



Amiata donkey milk chain: animal health evaluation and milk quality

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Abstract

This study presents a investigation into the animal health and quality of Amiata donkey milk for human consumption. Thirty one lactating dairy jennies were examined. The following samples were collected: faecal samples from the rectum of animals for parasitological examination; cervical swabs for the detection of bacteria causing reproductive disorders; and blood samples for serological diagnosis of main zoonotic (*Brucella* spp., *Leptospira* spp.) and donkey abortion agents (*Brucella* spp., *Leptospira* spp., *Salmonella abortus equi*, Equine viral arterithis virus, Equine herpesvirus type 1). In addition, individual milk samples were collected and analyzed for mastitis-causing pathogens and milk quality. Regarding animal health, we detected a high prevalence of strongyle parasites in donkeys. It is very important to tackle parasitic diseases correctly. Selective control programmes are preferable in order to reduce anthelmintic drug use. For dairy donkeys, withdrawal periods from anthelmintic drugs need to be carefully managed, in accordance with EU and national regulations. The isolation of *Staphylococcus aureus* in milk highlights the importance of preventing contamination during milking, by adopting appropriate hygiene and safety practices at a farm level. Amiata donkey milk lysozyme activity was high compared to cow's milk, contributing to the inhibitory activity against certain bacteria. Donkey milk was characterized by a high lactose content, low caseins, low fat, higher levels of unsaturated fatty acids compared to ruminant milks. Unsaturated fatty acids and omega 3 fatty acids in particular have become known for their beneficial health effect, which is favourable for human diet. These characteristics make it suitable for infants and children affected by food intolerance/allergies to bovine milk proteins and multiple food allergies as well as for adults with dyslipidemias and in the prevention of cardiovascular disease.

Introduction

The donkey are docile and rural equids, traditionally used as working animals and now also used in onotherapy (donkey assisted therapy) for children and hiking.

Interest in donkey milk has recently increased, especially in Europe, as it represents an alternative food in cases of bovine milk proteins allergy and in the prevention of metabolic pathologies (Trinchese *et al.*, 2015). Donkey milk has a somewhat similar chemical composition to human milk (Vincenzetti *et al.*, 2008). Recently it has been also highlighted the growing importance of milk production from species other than cattle, including donkeys, in tackling the growing global demand for milk (Faye and Konuspayeva, 2012).

The Amiata donkey is a local breed native to Mount Amiata (42°53'16"N 11°37'30"E-GPS coordinates) between the provinces of Siena and Grosseto, in the heart of Tuscany (Central Italy).

The Amiata population is a descendant of the North African donkey. It has its roots at a time when the Etruscan civilization was flourishing and occupied most of Central Italy (www.filieraippicatoscana.it). In 1800, Amiata donkeys were recognized as a homogeneous population. As a result of industrialization however, Amiata donkeys decreased dramatically in number and became endangered in the 1980s. In 1993, the breed was officially recognized (Martini and Salari, 2012), however programmes to protect and promote these donkeys have only recently been put in place enabling the population to reach the current number of about 2300 heads, more than double compared to 2005 (<http://www.anagrafeequidi.it/>).

An Amiata donkey milk supply chain was recently created in Tuscany, which represents a focus point for the protection of this breed. The creation of an innovative system focusing on donkey milk is particularly interesting for Central Europe and Mediterranean Countries, where native donkey breeds are strongly decreasing in numbers.

In addition, the lack of specific legislation for the production and marketing of donkey milk for human nutrition means that biological, chemical and physical evaluations have to be carried out in order to guarantee the nutritional quality and food safety.

In the light of the increasing interest in donkey milk, this study presents an investigation of some aspects of animals' health and quality characteristics of Amiata donkey milk for human consumption.

Materials and Methods

Animals and samplings

The sampling was carried out in a farm in Tuscany (Italy), within the native area of the Amiata donkey breed.

The donkeys were reared outdoors and were fed grass hay ad libitum and about 2.5 kg/d per head of commercial pelleted concentrate for dairy jennies. The jennies were routinely machine-milked and the foals were physically separated from their dams three hours before milking.

Thirty one lactating dairy jennies were examined. Pulse and respiration rates and body temperature were recorded, and body condition scores (BCS) were evaluated during the first examination (Svendsen, 2008). Due to the lack of information on the presence of infectious diseases in dairy donkeys, a sanitary screening protocol was designed encompassing udder health and the main reproductive, gastro-intestinal and respiratory disorders.

The following samples were collected from each jenny: one faecal sample for parasitological analysis; one cervical swab for detection of bacteria causing reproductive disorders, and one blood sample for serological diagnosis of the main zoonotic (*Brucella* spp., *Leptospira* spp.) and donkey abortion agents (*Brucella* spp., *Leptospira* spp., *Salmonella abortus equi*, Equine viral arteritis virus, Equine herpesvirus type 1).

In addition, during the different stages of lactation, four milk samples were collected from each animal (31 jennies). Two samples from each animal were taken in the period between the 2nd and 5th lactation month (31x2=62 samples), and 2 samples were taken between the 6th and 9th lactation month (31x2=62 samples). A total of 124 individual samples were analyzed for the detection of mastitis causative bacteria and for milk quality.

Animal health evaluation

Faecal samples were collected from the rectum, stored in sterile containers at 4°C until delivery to the laboratory, and analysed within 24 h as follows (Thienpont *et al.*, 1986): flotation test with a low density solution (sodium chloride (NaCl) saturated solution-s.g. 1.200) to detect intestinal helminths; McMaster assay with a sensitivity of 50 eggs per gram (EPG) to evaluate intestinal parasites count; Baermann technique to detect *Dyctiocaulus arnfieldi* larvae; faecal sedimentation to detect trematode eggs.

Cervical swabs were processed in order to detect *Taylorella equigenitalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus equi zooepidemicus* (OIE, 2008). The jennies who were positive for one of these bacteria were further tested after two weeks.

Blood serum was collected from the jugular vein using Vacutainer[®] tubes and stored at +4°C until delivery to the laboratory. The samples were obtained by centrifugation and stored at -20°C until testing. Sera were tested as shown in Table 1.

For the detection of mastitis causative bacteria, 10 ml of milk were aseptically collected from each half udder in sterile containers, stored at +4°C until delivery to the laboratory, and analyzed within 3 hours. Each milk sample was plated on Blood agar, MacConkey agar, Baird Parker agar and Edwards Medium agar and processed according to the Laboratory handbook on bovine mastitis (National Mastitis Council, 1999). Agar plates were incubated at 37°C and examined for growth at 24 and 48 h. Identification was performed by morphological analysis, Gram staining, and biochemical tests (API[®] system). Pathogens were also tested for antimicrobial susceptibility by the agar disk diffusion method (Bauer *et al.*, 1966). The tested antimicrobials were amoxicillin-clavulanate, cefoperazone, neomycin, penicillin, streptomycin and tetracycline.

Milk quality analysis

Individual milk samples were analysed in duplicate for dry matter (DM), fat and lactose by infrared analysis (Milkoscan, Italian Foss Electric, Padova, Italy); total proteins, caseins and ash content was also determined (AOAC, 2005)

The lysozyme activity was tested in all the milk samples by a fluorimetric method within six hours of milking (EnzCheck Lysozyme Assay kit, Invitrogen, Carlsbad, CA, USA). The method is based on the lysis of *Micrococcus lysodeycticus* marked with Fluorescein. The lysozyme activity was measured by a fluorometer (Salita, Thermo Labsystem FL, USA) using excitation of 494 nm and emission of 518 nm, and expressed as U/mL.

The diameter and the number of fat globules per mL of milk (μm) in each sample were measured by fluorescence microscopy following a direct method (Martini *et al.*, 2013). Milk fat extraction was performed following Rose-Gottlieb's method (AOAC, 2000). Fatty acid profile was assessed as follows: methyl esters (FAME) were prepared using methanolic sodium methoxide according to Christie (1982). A Perkin Elmer Auto System (Perkin Elmer, Norwalk, CT, USA) GC equipped with a flame ionization detector and a capillary column (30 m \times 0.25 mm; film thickness 0.25 mm; FactorFour Varian, Middelburg, Netherlands) was used. The helium carrier gas flow rate was 1 mL \cdot min $^{-1}$. The oven temperature program was as follows: level 1, 50°C held for 2 min, level 2, 50 to 180°C at 2°C \cdot min $^{-1}$ then held for 20 min, level 3, 180 to 200°C at 1°C \cdot min $^{-1}$ then held for 15 min, and finally level 4, 200 to 220°C at 1°C \cdot min $^{-1}$ then held for 30 min. The injector and detector temperatures were set at 270 and 300°C, respectively. The peak areas of individual fatty acids (FA) were identified using an FA standard injection (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) classes were calculated as follows:

SFA= \sum C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0;

MUFA= \sum C14:1, C15:1, C16:1, C17:1, C18:1 t9, C18:1 t11, C18:1 c9, C20:1, C22:1, C24:1;

PUFA= \sum C18:2 t9,12, C18:2 c9,12, C18:3 n6, C18:3 n3, C20:2, C20:3 n6, C20:4, C20:3 n3, C20:5, C22:2, C22:5, C22:6

Results and Discussion

Animal health evaluation

Clinical parameters are shown in Table 2.

All the jennies showed a good health status since the clinical parameters were consistent with the physiological range for donkeys (Svendsen, 2008).

Parasitological, bacteriological and virological assays results are shown in Table 3.

Strongyle eggs were found in 30 donkeys (96.77%), while *Oxiuris equi* eggs were found in one fecal sample (3.22%). Mean strongyle fecal egg count (FEC) was 886.67 eggs per gram (epg), with a minimum value of 50 epg and a maximum value of 2850 epg, and a standard deviation of 669,01 epg. *Oxiuris equi* FEC was 50 epg.

Dyctiocaulus arnfieldi was found in six donkeys (19.35%), while *Fasciola hepatica* was never detected.

There are only a few data available on the prevalence of donkey parasites. Moreover such studies have been carried out in extra-EU countries, where donkeys are reared for work purposes and live in a different environment and management conditions. The strongyle prevalence in different countries is: 95.2-97.1% in South Africa (Wells *et al.*, 1998), 99.5% in Ethiopia (Asefa *et al.*, 2011), and 80% in Mexico (Burden *et al.*, 2010). In Italy different studies have been also carried out, showing different prevalences: 77% (Giannetto *et al.*, 2008) and 93% (Veneziano, 2011).

The high prevalence of strongyles in the farm that we investigated may be related to the management conditions (free outdoor paddocks) and to the lack of parasite control programmes. In fact, a study on the efficacy of different parasite control strategies (selective and strategic therapies) is still in progress. It is important to plan a parasite control programme

in food-producing animals in order to guarantee animal welfare and animal health, taking into account a reduction in anthelmintic drug use.

The pharmacological control of donkey parasites can be difficult due to the lack of licensed anthelmintic products and the increasing prevalence of anthelmintic resistance (Matthews, Burden, 2013; Lawson *et al.*, 2015). This problem may be related to the application of therapeutic protocols designed for horses, using the same drug doses. Differences in pharmacokinetic and pharmacodynamic drug properties between horses and donkeys have been observed, as therapeutic doses used in horses has not been effective in donkeys (Grosenbaugh *et al.*, 2011).

A total of 27 of the tested animals showed a strongyle FEC above the cut-off value of 300 epg (Figure 1) (Matthews, Burden, 2013). Donkeys with a high FEC did not show any clinical signs of disease, as also described by Burden *et al.* (2010).

A selective or targeted therapy of strongyle infection, by treating only the animals with an FEC above the cut-off value, is preferable to a strategic therapy (Matthews, Burden, 2013).

The aims of a targeted therapy are to decrease selection pressure for anthelmintic resistance, to maintain animal health, and to ensure food safety.

Dyctiocaulus arnfieldi had the second highest prevalence in our study. None of the positive cases showed respiratory signs of disease, which confirms that this parasite does not cause any disease in healthy donkeys. However it is important to reduce *D.arnfieldi* infection, especially in old and immunocompromised animals or in mixed equine herds. Eprinomectin (EPM) administered as a pour-on preparation and at a dose rate recommended for cattle is effective against *D. arnfieldi* infection in donkeys (Veneziano *et al.*, 2011). The low level of excretion of EPM in donkey milk, which is lower than the maximum residue limit in bovine milk (20 µg/kg) (WHO/FAO, 1999), led to take into account the use of EPM in lactating donkeys at cattle dosage of 0.5 mg/kg bodyweight, thus avoiding any risk to food safety (Gokbulut *et al.*, 2012).

Microbiological analysis of cervical swabs showed the presence of *Streptococcus equi* subspecies *zoepidemicus* and *Klebsiella pneumoniae*, in three and two animals, respectively, with no clinical signs of reproductive diseases. *Streptococcus equi* subspecies *zoepidemicus* and *Klebsiella pneumoniae*, with *Pseudomonas aeruginosa*, represent commensal bacterial flora of genital system of equids (Samper, Tibary, 2006), so the isolation of these potential pathogenic bacteria alone is not sufficient evidence for diagnosis of endometritis. In stress conditions they can grow and proliferate, leading to clinical disorders. The positive females were tested again after two weeks and none of them were found to be positive for the previous isolated bacteria.

Serological tests showed no antibodies against the infectious agents tested. Brucellosis in equids is rare. In Europe there are no studies on the prevalence of this disease in donkeys, while in Nigeria a prevalence of 5% has been reported (Sadiq *et al.*, 2013).

All the individual milk samples collected during the first lactation period were negative for mastitic agents, while during the second lactation period *Staphylococcus aureus* and *Streptococcus equi* subsp. *zoepidemicus* were isolated in one and in two samples, respectively. These isolates were further tested by the agar diffusion method and none of them showed resistance to any of the antibiotics tested.

These results confirm the low prevalence of mastitic agents in donkeys (Pilla *et al.*, 2010) due to the peculiar anatomy of the equid udder which is well supported and sufficiently raised with respect to the ground. An additional reason is the antimicrobial activity of donkey milk due to the natural antimicrobial substances. A mastitic bacteria control programme is recommended however, because zoonotic bacteria isolation in apparently healthy animals can be a risk for end-consumers. This risk can be high if donkey milk is consumed raw, without any heat treatment. Methicillin resistant *Staphylococcus* spp. has been isolated in donkey milk in Italy (Naccari *et al.*, 2009).

Milk quality analysis

Table 4 shows the average composition of Amiata donkey milk.

The dry matter content is consistent with other donkey breeds, and lower than human and bovine milk (Martini *et al.*, 2014).

These differences between donkey vs human and cow milk are due to the milk nutritional components of the different species. Amiata donkey milk has a closer protein profile and lactose content to human than bovine milk. The lower total proteins compared to cow's milk, and above all the lower content of total caseins, makes donkey milk suitable for children allergic to cow milk proteins as also reported by Polidori and Vincenzetti (2013). The low casein content favours milk digestibility (Tidona *et al.*, 2011); in addition similarly to mare and human milk might determines the formation of a flocculent precipitate in the stomach, which decreases the bowel transit time (Malacarne *et al.*, 2002).

The low proteins contribute to a similar low renal solute load to breastfed children (Salimei, Fantuz, 2012). In fact, protein intake during infancy needs to be monitored due to the lifelong effect on kidney volume and function, and to obesity risk (Michaelsen and Greer, 2014).

The lactose content, which is similar to human milk, is much higher compared to bovine milk (Martini *et al.*, 2014). Lactose improves the palatability of donkey milk (Polidori, Vincenzetti, 2012) and stimulates the absorption of calcium in the intestine (Klobukowski *et al.*, 2014), contributing to calcium homeostasis. The calcium is considered a nutraceutical for geriatrics (Gupta and Prakash, 2015) and its bioavailability in donkey milk support this use of in geriatric nutrition.

The total minerals in Amiata donkey milk is intermediate between human and bovine (Martini *et al.*, 2014).

Similarly to the findings of Vincenzetti *et al.* (2008) in milk from other donkey breeds, Amiata milk also has an inhibitory activity against certain bacteria, probably due to the lysozyme content. The lysozyme content in donkey milk is very similar to that found in human milk, whereas only traces have been detected in bovine milk (Vincenzetti *et al.*, 2008; Uniacke-Lowe *et al.*, 2010). These differences are linked to the fact that the antimicrobial activity of human, horse and donkey milk is mainly determined by lysozyme and lactoferrin, whereas lactoperoxidase and immunoglobulins are the main defense systems in bovine milk (Uniacke-Lowe *et al.*, 2010; Hettinga *et al.*, 2011).

In our study the average lysozyme activity was high compared to cow's milk and similar to the lower value observed by Pilla *et al.* (2010) in the same donkey breed, whereas higher values have been reported in herds of crossbreeds, Martina Franca, and Ragusana donkeys (Cavallarin *et al.*, 2015). The differences with the study by Cavallarin *et al.* (2015) could be linked to endogenous and physiological factors (breed and lactation period) as well as the sample processing. In fact, although not significant, a progressive increased lysozyme activity has been found in raw milk during sample storage at 4°C (Addo and Ferragut, 2015). Amiata donkey milk could be considered as a diet food due to the low-fat content, compared to the average fat percentages of bovine milk and human breast milk. These characteristics along with the low energy value and hypolipidemic effects (Trinchese *et al.*, 2015) make donkey milk potentially exploitable for people suffering from obesity or cardio-vascular problems. n6:n3 ratio (1.65) in our study was lower compared to conventional and organic cow milk (5.77 and 2.28 respectively) (Benbrook *et al.*, 2013), this is a positive feature according the position of the Academy of Nutrition and Dietetics which recommend in adults diets an increased consumption of n3 fatty acids (Vannice and Rasmussen, 2014). However, the low fat may not make donkey milk nutritionally adequate for unweaned babies. This obstacle is still surmountable with appropriate lipid additions (Sarti *et al.*, 2016).

The analysis of the morphometric characteristics of donkey milk fat globules highlighted an average diameter of 2.12 μm , which is lower than human (3-5 μm) and cow milk (3.5-5.5 μm) (Martini *et al.*, 2016; Gallier *et al.*, 2015).

The small size of the fat globules favors the digestibility of the milk. Furthermore, the smaller globules have a higher surface/volume ratio compared to the larger globules, and contributes to a greater membrane intake in milk, which in turn contributes to the intake of various bioactive elements and polyunsaturated fatty acids (Martini *et al.*, 2013). In fact, in the milk analysed, the unsaturated:saturated fatty acid ratio was more similar to human (1.1) (Mäkelä *et al.*, 2013) than bovine (2.06) milk (Soyeurt *et al.*, 2008)

Conclusions

Regarding animal health, we confirmed the high prevalence of strongyle parasites in donkeys. It is very important to tackle parasitic diseases correctly, by proper diagnosis and pharmacological therapy with a suitable drug dosage. Selective control programmes are preferable in order to reduce anthelmintic drug use. For dairy donkeys, withdrawal periods from anthelmintic drugs need to be carefully managed, in accordance with Regulation 470/2009/EC (European Commission, 2009) and Regulation 37/2010/EC (European Commission, 2010).

The isolation of *Staphylococcus aureus* in milk highlights the importance of preventing contamination in the primary production, by adopting appropriate hygiene practices that ensuring the achievement of the objectives of Regulation 852/2004/EC (European Commission, 2004).

Donkey milk was characterized by the high lactose content, low fat, higher levels of unsaturated fatty acids compared to ruminant milk. Unsaturated fatty acids and omega 3 fatty acids in particular have become known for their beneficial health effect. A good unsaturated:saturated ratio and n6/n3 ratio can be considered favourable for human diet. Milk quality characteristics support its use in infants and children affected by food intolerance/allergy to bovine milk proteins and multiple food allergy and also in adults and in the elderly with dyslipidemias and in the prevention of cardiovascular disease.

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Table 1. Infectious agents investigated by serological analysis.

Infectious agents	Tests	References
<i>Brucella</i> spp.	Rose Bengale test	OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 6th ed., chap 2.4.3 part 2.a
Equine Viral Arteritis virus	Virus neutralisation test	OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 6th ed., chap 2.5.10 part 2.a
Equine Herpes Virus type I	Virus neutralisation test	OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 6th ed., chap 2.5.9 part 2.1
<i>L. interrogans</i> (Bratislava, Pomona, Icterohaemorrhagiae and Saxkoebing)	Microscopic agglutination test	OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 6th ed., chap 2.1.9 part 2.1
<i>Salmonella abortus equi</i>	Direct agglutination test (micromethod)	OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 6th ed., chap 2.9.9

Table 2. Mean, standard deviation, minimum and maximum values of clinical parameters.

	Mean±SD	Min	Max
BCS	3.6±0.506	3	4
Temperature (°C)	37.06±0.265	36.6	37.5
Pulse (beats/min)	40.52±4.589	32	48
Respiration (breaths/min)	21.16±3.891	16	32

SD, standard deviation.

Table 3. Parasitological, bacteriological and virological assays and prevalence.

Classes of disease		Sample	Agents	Prevalence (%)
Parasites	Intestinal	Faeces	Strongyles	96.77
			<i>Oxiuris equi</i>	3.22
	Respiratory		<i>Dyctiocaulus arnfieldi</i>	19.35
	Hepatic		<i>Fasciola hepatica</i>	0
Reproductive bacterial disorders		Cervical swab	<i>Taylorella equigenitalis</i>	0
			<i>Klebsiella pneumoniae</i>	6.44
			<i>Streptococcus equi zooepidemicus</i>	9.68
			<i>Pseudomonas aeruginosa</i>	0
Infectious diseases		Blood serum	<i>Brucella</i> spp.	0
			<i>Leptospira</i> spp.	0
			<i>Salmonella abortus equi</i>	0
			Equine Arteritis Virus	0
			Equine Herpesvirus type 1	0
Udder health	First sample	Individual milk	Mastitic agents	0
	Second sample		<i>Staphylococcus aureus</i>	3.22
			<i>Streptococcus equi zooepidemicus</i>	6.44

Table 4. Amiata donkey milk composition (n=124).

Gross composition (g/100mL)	
Protein	1.57±0.248
Casein	0.75±0.030
Fat	0.40±0.196
Lactose	7.23±0.243
Ash	0.37±0.061
Dry matter	9.38±0.546
Milk fatty acid (FA) classes and globule diameter	
Saturated FAs (g/100g of total FAs)	56.65±8.304
Monounsaturated FAs (g/100g of total FAs)	22.17±8.184
Poliunsaturated FAs (g/100g of total FAs)	21.18±4.051
Unsaturated:saturated FA ratio	0.80±0.347
n6:n3 ratio	1.65±0.185
Diameter of the milk fat globules (µm)	2.12±0.712
Lysozyme	
Activity (U/mL of milk)	3986.21±277.80
Somatic cell count	
Number of cells per mL	9.06·10 ³ ±3.67·10 ³

Values are expressed as means±standard deviation.

Figure 1. Stratification of animals (n) in relation to fecal egg count.

