acid has been shown to induce ROS generation and might also modulate PPARγ activity indirectly via the ROS signaling pathways (Guo et al., 2006). Accordingly, digested milk from ruminant species showed a mean content of free caprilic acid higher than FM and HM (6.69, 12.22, 5.17 μg/ml for bovine, ovine and caprine respectively, vs. 4.85, 3.5 μg/ml of extract for formula and human milk respectively), and this could partly explain the lower ROS content in the former samples. Palmitic acid also represents an inflammatory mediator in the adipose tissue inducing white adipose tissue expansion and increasing inflammation through oxidative stress (Kennedy et al., 2009), palmitic acid being found in high levels in digested donkey milk. On the other hand, many natural lipid compounds with anti-inflammatory and antioxidant effects have been used to treat obesity such as n-3 PUFA, EPA and DHA, MUFA and CLA (Kusunoki et al., 2013; Chang et al., 2015). Here we observed higher free oleic and linoleic acid levels in digested human and formula milk; oleic acid 1.5- and 6-folds higher and linoleic acid 2- and 5.5-folds higher than that found in milk from ruminant species, respectively.

Gene expression of NF-kB p65 and HMGB1

Since ROS are key signaling molecules that play an important role in the progression of inflammatory disorders (Mittal et al., 2014), NF-kB p65 and HMGB1 were evaluated. The effect of digested milk source on the expression of NF-kB p65 and HMGB1 in 3T3-L1 mature adipocyte is shown in Figure 4a and 4b, respectively. All digested milk elicited a significant over-expression of NF-kB p65 in 3T3-L1 adipocytes compared to the control. Among treatments, the lowest gene expression was found in HM, BM, OM and CM, the highest in FM and an intermediate behavior was shown in DM. The p65 subunit of NF-kB is readily activated when cells are stimulated with various agents such as inflammatory cytokines, LPS, oxidative or shear stress (Yang et al., 1999), and exogenous administration of SFA was reported to exert a pro-inflammatory effect that plays a major role in the activation of NF-kB (Suganami et al., 2007). Inflammation induced in adipocytes by fatty acids is rather specific, in particular palmitate activates the NF-kB transcription factor in 3T3-L1 adipocyte and DHA and linoleate disrupt and prevent the NF-kB activation by palmitate (Ajuwon and Spurlock, 2005; Kennedy et al., 2009). The gene expression of HMGB1 was significantly up-regulated in HM, FM, CM and in DM with the highest gene expression, while it was comparable to the control in BM and OM. HMGB1 is a group of non-histone DNA-binding proteins with the role of stabilizing the nucleosomes, to help DNA binding and then to take part in DNA replication, transcription and repair (Wang et al., 2016). Nevertheless, under signals like stress, cell death, infection or inflammation, HMGB1 is found to be released from adipose tissue especially from obese persons (Gunasekaran et al., 2013). Excessive consumption of SFA and in particular palmitate expands adipose tissue and contributes to inflammation and weight gain (Kennedy et al., 2009). HMGB1 promotes inflammation and its receptors interact with NF-kB p65 forming a positive feedback loop to sustain inflammatory conditions (Wang et al., 2016). Both NF-kB p65 and HMGB1 expression in 3T3-L1 mature adipocyte treated with digested donkey milk could be an outcome of the free fatty acid profile

characterized by the highest content of SFA and palmitic acid, the absence of DHA, and low content of linoleic acid.

Gene expression of SRBP-1c and FAS

The effect of digested milk treatments on the expression of SRBP-1c and FAS in 3T3-L1 mature adipocyte is shown in Figure 1c and 1d, respectively. All digested milk treatments influenced the gene over-expression of SRBP-1c with FM and HM showing the highest levels. For FAS expression, BM showed the highest level, OM and CM the intermediate and FM, HM and DM the lowest levels, however HM and DM had comparable levels to the control. These two genes are closely related although their expression is sequential. SREBP-1c is one of the transcription factors that are implicated in adipose tissue differentiation, also involved in lipid metabolism and regulation of lipid homeostasis by controlling the expression of genes required for fatty acids and lipids production such as FAS (Shimomura et al., 1998; Jang et al., 2017).

In conclusion, all digested milk treatments decreased cell viability in mature adipocytes and induced cell death, partially due to apoptotic event, although no differences were observed among milk sources. In all digested milk treatments ROS level was increased relative to the control, however, 3T3-L1 mature adipocytes treated with digested human and formula milk showed lower levels of ROS probably due to the peculiar free fatty acid profile of these milks, yielding lower free caprilic acid after digestion. All digested milk treatments exerted a pro-inflammatory effect in mature adipocytes through over expression of HMGB1 and NF-kB p65 although lower gene expression was found in human milk and milk from ruminant species treatments. Finally, the study of SRBP-1c and FAS genes demonstrated a slight expression of the adipogenic pathway in adipocytes stimulated with different digested milk sources.

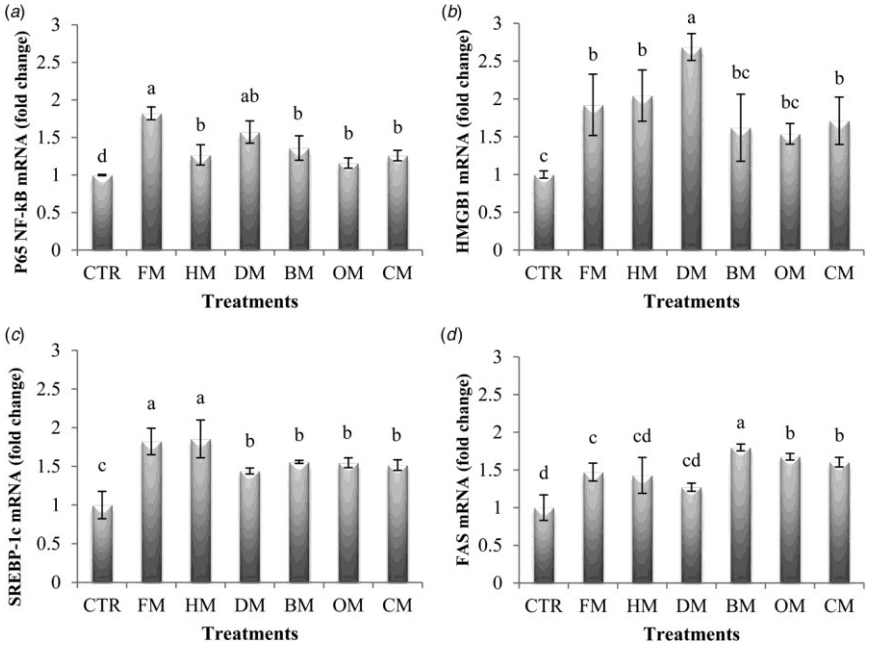

Figure Effect of digested milk treatments on the expression of NF-kB p65, HMGB1, SREBP-1c and FAS in 3T3-L1 mature adipocyte. The mRNA levels of (a) NF-kB p65, (b) HMGB1, (c) SREBP-1c and (d) FAS were measured by reverse transcription quantitative polymerase chain reaction. The mRNA expression levels of target genes were normalized using β-actin and GAPDH. Treatments not sharing a common letter differ significantly from one another ($P < 0.05$). CTR=control treatment, FM= digested formula milk, HM= digested human milk, DM= digested donkey milk, BM= digested bovine milk, OM= digested ovine milk, CM= digested caprine milk.

Conclusion

All digested milk treatments decreased cell viability in mature adipocytes and induced cell death, partially due to apoptic event, although no differences were observed among milk sources. In all digested milk treatments ROS level turned out to be higher than the control, however 3T3-L1 mature adipocytes treated with

digested human and formula milk showed lower levels of ROS probably due to the peculiar free fatty acids profile of milk yielding lower free caprilic acid after digestion. All digested milk treatments exerted pro-inflammatory effect in mature adipocytes through over expression of HMGB1 and NF-kB p65 although lower gene expression was found in human milk and milk from ruminant species treatments. Finally the study of SRBP-1c and FAS genes evidenced a slight expression of the adipogenic pathway in adipocytes stimulated with different digested milk sources.

Productive sector may benefit from improved knowledge of the effects of acidic profile of milk from different species on adipose tissue for exploitation of alternative milk to human milk for infant feeding. Overall this may be valorized by the dairy industry for the design of novel formulations characterized by a nutritional profile able to promote a correct development and functioning of adipose tissue suitable for sustaining human health.

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5. OVERALL CONCLUSIONS

Every single trial showed specific results and conclusion but in an overall vision it is possible to conclude that each milk has a peculiar effect on human health.

The trial about the fatty acids profile before and after *in vitro* digestion of milk from different sources, highlighted that the percentage distribution of fatty acids liberated upon gastrointestinal digestion did not reflect the patterns found in the corresponding not digested milk sources. This is likely due to the different susceptibility of milk sources to the action of the gastrointestinal enzymes used during the *in vitro* digestion process. The fatty acids composition of not digested milk is in accordance with the general scientific knowledge; the groups of short, medium chains and the saturated fatty acids, were major represented in ruminant milk than in human, donkey and formula milk which were more abundant in the long chain, mono- and polyunsaturated fatty acids groups. The amount of total free fatty acids liberated by digestion was highest in human milk, lowest in formula milk and intermediate in donkey and ruminants milk; the percentage contribution into the total free fatty acids amount was given main by saturated fatty acids that seem to be liberated more easily during digestion, in comparison to mono- and polyunsaturated fatty acids. It is worth to note that after digestion ovine and caprine milk showed a concentration of EPA and DHA comparable to ones observed in human milk; and concerning linoleic acid, donkey and caprine milk showed a major concentration compared to all other milk. This study about fatty acid pattern liberated upon milk digestion trying to exploit an alternative milk source for designing an innovative substitute in infant nutrition.

In whom to concern the two studies for evaluate the effect of whole milk and protein fractions from bovine, caprine, and ovine milk on production of cytokines and ROS and RNS by cultured PMBC from infants with generalized epilepsy, the results were most complex. Both in the first and in the second study, pro- and anti-inflammatory cytokines produced *ex vivo* from PBMC isolated from children with generalized epilepsy were influenced by stimulation with milk and milk protein fractions from different milking species. The differences in cytokine responses may be associated with genetic polymorphisms of the milk proteins. Evaluating

the production of cytokines by PBMC after exposure to different milk protein fraction is interesting to identify potential antigens in milk. The ability of PBMC to secrete cytokines in response to stimulation by milk and protein fractions may be used like a predictor of the secretion of pro- and antiinflammatory cytokines in the bloodstream of challenged patients. Detection of TNF-α and ROS/RNS *in vitro* may provide information on inflammatory status and oxidative damage occurring in infants with generalized epilepsy. Cytokines might be useful biomarkers to discriminate the effects of foods on the inflammatory response; dietary strategies could help in alleviating the negative effect of epilepsy in infants. Further investigations on milk components are needed to better clarify the mechanisms affecting immunological status in infant with generalized epilepsy.

In the last trial, emerged that all digested milk treatments decreased cell viability in mature adipocytes and induced cell death, partially due to apoptic event, although no differences were observed among milk sources; also the level of ROS in all digested milk treatments turned out to be higher than the control, however 3T3-L1 mature adipocytes treated with digested human and formula milk showed lower levels of ROS probably due to the specific free fatty acids profile of milk yielding lower free caprilic acid after digestion. Every digested milk treatments exerted pro-inflammatory effect in mature adipocytes through over expression of HMGB1 and NF-kB p65; although among milk source, the lower gene expression was found in human milk and milk from ruminant species. Finally, the study of SRBP-1c and FAS genes evidenced a slight expression of the adipogenic pathway in adipocytes stimulated with different digested milk sources.

Productive sector may benefit from improved knowledge of the effects of acidic profile of milk from different species on adipose tissue for exploitation of alternative milk to human milk for infant feeding. Overall this may be valorized by the dairy industry for the design of novel formulations characterized by a nutritional profile able to promote a correct development and functioning of adipose tissue suitable for sustaining human health.

A new approach was used in the Ph.D. research project to highlight the impact of animal management not only on the milk quality but studying this matrix as a food using in vitro digestion, which represents the complex phenomenon that transforms milk into a nutritional and functional matrix.