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Review

Proteins and bioactive peptides from donkey milk: The molecular basis for its reduced allergenic properties

Vincenzo Cunsolo*, Rosaria Saletti, Vera Muccilli, Serafina Gallina, Antonella Di Francesco, Salvatore Foti

Department of Chemical Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

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ABSTRACT

The legendary therapeutics properties of donkey milk have recently been supported by many clinical trials who have clearly demonstrated that, even if with adequate lipid integration, it may represent a valid natural substitute of cow milk for feeding allergic children. During the last decade many investigations by MS-based methods have been performed in order to obtain a better knowledge of donkey milk proteins. The knowledge about the primary structure of donkey milk proteins now may provide the basis for a more accurate comprehension of its potential benefits for human nutrition. In this aspect, experimental data today available clearly demonstrate that donkey milk proteins (especially casein components) are more closely related with the human homologues rather than cow counterparts. Moreover, the low allergenic properties of donkey milk with respect to cow one seem to be related to the low total protein content, the low ratio of caseins to whey fraction, and finally to the presence in almost all bovine IgE-binding linear epitopes of multiple amino acid differences with respect to the corresponding regions of donkey milk counterparts.

1. Introduction

Since its domestication, about 5000 years ago, donkey (*Equus asinus*) has been used principally as pack and riding animal and for millennia its main activity was to carry weights and pull carts. At the end of XX century, donkey was reassessed and for the first time was used as a partner in pet-therapy projects and “onodidattica” (education through donkeys) activities for children with and without disabilities. So that, with the beginning of XXI century an ancient knowledge was rediscovered: the value of donkey milk (DM). Indeed, since ancient times DM has been used for infant nutrition and its therapeutic properties were widely known. Hippocrates as well as Pliny the Elder believed that it could act therapeutically in numerous cases, such as liver problems, infectious diseases, fevers, asthma, etc. (Hippocrates; translated by Adams, 1843; Pliny the Elder, translated by Bostock, & Riley, 1855). The use of DM as a moisturiser in cosmetics is even more fabled. Cleopatra and other privileged ladies of ancient times were taking their bath in DM in order to keep their skin fresh and shiny. Nowadays, the alleged therapeutic and cosmetic properties of DM seem to be validated by many trials (Brumini, Criscione, Bordonaro, Vegarud, & Marletta, 2016). DM is rich in vitamins and polyunsaturated fatty acids (Aspri, Economou, & Papademas, 2016) and contains anti-ageing, anti-oxidant and regenerating compounds, which are described as naturally active in skin hydration and skin ageing prevention. The lipid fraction is characterized by high levels of essential fatty acids and low saturated fatty acids (Gastaldi et al., 2010). Compared with ruminants' milk, the considerable presence of unsaturated fatty acids found in DM make it very useful in the prevention of the cardiovascular, auto-immune and inflammatory diseases (Martemucci & D'Alessandro, 2012; Martini, Altomonte, & Salari, 2014). In this aspect, the high content of ω3-polyunsaturated fatty acids, characteristic constituents of the fish oils, can counteract the above-mentioned pathologies through the synthesis of anti-inflammatory, anti-aggregant and non-immunosuppressant substances, like lipidic prostaglandins, leukotrienes, cytokines or interleukins (Salimei & Fantuz, 2012). Moreover, like human milk (HM), DM contains a substantial amount of lactose (about 7%), which determines its flavour and facilitates calcium absorption. The high lactose content together with the low protein amount, the low casein (CN)/whey protein (WP) ratio and the high level of the non-protein nitrogen (NPN) fraction reveal that DM differs considerably from that of the principal dairying species (i.e. cow, goat and sheep), whereas it is the most similar to breast HM and mare milk as well (Table 1). However, it should be noted that DM, with respect to breast HM, has a low caloric value and relatively low lipid content. So that, DM is inadequate as exclusive food in infants for the first year of life, and therefore adequate lipid integration is needed for toddlers' diet (D'Auria, Mandelli, Ballista,

* Corresponding author.
E-mail address: vcunsolo@unict.it (V. Cunsolo).

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

	Donkey	Mare	Human	Cow	Goat	Sheep
Proteins[b]	16	23	13	34	35	56
Fat[b]	8	11	40	36	38	69
Lactose[b]	66	63	66	50	41	49
Caseins/Whey Ratio	1.3	1.1	0.4	4.6	3.5	3.2

[a] Values are reported as average of different literature data (see text for bibliography).
[b] Values are expressed as g/kg milk.

Di Dio, & Giovannini, 2011). Compared with mare and cow milk (CM), DM has a lower AA content, probably related to its lower protein content. On the other hand, the overall amount of essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, and valine) in DM is higher than that in bovine milk. Moreover, DM shows noticeably higher levels of serine, glutamic acid, and arginine, and a lower value of cysteine with respect to CM. An exhaustive description of the gross composition of DM is beyond the scope of this review. However, an excellent survey about this topic and a comparison among different mammalians' milk can be found in some review articles (Guo et al., 2007; Medhammar et al., 2012; Uniacke-Lowe, Huppertz, & Fox, 2010). DM is known also to possess natural protective antimicrobial factors and a specific epidermal growth factor (EGF) that suggest its beneficial impact on gastrointestinal mucosa health and integrity; a claim particularly valuable for children, the elderly, and convalescents, who have a reduced immune defense system (Brumini et al., 2016; Scafizzari et al., 2009). In fact, many clinical studies have clearly demonstrated that DM may be very useful in the prevention of atherosclerosis (Tafaro et al., 2007), has an effect on the osteogenesis process, as well for the rehabilitation of patients with coronary heart disease or premature senescence, and in hypocholesterolemic diets (Chiofalo, Salimei, & Chiofalo, 2001). In raw DM the antimicrobial activity is mainly attributed to the very high content of lysozyme (Uniacke-Lowe et al., 2010) that, working together with lactoferrin and immunoglobulis, inhibits microbial growth in the digestive tract and contributes to reduce the incidence of gastrointestinal infections, especially during infancy and in childhood (Baldi et al., 2005; Gubić et al., 2016). Finally, taking into account the very similar composition between DM and HM, an increasing number of literature data shows that DM may represent, for a large number (about 90%) of the investigated subjects, the best natural substitute of CM for children affected by Cow Milk Protein Allergy (CMPA) (Bertino et al., 2010; Carroccio et al., 2000; Monti et al., 2007; Monti et al., 2012). As a consequence, in the last decade the interest of researchers, above all paediatric allergologists, towards DM has increased. Many investigations have been performed in order to obtain a better knowledge of DM components, particularly proteins, to detect unintended contaminations, improve dairy productions, and to accomplish a more accurate comprehension of its potential benefits for human nutrition (Claeys et al., 2014; Cunsolo, Muccilli, Saletti, & Foti, 2013; Medhammar et al., 2012). In this aspect, the continuous and rapid evolution of mass spectrometry-based techniques, which arguably represent the core tools in proteomic approaches, has provided an efficient platform for the characterization of food-derived proteins (Cunsolo, Muccilli, Saletti, & Foti, 2014).

In this review the main results of research oriented to the elucidation of donkey milk proteins characterization will be summarized. Moreover, structural differences between proteins of donkey and other mammalian milks, their significance in human nutrition and potential cross-reactivity of milk proteins from different species in relation to the treatment of CMPA will be discussed. This overview will be preceded by a brief description of the most common MS-based proteomic techniques.

2. MS-based approaches in milk proteomics

No one can deny the fact that the bovine is certainly the primary dairy animal species dominating global milk production and consequently, among mammalian milks, this brand has been the longest and the broadest investigated (D'Alessandro, Zolla, & Scaloni, 2011). On the contrary, although extensive reviews about the composition, physicochemical and nutritional properties of DM have been published, the characterization of the protein fraction until ten years ago was very scanty and limited to a handful of whey proteins, whereas the knowledge of the donkey caseins was totally absent (Cunsolo, Saletti, Muccilli, & Foti, 2007). In the last years, an extensive investigation of DM aimed to the characterization of its molecular composition, allowed to expand the information regarding the most abundant whey proteins and to identify and characterize the casein components previously completely unknown (primary structure, disulphide bridges and other post-translational modifications such as phosphorylation and glycosylation). An important step forward has occurred in 2011 when an in-depth proteomic investigation described the presence of about one hundred proteins in DM (Cunsolo et al., 2011). The recent advances in the proteomic analysis of DM have been possible because the continuous and rapid evolution of techniques usually employed for the investigation and characterization of complex protein mixtures. Particularly, the impressive increasing in performance and versatility of the MS-instrumentation has contributed to the development of new analytical strategies for proteins identification and structure determination, evidencing how mass spectrometry arguably represents an indispensable tool in proteomics. On the other hand, concurrent optimization of the separation techniques and the availability of efficient methods for gene sequencing, resulting in a extensive enlargement of the protein sequences database, have provided an efficient platform for large scale protein investigation. In the current proteomic approaches, the characterization of protein mixtures is usually performed combining highly efficient separation techniques, such as reversed-phase ultra high performance liquid chromatography (RP-UHPLC), mono- (SDS-PAGE) or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with biochemical methods (e.g. to immunospecific staining), enzymatic digestions and mass spectrometric analyses in order to obtain data suitable for searching, by specific software, protein databases. Taking into account that the general strategy applied depends on the experimental question and the scope of the research, two complementary MS-based approaches are currently used in proteomics: the so-called “bottom-up” and “top-down”, each of them having advantages and drawbacks (Kelleher et al., 1999). Briefly, bottom-up strategy represents the most widespread proteomic workflow and can be carried out by different ways; as example, in “break-then-sort” approach, the protein mixture is directly digested into a collection of peptides, which are separated by multidimensional chromatography (e.g. strong cation exchange followed by RP-HPLC) on-line coupled to ESI tandem MS analysis. Instead, in the so-called “sort-then-break” approach, proteins are firstly separated by 2D electrophoresis; then, the isolated proteins are subjected to enzymatic digestion followed by LC-MS/MS analysis. If the “bottom-up” represents the traditional approach, the so-called “top-down” represents the emerging MS-based strategy in proteomic studies, providing information on both intact protein mass and its amino acid sequence. The top-down proteomics approach utilizes molecular and fragment ion mass data obtained by the ionization and fragmentation of a protein in the mass spectrometer, without prior proteolytic digestion. In particular, the proteins present in a complex mixture are firstly fractionated and separated into pure single proteins or less complex protein mixtures. This step is followed by off-line static infusion or by on-line LC/MS analysis of sample into the mass spectrometer for high resolution mass measurement of intact protein multi-charged molecular ions. MS/MS of mass-selected multi-charged ions then provides fragment mass values for its structural characterization (McLafferty et al., 2007). Top-down and bottom-up