Dairy Sci. & Technol. (2016) 96:1–14
DOI 10.1007/s13594-015-0246-1

REVIEW PAPER

Whey proteins and their antimicrobial properties in donkey milk: a brief review

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Received: 3 April 2015 / Revised: 1 July 2015 / Accepted: 2 July 2015 /
Published online: 23 July 2015

Abstract Milk is a source of bioactive compounds essential for health and growth of newborns. Donkey milk, rich in lactose and whey proteins, has been proven to be a good breast milk substitute during infancy and adequate nourishment for patients with cow milk protein allergy. Beside, this milk is gaining a growing interest for human nutrition because of some other alleged health benefit. It shows antibacterial activity toward a wide range of Gram-positive and Gram-negative bacteria, stimulates immune system in convalescence, regulates gastrointestinal flora, and prevents inflammatory and autoimmune diseases. As regards its antimicrobial properties, although all the milk components might contribute to this activity, the whey protein fraction of donkey milk is generally believed to play the main role. The aim of this review is to highlight the antimicrobial properties of donkey milk with a special focus on the whey protein fraction. The effects of preservation and processing treatments on whey protein content and antimicrobial activity are also discussed.

Keywords Donkey milk · Whey protein · Antimicrobial properties · Processing treatment

1 Introduction

In the first months of life, colostrum and milk provide to the newborn a variety of compounds able to fulfil all nutritional requirements and perinatal passive immunization. In addition, milk is crucial for setting up the oral tolerance to nutrient molecules in

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the newborn, allowed through the passage of milk proteins and peptides, which, after digestion, stimulate the mucosa immune system of the infant (Baldi et al. 2005). When the mother is not able to breast feed, or after weaning, it becomes important to find an adequate alternative nourishment. Equidae milk has the most comparable protein composition to human milk among different species (Table 1); in particular, donkey milk (DM) often represents a good natural breast milk substitute during early infancy. It has a peculiar protein composition, and it is rich in polyunsaturated fatty acids, essential amino acids, and lactose (Guo et al. 2007). Donkey milk contains a low level of total protein (about 1.7%) and a low casein/whey protein ratio (on average 1.3) with values ranging from 0.66 to 1.33 in individual milk along the lactation (Tidona et al. 2011a). This ratio is believed to play a crucial role in the sensitization to cow milk protein fraction, reducing the allergenic capacity: the lower the value, the lower the allergenic capacity (Lara-Villoslada et al. 2005). Although thorough studies would have to be conducted to achieve more conclusive results (Alessandri and Mari 2007), in vivo donkey milk showed to be well tolerated by children with cow milk protein allergy (CMPA) in terms of clinical tolerability (Restani et al. 2002; Monti et al. 2007; Swar 2011). In vitro monoclonal and polyclonal antibodies produced against cow milk proteins showed a very mild cross-reaction with donkey milk (Restani et al. 2009; El-Agamy et al. 2009).

Furthermore, donkey milk is known 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 (Scafizzari et al. 2009).

For these reasons, in recent years, donkey milk has been considered also a functional food because it contains healing properties above and beyond its basic nutritional value. The high content of lactose gives a good palatability and makes it a good growth medium for potentially probiotic strains of *L. rhamnosus* (Coppola et al. 2002) and exerts a good effect on gut health.

In raw donkey milk, the foodborne pathogens are generally absent and the somatic cells and total bacterial count are often low suggesting it could be a safe food, if the mammary gland is healthy and the animals are milked in proper hygienic conditions (Pilla et al. 2010). On the other hand, the high value of antimicrobial components as well as the lysozyme confers on donkey milk a long natural shelf life and bactericidal

Table 1 Milk gross composition (%) of different species

	Total solid	Protein	Fat	Lactose	Ash	Casein/whey protein ratio	Reference
Human	11.9	0.9	3.8	7.0	0.2	0.4:1	Hambræus (1984)
Donkey	9.4	1.7	0.4	6.9	0.4	1.3:1	Salimei et al (2004)
Horse	10.1	2.1	1.2	6.4	0.4	1.1:1	Malacarne et al. (2002)
Cow	12.6	3.4	3.7	4.8	0.7	4.7:1	Jenness (1974)
Goat	12.2	3.5	3.8	4.1	0.8	3.5:1	Park (2006)
Camel	13.3	3.5	4.0	4.9	0.9	3.3:1	Shamsia (2009)

action on pathogen or potentially pathogen bacteria (Zhang et al. 2008; Nazzaro et al. 2010b).

Recently, donkey milk was shown to have positive effects on the regulation of immune response in healthy elderly consumers (Jirillo et al. 2010). In addition, its antimicrobial activity has been tested and proved effective against bacteria and viruses often associated with intestinal infection (Nazzaro et al. 2010a; Tidona et al. 2011b; Šarić et al. 2012; Brumini et al. 2013a).

The aim of this brief review is to collect the first reports on antimicrobial properties of fresh donkey milk, with a special focus on its whey proteins. The effect of some processing (heating, fermentation, digestion) on whey protein content and antimicrobial activity is also discussed.

2 Donkey whey proteins with antimicrobial activity

Milk has a natural protective function: it can increase resistance to infection and inhibit the action of potentially pathogenic microorganisms. Antimicrobial activity in milk is mainly attributed to some minor whey proteins: lactoferrin (Lf), lysozyme (Lyz), immunoglobulins (Igs), and lactoperoxidase (LP) (Baldi et al. 2005; Yamauchi et al. 2006); their content may vary largely between species, breeds, and individuals because of genetic or breeding variants.

The whole whey protein fraction of donkey milk is considered to be responsible for the low total bacterial count reported in literature (Chiavari et al. 2005; Šarić et al. 2012); the antimicrobial activity is mainly attributed to Lyz and, to a lesser extent, to Lf (Uniacke-Lowe et al. 2010).

Some studies suggest that Lyz, Lf, and Igs, working together in donkey milk, create synergy with potential function in the digestive tract to inhibit microbial growth and contribute to reduce the incidence of gastrointestinal infections, especially during infancy and in childhood (Baldi et al. 2005; Businco et al. 2000).

In donkey milk, particularly rich in lysozyme, a synergistic activity of Lyz with Lf and some fatty acids with known antibacterial activity as well as linoleic, lauric, and oleic acid which is the most abundant could also contribute to donkey milk's strong overall antibacterial activity against both Gram-positive and Gram-negative bacteria (Šarić et al. 2014a). In fact, the interaction of Lf with the lipopolysaccharidic (LPS) layer is reported to cause disruption of the outer membrane and enhance susceptibility of Gram-negative bacteria to the lysozyme by increasing in membrane permeability (Ellison and Giehl 1991; Farnaud and Evans 2003; Benkerroum 2008).

Whey proteins content in donkey milk is about 4.9–9.6 $mg.mL^{-1}$ (Table 2) and is mainly made up of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), lysozyme (Lyz), immunoglobulins (Ig_s), serum albumin (SA), and lactoferrin (Lf). Some variation of the protein composition in donkey's milk has been observed during lactation (Tidona et al. 2011a; Guo et al. 2007). In Sicilian Ragusano donkey breed, the milk showed a constant reduction of total protein content, due to the decrease of casein fraction (about 0.63 $g.100\ g^{-1}$); as a consequence, the casein/whey protein ratio ranged from 1.33 to 0.60 from early to late lactation (Tidona et al. 2011a). Within the whey protein fraction, β-Lg, α-La, and Lyz decrease, whereas Igs increase and Lf maintains around the same amount during lactation (Guo et al. 2007; Vincenzetti et al. 2008).

Table 2 Whey protein content in different milks/species

Content ($g.L^{-1}$)	Human	Donkey[a]	Horse	Cow	Goat	Camel[b]
Total whey protein	6.2–8.3	4.9–9.6	7.4–9.1	5.5–7.0	3.7–7	5.9–8.1
β-lactoglobulin	—	1.3–5.5	2.55	3.2–3.3	1.5–5.0	—
α-lactalbumin	1.9–3.4	0.8–2.7	3.37	1.2–1.3	0.7–2.3	0.8–3.5
Serum albumin	0.4–0.5	0.4	0.37	0.3–0.4		7–11.9
Immunoglobulins	0.96–1.3	1.30	1.63	0.5–1.0		1.5–19.6
Lactoferrin	1.5–2	0.005–0.05	0.1–2.0	0.02–0.5	0.02–0.2	0.02–7.28
Lysozyme	0.1–0.89	0.67–4.00	0.5–1.33	$(70–600)\times 10^{-6}$	250×10^{-6}	$(60–1350)\times 10^{-6}$

Data from Claeys et al. (2014)

[a] Data from Salimei et al. (2004); Chiavari et al. (2005); Coppola et al. (2002); Vincenzetti et al. (2008, 2010 and 2011). Gubić et al. (2014); Tidona et al. (2011a, b); Guo et al. (2007); and Šarić et al. (2014c)

[b] Data from Shamsia (2009)

2.1 β-lactoglobulin

β-lactoglobulin (β-Lg) is the major whey protein except for human, camel, lagomorphs (hares, rabbits and pikas), and rodent milk (Uniacke-Lowe et al. 2010). It is a globular soluble protein with a molecular weight of about 18 $kg.mol^{-1}$ (Farrell et al. 2004) which is believed to play several biological roles (Sawyer 2003).

In donkey milk, the β-Lg showed a molecular weight of about 18.4 $kg.mol^{-1}$ and a mean content of 1.3–5.5 $mg.mL^{-1}$. Recently, Gubić et al. (2014) reported a very low content of β-Lg (from 139 to 263 $mg.L^{-1}$) in ten Balkan donkeys together with a high level of α-lactalbumin.

Donkey β-Lg consists of two components, the major β-Lg I of 162 amino acid (aa) residues and the minor β-Lg II of 163 aa, deriving from the insertion of a Gly between the 116th and the 117th residues, as occurs in mare's milk. β-Lg I (gij125913) represents about 80% of total β-Lg and has only two variants, A and B: the former is of 18,528 $g.mol^{-1}$, the latter is of 18,514 $g.mol^{-1}$ (Godovac-Zimmermann et al. 1990). In contrast, β-Lg II (gij125904) (Mw 18,200 $g.mol^{-1}$) that represents the remaining 20% of the β-Lg fraction has five genetic variants. The variants A, B, C (Godovac-Zimmermann et al. 1990; Herrouin et al. 2000), D (Cunsolo et al. 2007), and E (Chianese et al. 2013) showed molecular weight ranging from 18,227 (variant B) to 18,311 $g.mol^{-1}$ (variant D).

β-Lg, absent in human milk, is generally considered to be, together with casein fraction, one of the main causes of CMPA (Restani et al. 2009) because it is able to elicit an allergic reaction in sensitive subjects (Uniacke-Lowe et al. 2010). In this light, the identification of animals producing milk lacking in β-Lg II protein (Criscione et al. 2009) appears promising to solve potential residual cases of reactivity (Wal 2002).

Moreover, though the β-Lg is generally resistant to human gastrointestinal enzymes, probably because of the binding of fatty acids in the barrel of the structure, resistance to peptic digestion is different among species, so that ovine β-Lg is far more digestible than bovine counterpart (El-Zahar et al. 2005). Interestingly, donkey β-Lg is highly degraded (70%) in vitro by human gastric (HGJ) and duodenal juice (HDJ) (Tidona et al. 2014) that is twice as much in comparison to cow counterpart (Inglistad et al. 2010). This higher degradability of donkey's β-Lg could enhance β-Lg tolerability and the yield of derived bioactive peptides in gut with potential antimicrobial activity (Tidona et al. 2011b).

In general, quantitative variation β-lactoglobulin has an effect on β-lactoglobulin digestibility; in fact, two rare samples lacking the β-lactoglobulin II showed a more rapid and complete degradation of the β-lactoglobulin fraction, particularly during the gastric digestion step (Tidona et al. 2014).

2.2 α-lactalbumin

α-lactalbumin (α-La) is a globular small calcium metalloprotein, homologous with the well-characterized C-type lysozyme. Synthesized in the rough endoplasmic reticulum of the mammary gland, it is transported to the Golgi apparatus where it carries out its primary physiological function of the lactose synthesis regulation. In donkey milk, the α-La content showed a marked increase 3 months after parturition to reach a stable value of 1.8–2.1 $mg.mL^{-1}$. A recent paper reports that α-La is the major whey protein in Balkan donkey's milk, with a content ranging from 2.7 to 1.3 $g.L^{-1}$ from early to late lactation (Gubić et al. 2014). According to the literature, the content ranges from 0.8 to 2.7 $g.L^{-1}$ (Table 2). Donkey α-La contains 123 amino acid residues and has a molecular weight of 14,215 $g.mol^{-1}$ (Giuffrida et al. 1992). Only one α-La genetic variant (gij262063) with two isoforms characterized by different pIs (4.76 and 5.26, respectively) have been identified so far (Cunsolo et al. 2007; Vincenzetti et al. 2012). The high similarity between donkeys and other species α-La suggests that the presence of the same molecular structure with four intramolecular disulfide bridges is highly likely.

Donkey α-La is resistant to gastric and duodenal enzymes, since the 95% is undigested after a two-step in vitro test (Tidona et al. 2014), so that it reaches the gut relatively intact as already reported for raw equine, cow, and human milk (Inglistad et al. 2010). Even though it shows a striking homology, in terms of amino acid sequence, with C-lysozyme, which is a known powerful antibacterial agent (Lopez-Exposito and Recio 2008), there is no direct evidence of antimicrobial activity of donkey α-La and/or its derived peptides, so far.

2.3 Immunoglobulins

Immunoglobulins (Igs) in milk are an important defense family of proteins for the newborn naturally protecting the gut mucosa against pathogenic microorganisms. They inactivate bacteria by binding to specific sites on the bacterial surface: their role is to confer passive immunity to the newborn while its own immune system is developing (Gapper et al. 2007). Three classes of immunoglobulins are commonly found in milk: immunoglobulin G (IgG)—the principal immunoglobulin in equine colostrum—A (IgA)—the main form in equine milk (Uniacke-Lowe et al. 2010)—and M (IgM); IgG is often subdivided into two subclasses, IgG_1 and IgG_2 (Hurley 2003).

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

In donkey, total IgG content increases in milk during lactation, as reported by Guo et al. (2007); Gubić et al. (2014) confirmed this trend but only after a peak observed at 100th day of lactation. In general, a high amount of Igs in colostrum and in mature milk inhibit microbial growth and contribute to the immunity of the neonate; this feature is often used as an argument for attributing health beneficial effects to a given milk type or for consuming raw milk (Claeys et al. 2014). Milk Igs surely act in natural immune-passive system; besides, about 19% of ingested bovine IgG and IgM was found to retain immunological activity in the ileum of healthy human adults (Roos et al. 1995; Möller et al. 2008), so that they are also believed to exert their antimicrobial action directly in the intestine, as well as Lf and lysozyme. In addition, a recent paper showed Igs of donkey milk were highly degradated by HGJ and HDJ, but the 33% left undigested in an in vitro simulated human digestion (Tidona et al. 2014). Further investigation is needed.

2.4 Lactoferrin

Donkey lactoferrin (Lf) is an 80-kg.mol^{-1} (Vincenzetti et al. 2012) iron-binding glycoprotein of the transferrin family (Yamauchi et al. 2006). This multifunctional protein exerts several biological activities that can be different among species. However, human Lf has a significant antimicrobial bacterial activity in vivo and in vitro (Arnold et al. 1980; Sanchez et al. 1992; de Araujo and Giugliano 2001), and bovine lactoferrin, that has been deeply investigated, is associated with antimicrobial, antifungal, antiviral, immune-modulatory, and anti-carcinogenic activity (Tomita et al. 2009 for a review). The antibacterial activity of lactoferrin against a broad range of Gram-positive and Gram-negative pathogens seems to be dependent on its ability to sequester iron, producing an iron-deficient environment that limits microbial growth, and also dependent on the permeabilization of bacterial cell walls by binding to the lipopolysaccharides through its N-terminus (Valenti and Antonini 2005). The genetic polymorphism can also affect antibacterial activity. Caprine Lf was observed to exert high antibacterial activity as well (Recio and Visser 2000), also in relationship with its genetic polymorphism (Lee et al. 1997).

In general, Lf in donkey milk is higher than in ruminant milk, but much lower than in mare and in human milk (Kanyshkova et al. 2001). The average amount of lactoferrin in donkey showed a very wide range of variation (Table 2); it was 0.080± 0.0035 and 0.0048 g.L^{-1} in Italian (Vincenzetti et al. 2012) and Balkan donkeys (Šarić et al. 2012), respectively. More recently, in Balkan donkeys, Šarić et al. (2014c) reported Lf has undetectable but also a very high value (0.054 g.L^{-1}), whereas Gubić et al. (2014) reported a high concentration of Lf with a decreasing trend ranging from 0.041 to 0.006 g.L^{-1} from the 40th to 200th day of lactation. These findings deserve to be further investigated.

Some experimental evidences seem to suggest that also in donkey milk, Lf and Lyz work synergistically to effectively eliminate Gram-negative bacteria (Tidona et al. 2011b; Šarić et al. 2012). In fact, Lf can bind different components in the outer bacterial membrane, thereby opening “pores” for Lyz to disrupt glycosidic linkages in the interior of the peptidoglycans (Ellison and Giehl 1991; Leitch and Willcox 1999).

Donkey's milk Lf is quite easily digested by gastric and duodenal juice (Tidona et al. 2014). This evidence suggests that Lf can play its biologic role directly in the gut (Brock 2002) as well as through its bioactive peptides called lactoferricin (Lfcin) and lactoferrampin (Lfampin) observed in cow milk produced by commercial pepsin. In this light, it is noteworthy that bovine Lfcin has not been identified after digestion by human gastrointestinal enzymes (Furlund et al. 2013).

Tidona et al. (2011b) found that the antimicrobial activity of native and digested donkey milk against different bacteria could be due to Lf both intact protein and its peptides yielded by digestion. Recently, Brumini et al. (2013b) tested the antiviral effect of donkey milk and its whey protein on *Echovirus type 5*, an enterovirus which infects the human gastrointestinal tract; all the fractions (whole DM, skimmed DM, DM digested by human gastrointestinal juice, and DM whey protein fraction) showed a severe inhibition of the virus replication, with the highest antiviral effect observed for the whey protein fraction, probably because of the presence of Lf. Enzymatic digestion did not enhance the antiviral effect of donkey milk. Afterwards, it was confirmed that the main antiviral on *Echovirus* in DM may be attributed to high molecular mass whey proteins (Lf, LP, and Igs), even if a synergic action of the diverse proteins in the whey cannot be excluded (Brumini et al. 2013b).

2.5 Lysozyme

Lysozyme (Lyz) acts as a natural preservative, conferring a lengthy shelf life to raw donkey's milk (Zhang et al. 2008; Šarić et al. 2012).

It is a powerful antibacterial protein (Lopez-Exposito et al. 2008) with an important role in the intestinal immune response. Lyz splits the bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of the peptidoglycan leading to fragments with high and low molecular weight, which are the agents responsible for the specific immune cell activations. Because of this mechanism of action, Gram-negative bacteria are less sensible to Lyz than Gram-positive. As a consequence, antibacterial action against some Gram-negative bacteria can be explained by a synergistic action of Lyz with Lf, according to Ellison and Giehl (1991).

Donkey milk lysozyme, as well as equine and canine counterpart, belongs to C-type calcium-binding lysozyme and is able to bind calcium ions; this binding leads to more stable complex with an enhanced antimicrobial activity (Wilhelm 2009). Recently, Šarić et al. (2014b) report that donkey milk shows a calcium-dependent activity against *E. coli*.

In donkey milk, two variants of Lyz (A and B), both containing 129 amino acids (gij126613; gij126614), and with molecular weight 14,632 g.mol^{-1} that differ in three amino acid substitutions at positions 48, 52, and 61 have been described so far (Herrouin et al. 2000; Cunsolo et al. 2007). Lysozyme is present with high value in donkey milk, ranging from 0.67 to 3.74 g.L^{-1} in Italian and Domestic Balkan donkey (Vincenzetti et al. 2011; Šarić et al. 2012; Šarić et al. 2014c), and maintains the same high percentage (13.13–15.44) over the total protein during 150 days of lactation (Guo et al. 2007). In contrast, a decreasing trend was described in Balkan donkeys with values of 2.9 and 1.7 g.L^{-1} from 40 to 200 days of lactation (Gubić et al. 2014).

Donkeys' milk showed a significant antibacterial activity against *Listeria monocytogenes* and *Staphylococcus aureus* (Šarić et al. 2014a) that was also put in relationship with the high content of Lyz that ranged from 0.67 to 3.54 $g.L^{-1}$ together with good amount of some fatty acids, namely linoleic, lauric, and oleic acid, with well-known antibacterial activity toward Gram-positive bacteria (Galbraith et al. 1971; Galbraith and Miller 1973).

Donkey milk's Lyz is resistant to digestion: only the 25% of the total protein is digested in vitro by gastrointestinal juice (Tidona et al. 2014). It is also thermostable even after a thermal treatment of 63 °C for 30 min (Coppola et al. 2002; Di Cagno et al. 2004; Chiavari et al. 2005).

2.6 Lactoperoxidase

Lactoperoxidase (LP) is an oxidoreductase enzyme with protective function against microorganism infections. The enzyme contains a heme group, carbohydrates (about 10%), and a calcium ion. LP is a major antibacterial agent in bovine colostrum (de Wit and van Hooydonk 1996). The amount of lactoperoxidase in donkey milk has been scarcely investigated. According to Vincenzetti et al. (2012), it was 0.11 ± 0.027 $mg.mL^{-1}$, closer to human milk (0.77 $mg.mL^{-1}$) (Shin et al. 2001) than bovine milk (0.03 ± 0.1 $mg.mL^{-1}$) (Janet and Tanaka 2007).

Beghelli et al. (2011), testing antioxidant properties of donkey milk, detected a lactoperoxidase activity equivalent to about 0.11 $mg.mL^{-1}$. A very low peroxidase activity (4.83 ± 0.35; 1.39 ± 0.23; 2.88 ± 0.51 $mU.mL^{-1}$) was found in fresh, frozen, and powered donkey milk, respectively (Mariani 2010). The low activity confirms the small concentration of LP in fresh donkey milk.

In contrast, this enzyme could be of significant nutritional interest in raw fresh milk and, since it works in synergy with Lf and Lyz, could contribute to enhance the natural preservative action of donkey milk and may function in the infant's digestive tract to reduce the incidence of gastrointestinal infections (Businco et al. 2000). In fact, the total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and non-immunoglobulin defense proteins. This is thought to be for their synergy or for the presence of natural bactericidal peptide (Clare and Swaisgood 2000).

3 Influence of different processing treatments on whey protein content and antimicrobial activity of donkey milk

Fresh raw donkey milk is gaining an increasing popularity as "healthy food." When produced under standard conditions, donkey milk shows a low total bacterial count (about 4×10^4 $CFU.mL^{-1}$) (Coppola et al. 2002) and keeps a low value even during about 4 days of storage (Chiavari et al. 2005; Šarić et al. 2012). The high content of Lyz and other non-specific antimicrobial compounds gives to raw fresh donkey milk a long natural shelf life and makes a prolonged heat treatment unnecessary, which is normally required for preserving hygienic quality and nutritional properties. However, it still needs thermal treatments in order to guarantee its healthiness and quality over time.

The most common method of sanitization is the pasteurization at 63 °C, which generally is not expected to affect the main nutritional and health properties (Coppola et al. 2002; Chiavari et al. 2005). Other preservation methods, such as drying (spray-drying and freeze-drying) or fermentation, allow a longer storage but can significantly affect milk protein composition and their functional properties.

Polidori and Vincenzetti (2010) evaluated the effect on the whey fraction of donkey milk of two technological processes, freezing and spray-drying, as a two-step process which includes a strong thermal treatment to produce powdered milk. Total whey protein and lysozyme contents were almost similar in fresh (7.5 and 1 $mg.mL^{-1}$), frozen (7.2 and 1 $mg.mL^{-1}$), and spray-dried/powdered (6.7 and 0.94 $mg.mL^{-1}$) donkey milk. Lysozyme activity remained the same in fresh and frozen donkey milk ($U.mL^{-1}$=0.035) also after 1 month of storage at 4 °C, in the case of fresh donkey milk. In contrast, powdered donkey milk retained only the 30% of the lysozyme activity; thermal denaturation started at 70 °C.

Lyophilisation, or freeze-drying, is another powdering process obtained by low temperature and vacuum. Fresh and freeze-dried donkey milk show a similar protein composition in terms of caseins and total proteins, except for α-La; on the contrary, a significant reduction of these fractions can be observed in frozen milk after 1 and 2 months of storage (Vincenzetti et al. 2011). In the same experimental conditions, Lyz maintained its high level and enzymatic activity.

The fermentation process increases the good lactic acid bacteria (LAB) in milk and at the same time reduces the content of pathogen bacteria. As a consequence, fermented milk enhances gut health preventing and/or reducing the most common infections supported by bacteria and viruses. A general concern on donkey milk is that the high level of Lyz inhibits the growth of some lactobacilli or can cause a severe selection on several LAB strains. On the other hand, fresh donkey milk is a rich natural resource of probiotic bacteria which are capable to colonize the colon, acting directly in the gut against outcome pathogen bacteria and also stimulating the immune system (Nazzaro et al. 2012). Recently, eight bacterial strains belonging to *Lactobacillus* genus have been isolated from donkey milk; they might be used in fermented milk or yogurt (Nazzaro et al. 2012). Besides, if fermented, donkey milk will yield metabolites such as acetate and lactate that inhibit the growth of potential enteropathogens (Fooks and Gibson 2002) and others with several healthy functions including antibacterial activity (Nazzaro et al. 2008). Lactic acid present at the end of the fermentation could also decrease the pH value in the intestine acting as bacteria antagonist and facilitate calcium absorption (Lan et al. 2007). The synergism among natural compound of milk, microorganisms, and metabolites positively influences the intestinal flora composition and the defense system mechanisms of the host (Fooks and Gibson 2002).

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

The digestion process, as well as fermentation, is able to breakdown the proteins to shorter peptide chains. Besides native proteins, the biological action could be exerted by bioactive peptides; endowed sequences, inactive while part of the protein, that after the passage through the stomach and intestine, are partially or totally hydrolyzed into fragments (Hill et al. 2000; Yalcin 2006).

In a recent study, donkey milk revealed to be a good source of antimicrobial bioactive peptides, released during in vitro simulated gastrointestinal digestion processes, able to inhibit the replication of three enterobacteria (Tidona et al. 2011b). β-Lg and Lf are the highest digested fractions, while α-La and Lyz are the most resistant proteins; however, since part of those proteins remain undigested, continuing to perform their action intact (Tidona et al. 2011b), the antimicrobial activity might result from a synergism of the intact proteins and peptides.

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

4 Conclusions and future perspective

In the light of these first findings, the antimicrobial properties of donkey milk whey proteins against bacteria, both Gram positive and Gram negative, and viruses (Table 3) seem very promising. In many case, the role of the single whey protein (Lf, Lyz, LP, Igs) as well as its mechanism of action remain to be fully clarified. Nonetheless, these arguments reinforce the claim of donkey milk as functional food with diverse bioactivities. It surely represents a valuable natural breast milk substitute, useful both to strengthen the host's immune defense systems and to preserve gut health in infancy.

Table 3 Antimicrobial activity of donkey milk against/toward different microorganisms

Microorganism	Type of Sample	Reference
Bacillus cereus DSM4384	Hydrolyzed donkey milk	Nazzaro et al. 2010a
Bacillus cereus RT INF01	Digested donkey Milk	Tidona et al. 2011a, b
Echovirus type 5[a]	Whey proteins of donkey milk	Brumini et al. 2013a, b
Enterococcus faecalis DSM2352	Hydrolyzed donkey milk	Nazzaro et al. 2010a
Escherichia coli ATCC 10536	Donkey milk	Šarić et al. 2012; Šarić et al. 2014b
Escherichia coli ATCC 8739	Donkey milk	Šarić et al. 2014b
Escherichia coli (EPEC) 10208355	Digested donkey Milk	Tidona et al. 2011a, b
Listeria monocytogenes ATCC 19111	Donkey milk	Šarić et al. 2014a
Listeria monocytogenes 2230/92	Digested donkey Milk	Tidona et al. 2011a, b
Salmonella choleraesuis (CGMCC 1.1859)	Donkey milk	Zhang et al. 2008
Salmonella enteritidis ATCC 13076	Donkey milk	Šarić et al. 2012; Šarić et al. 2014c
Salmonella livingstone	Donkey milk	Šarić et al. 2014c
Salmonella typhimurium ATCC 14028	Donkey milk	Šarić et al. 2014c
Shigella dysenteriae (CGMCC 1.1869)	Donkey milk	Zhang et al. 2008
Staphylococcus aureus ATCC 25923	Donkey milk	Šarić et al. 2014a
Staphylococcus aureus DSM25923	Hydrolyzed donkey milk	Nazzaro et al. 2010a

[a] Virus

Furthermore, natural antimicrobial activity of donkey milk can strengthen host defense, stimulates the immune system, regulates gastrointestinal flora, and prevents inflammatory and autoimmune diseases in children and adults.

Therefore, a wider consumption of fresh donkey milk could be definitely advisable not only for babies or for patients affected by CMPA but also for different categories of consumers, including the elderly and convalescents, with reduced immune defense system. Nevertheless, despite the recent expansion of specialized donkey breeding, donkey milk is still a "niche product," which often can only be retailed in farms for direct consumption. Fermented milk can represent a good alternative, suggested by some authors. Moreover, the application of some new technologies, such as lyophilisation and, to a lesser extent, microencapsulation could enable a better exploitation of this product. However, various factors have to be taken into account such as the stability, the biological activity, and the cost.

Further studies are needed to identify the components with higher antimicrobial properties and to evaluate other bioactivities, both in vitro and in vivo, in order to promote the potential use of donkey milk as functional food or as bioactive compound to formulate functional foods and drinks. Genomics and proteomics could provide cutting edge approaches in this topic.

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

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