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Donkey Milk Proteins: Digestibility and Nutritional Significance

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Abstract

Donkey milk is particularly recommended for infant nutrition as substitute of cow milk in case of sensitive neonates (showing cow milk protein allergies). Its protein composition and the ratio between caseins and whey proteins reveals a high similarity with human milk, thus, in the last 10 years, an increasing interest arose to obtain a full characterisation of donkey milk proteins, here acknowledged. Digestibility data, mainly derived *in vitro* with human gastrointestinal enzymes, showed the high digestibility of donkey caseins and major whey proteins, except lysozyme and α -lactalbumin which proved to be quite resistant. The reported antimicrobial properties of donkey milk open concrete possibilities to use donkey milk as natural food preservative. Due to its attractive healthy properties, donkey milk was investigated for useful applications or to develop novel foods characterised by a high nutritional profile.

Keywords: donkey milk, proteins, nutritional value, digestibility, novel food

1. Introduction

A newborn who is fed colostrum (milk of the first 3–5 days of lactation, reinforced with transfer immunity factors) and then mature milk greatly benefits for a healthy growth, fulfilling all nutritional requirements and perinatal passive immunisation. The early feeds should be easily digestible and well tolerated to achieve a suitable intake and reduce the risk of diseases, mainly favoured by the passage of milk proteins and peptides which, after digestion, stimulate the mucosa immune system of the infant [1]. The milk of each species is designed to meet the specific needs of the neonate; however, when breastfeeding is not possible, or after weaning, it becomes

important to find an adequate alternative nourishment. This is particularly challenging for the infants affected by cow milk protein allergies (CMPA) that, occasionally, are associated with clinical cross-reactivity between milks of other ruminants [2]. In addition, if multiple milk protein allergies appear, the use of soy-based formulas or extensively hydrolysed protein formulas is not recommended to avoid the risk of cross-sensitisation [3]. Contrarily, equid milk, especially donkey milk, showed to be well tolerated by children with CMPA in terms of clinical tolerability [4, 5], likely associated to the comparable protein fraction composition between donkey and human milk. With the exception of a notably lower fat content, donkey milk is characterised by a gross composition similar to human milk, with a close ratio between casein/whey proteins which is believed to play a crucial role in the sensitisation to cow milk protein fraction, reducing the allergenic capacity [6]. Although further studies, including *in vivo* tests, are desirable to achieve more consistent results on its hypoallergenic properties, an increasing interest is arising around donkey milk. Indeed, recent investigations on the protein and fatty acid profile [7, 8], main mineral composition [9], some vitamin contents, microbiological and hygienic aspects [10, 11], greatly contributed for a wider knowledge on this milk. The majority of surveys on the quality of donkey milk have been conducted in Italy, but some data are also available from the milk of Chinese and Balkan donkey breeds [12]. A distinctive value of donkey milk is especially related to its antimicrobial properties which were more extensively documented [13–15]. Furthermore, numerous evidences reported several health-promoting properties such as antioxidant activity [16], the regulation of immune response in healthy elderly consumers [17] and anti-proliferative and anti-tumour *in vitro* effects on human lung cancer cells [18]. Besides infant diet therapy, the attractive nutritional features shown by donkey milk could also be addressed to other categories (e.g. elderly population) or employed in alternative food formulations.

This chapter will focus on the mainly characterised proteins of donkey milk, showing their nutritional value, the related impact on the human digestive system, and some potential applications in the dairy field.

2. Donkey milk caseins

Total protein content of donkey milk ranges between 15 and 18 g/L and the casein fraction represents about 35–45%, much lower than the milk of ruminants (>70%) but more similar to human milk (<30%). The available knowledge on donkey milk caseins is limited, compared to conventional dairy species and a full characterisation was also complicated by their heterogeneity, partly due to post-translational processes, genetic polymorphism, non-allelic deleted forms [19]. A combination of electrophoretic, chromatographic, and proteomic-based methods allowed the identification of the four casein fractions (α_{s1} -, α_{s2} -, β -, and k -casein). Casein (CN) distribution of donkey milk showed β -casein as the predominant one, followed by the α_{s1} -casein whereas α_{s2} -casein was detected as minor component [7, 19]. k -casein was only found in traces and is reported to be the most heterogeneous individual casein, likely due to different levels of glycosylation [19]. Donkey CSN2 gene has been sequenced (GenBank FN598778.1) and β -casein's primary structure of donkey milk has been completely characterised [20], using

mare's β -CN derived from cDNA, as reference. It is constituted of 226 amino acids and it has a molecular weight of 25,529 Da, containing seven potential phosphorylation sites, together with two additional ones (located at Thr12 and Thr207), analogously to the homologous mare's β -CN. According to Chianese et al. [19], the full-length β -CN and its deleted form were both found equally phosphorylated with 5, 6, and 7 P/mole, together with a novel β -CN variant which showed the same phosphorylation pattern but a higher molecular weight (>28 mass units) than the most common β -casein.

Donkey α_{s1} -CN contains 202 amino acids and has a molecular mass of 24,406 Da, prior to post-translational modifications [21]. The related CSN1S1 gene has been sequenced (GenBank Acc. Num. FN386610) and a rare mutation was reported to be associated (Marletta et al., personal communication) with the apparent absence of this fraction in the milk of a jennet belonging to Ragusano breed [22]. A remarkable heterogeneity of this protein was assigned to either discrete phosphorylation (5, 6, and 7 P/mole) or non-allelic deleted forms, generated by incorrect RNA splicing as already shown in the homologous goat and sheep casein [19]. α_{s1} -CN of donkey and cow shows a low homology and their difference in the amino acid sequence of the IgE-binding linear epitopes may be responsible for the hypoallergenic properties [23]. Regarding the α_{s2} -CN of donkey, the CSN1S2 gene (GenBank Acc. Num. CAX65660.1) has 2 forms (I and II) that differ in structure and encoded protein sequence. The major form (CSN1S2 I) consists of 19 exons and encodes a 221 amino acid protein, whereas the CSN1S2 II form is shorter (16 exons and 168 encoded amino acids). The existence of different splicing isoforms has been also suggested [24, 25], whereas three main phosphorylated components have been described for α_{s2} -CN, each accounting for 10, 11, and 12 P/mole [19]. All these structural variations may influence protein allergenicity [26]. Finally, the CSN3 gene sequence has also been determined in donkey (GenBank Acc. Num. FR822990.1) but only a specific immunostaining was able to detect *k*-casein in donkey milk; it was surely due to the low amount of this fraction and to its great heterogeneity, so that eleven *k*-casein components were found in an individual sample [19].

3. Donkey milk whey proteins

Donkey milk is characterised by a high proportion of whey protein. Most of the alleged nutritional properties of this milk can be attributed to this fraction, that is mainly composed of β -lactoglobulins (β -Lg) α -lactalbumin (α -La) and lysozyme (Lyz). The other three minor proteins immunoglobulins (Igs), serum albumin (SA), and lactoferrin (Lf) are also present. Even if the whole whey protein fraction is considered to be responsible for the low bacterial count of donkey milk [15, 27], the antimicrobial activity is mainly attributed to Lyz and, to a lesser extent, to Lf [28]. These minor proteins, together with Igs, are believed to work in synergy, for inhibiting microbial growth and reducing the incidence of gastrointestinal infections [1, 29, 30].

Donkey β -Lg, that is the most abundant whey protein, consists of two components: a major β -Lg I of 162 amino acid residues and a minor β -Lg II of 163 residues, which possesses an additional Glycine between the 116th and the 117th residue, as it occurs in mare's milk. β -Lg I

(gij125913) represents about 80% of total β -Lg and presents only two variants, A and B: the former is of 18,528 Da, the latter is of 18,514 Da [31]. In contrast, β -Lg II (gij125904) (Mw 18,200 Da), representing only the remaining 20%, has five variants: A, B, C [31, 32], D [33], and E [34] with molecular weights ranging from 18,227 (variant B) to 18,311 (variant D). Very recently, a new variant, with a predicted molecular weight of 18,315, has been identified in Ragusano donkey breed by direct DNA sequencing (Marletta et al., personal communication). β -Lg, absent in human milk, is generally considered to be one of the main causes of CMPA [35] because it can elicit an allergic reaction in sensitive subjects [28]. To this point, the identification of animals producing milk lacking in β -Lg II protein [22] appears promising to solve potential residual cases of reactivity [36]. Interestingly, donkey β -Lg was found to be highly degraded (70%) *in vitro* by human gastric and duodenal juice [37] in comparison to cow counterpart [38]; this feature could enhance the formation of derived bioactive peptides in gut, with potential antimicrobial activity [14]. Donkey α -lactalbumin (α -La) contains 123 amino acid residues and has a molecular weight of 14,215 Da [39]. Only one α -La genetic variant (gij262063) with two isoforms, characterised by different isoelectric points (pI 4.76 and 5.26, respectively), have been identified so far [33, 40]. Even though donkey α -lactalbumin shows a striking sequence homology with C-lysozyme, which is a known powerful antibacterial agent [41], there is no direct evidence of antimicrobial activity of donkey α -La and/or its derived peptides, so far. Lysozyme (Lyz), whose content is particularly high in donkey milk (up to 4 g/L), has two variants (A and B) both containing 129 amino acids (gij126613; gjj126614) and a molecular weight of 14,632 Da, which differ in three amino acid substitutions at positions 48, 52, and 61 as previously described [32, 33]. In general, lysozyme has an important role in the intestinal immune response since it acts as a powerful antibacterial protein, splitting the bonds between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan [42]; thus, Gram-positive bacteria are more sensible to Lyz than the Gram-negative. However, a synergistic action with lactoferrin is supposed to enhance antibacterial action against also towards some Gram-negative bacteria [15]. These natural preservative properties could be the reason for lengthy shelf-life reported for raw donkey milk [13, 15].

Immunoglobulins (IgGs) of donkey milk show a high content in comparison with human and bovine counterparts [28] since they are supplied to the foal only after parturition to fortify the natural immunopassive system of the neonate. The presence of IgGs in colostrum and then (in lower amount) in mature milk is still a matter of debate for attributing health beneficial effects to a given milk type or for consuming raw milk [43]. Donkey lactoferrin (Lf) is an 80-kg/mol iron-binding multifunctional glycoprotein that exerts several biological activities [40, 44]; it is generally associated with antimicrobial, antiviral, immunomodulatory, and anticarcinogenic activity [[45] for a review], although its content in donkey milk is relatively low compared to lysozyme. Finally, lactoperoxidase (LP), an oxidoreductase enzyme with protective function against microorganism infections, is found at a small concentration in fresh donkey milk [46, 47]. LP is known to be inactivated by high temperature but this enzyme could be of significant nutritional interest in raw-fresh milk, because working in synergy with lactoferrin and lysozyme could contribute to enhance the natural preservative action of donkey milk [15].

4. Remarks on donkey milk digestibility

Digestibility of donkey milk proteins was firstly assessed by Tidona et al. [14] in a simulated gastrointestinal digestive process using human gastric and duodenal juices; donkey caseins proved to be rapidly digested since after 1 hour of digestion only about 7% remained intact. The acid coagulum observed in the acidic conditions of gastric digestion (pH ~2) was very fine and the formation of a soft precipitate was also reported in equine and human milk, which is physiologically more suitable for infant nutrition than the firm coagulum formed by bovine milk [48]. Casein micelle size of donkey milk (about 298 nm) was found to be much larger than the one of human milk (64 nm), as it is inversely related to the *k*-casein content [49]; this condition, together with the relative abundance of β -casein, may be the reason for the high susceptibility to hydrolysis by gastrointestinal enzymes [38]. Certainly the low protein and casein content of donkey milk might favour the fast digestible caseins compared to high casein predominant milk of other species.

Regarding the whey proteins, β -lactoglobulins showed to be quite resistant to gastric enzymes (mainly pepsin) but were highly degraded by human duodenal juice (~30% remained undigested) contrarily to what was reported from cow and goat milk [38]. Digestibility of β -Lgs could be even enhanced in the individual milk of donkeys lacking β -lactoglobulin type II, achieving a higher rate of degradation [37]; this is nutritionally relevant since human milk is typically devoid of β -lactoglobulins. Donkey α -lactalbumin is the most resistant protein, since the 95% was found undigested after 1 hour of *in vitro* digestion [37], so that it reaches the gut relatively intact as already reported for milk of other species [38]. Similarly, lysozyme was quite resistant to human gastrointestinal enzymes, although at a lower extent (~75%) compared to α -lactalbumin, and was also found to be thermal stable after high pasteurisation treatment [27, 50]. Particularly interesting is the high digestibility of Donkey Lf by gastric and duodenal juice [37]. This evidence suggests that lactoferrin might play a further biologic role directly in the gut [51] as well as through its bioactive peptides, called lactoferricin (Lfcin) and lactoferrampin (Lfampin), as already observed in cow milk. The presence of these proteins and the peptides derived (so far unidentified) during digestion could still inhibit sensitive bacteria in the intestine as the antimicrobial activity exhibited by the digested donkey milk was even enhanced [14]. Among the other minor whey proteins (such as immunoglobulins, serum albumin, and lactoperoxidase), weak data are reported given their low concentration in donkey milk, but warrant further investigations to evaluate how the natural immunepassive system may be affected by the digestion process.

5. Nutritional value and potential applications

Since ancient times, queens and empresses like Cleopatra and Poppea used to take a bath in donkey milk, experiencing its smoothing and lenitive properties. Nowadays, donkey milk is still used in cosmetic preparations but the renewed interest shown by the scientific community is mostly oriented for feeding purposes. Namely, donkey milk was defined as “pharma-food” for its nutritional, nutraceutical, and functional properties [16]. To enhance the nutritional value, donkey milk was considered a suitable matrix as carrier of probiotic bacteria,

when supplemented in concentrated freeze-dried cells, reporting a high viability of *L. rhamnosus* and *L. paracasei* in donkey milk [52], which could be useful for the prevention and treatment of antibiotic-associated diarrhoea [53].

The peculiar high lysozyme content and the antimicrobial activity shown by native donkey milk were tested by its addition to goat milk, which is often used as hypoallergenic drinkable milk, to evaluate the inhibition of pathogen bacteria under refrigerated storage conditions; an inhibited growth rate was especially evident for *Staphylococcus aureus*, *Listeria monocytogenes*, and *Campylobacter jejuni* [54]. Moreover, the employment of donkey milk was recently proposed as an alternative to egg lysozyme, in order to prevent blowing defects in long seasoned cheeses [55]: the addition of donkey milk (1.1% v/v), significantly contributed to reduce the presence of coliforms in cheese.

Despite the high lysozyme content, which tends to inhibit several bacteria, some less sensitive lactic acid bacteria were used to ferment donkey milk, which resulted in a non-conventional ingredient to deliver fermented milks of new generation [56]. Recently, a fermented beverage based on donkey milk emulsified with sunflower oil was developed, showing an increased folic acid content, a higher level of polyunsaturated fatty acids, and more favourable lipid quality indexes (atherogenicity and thrombogenicity index) than donkey milk itself [57]. The manufacture process for yogurt production with donkey milk was also attempted with the addition of two adjunct probiotic strains: the resulted product presented a higher antioxidant activity and a lower lactose content, which aims to meet nutritional requirements of certain consumers groups [16]. Besides, rheological aspects related to donkey milk fermented products are limited by the low protein and casein content because the acid-induced coagulation of equine caseins behaves more as a micellar flocculation rather than gelation [28] and a high tendency to syneresis [57]. Indeed, the protein content (low in caseins) and profile (poor in *k*-casein) of donkey milk are not very suitable for transformation as the use of bovine chymosin leads to a very weak gel, without curd formation. Yet, raw donkey milk was surprisingly employed in cheese-making using a new coagulant, a pure camel chymosin which increases the substrate binding and determines a higher milk clotting activity [58]; the result was a soft cheese that allowed merely 3.3% of cheese yield. The manufacture of the cheese was only obtained with fresh donkey milk, since any heat treatment prevented coagulation. Thus the mechanism underlying the enzymatic coagulation of donkey milk needs further research.

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