

ORIGINAL
RESEARCH

Effect of cactus pear (*Opuntia ficus-indica* (L.) Miller) on the antioxidant capacity of donkey milk

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The cladodes of Opuntia ficus-indica (L.) Miller were fed to lactating donkeys as an alternative food, in order to evaluate effects on the chemical composition and antioxidant activity of their milk. Lactating donkeys were divided into two groups: standard feeding and alternative feeding. With regard to chemical composition, no differences were observed. Total antioxidant capacity was constant in the standard feeding group, while it increased in the alternative feeding group: the radical scavenging activity increased from 95.53% to 96.62% in the ABTS test and from 28.40% to 33.33% in the DPPH test. This indicated that alternative feeding affects the antioxidant capacity of milk.

Keywords Cactus pear, Milk, Donkey, Antioxidant activity.

INTRODUCTION

A trend in recent years is to use natural products such as fruits, herbs, oil seeds and vegetables as functional foods and sources for nutraceutical antioxidants (Gallegos-Infante *et al.* 2009). In addition, the interest of food industry has been increasing for natural antioxidants, to produce functional foods with beneficial effects for human health. In this contest, many researchers have focused their attention on *Opuntia ficus-indica* (L.) Miller (cactus pear or prickly pear) for its radical scavenging activity.

Moreover, cactus pear fruits and stems have been traditionally used for medicinal and cosmetic purposes, as forage, building materials and as a source of natural colours (Stintzing *et al.* 2005). Some research has been conducted on the introduction of this species in livestock husbandry as conventional substitute feeding. Costa *et al.* 2010 investigated the use of cladodes of the cactus pear (*O. ficus-indica* (L.) Miller) as a substitute for an energy concentrate in diets for lactating goats and their influence on milk quality. Cladodes are rich in pectin, mucilage, minerals, malic acid, vitamins and antioxidants (Gallegos-Infante *et al.* 2009; Sáenza

et al. 2004; Sepúlveda *et al.* 2007). Fermentation of the opuntia pectin results in an improvement of the rumen environment increasing acetate production (Berchielli *et al.* 2005). However, there is no more information about its use for the feeding of monogastric animals such as donkeys, and also on its contribution towards the chemical modification of milk product from these animals. Equine milk composition differs from that of other mammals with regard to its nutritional value (Chiavari *et al.* 2005), and also, donkey milk became interesting for researchers because of its high concentration of lysozyme, which is only present at low levels in bovine, caprine and ovine milk (Bucevic-Popovic *et al.* 2014; Vincenzetti *et al.* 2008). Lately, the research interest in donkey milk has increased in Europe, especially in Italy, because its composition is similar to that of human milk. This milk has a lower content of fat and protein, and higher content of lactose compared to bovine milk. Its low casein content, high percentage of essential amino acids and protein, and lipid profiles are similar to those of human milk, which favours its use as a potential new dietetic food. In fact, it is used in the paediatric sphere for children affected by cow milk protein allergies

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(CMA) and intolerance and as an alternative to human milk (Cosentino *et al.* 2012; Giosuè *et al.* 2008). Moreover, because of its high concentration of polyunsaturated fatty acids, calcium, vitamins A, B, C, and low cholesterol level, donkey milk is indicated for patients affected by heart disease, osteoporosis and atherosclerosis (Giosuè *et al.* 2008; Polidori and Vincenzetti 2010).

This interest has extended to its use in cosmetic products following greater consumer demands for 'natural' products (Cosentino *et al.* 2011, 2015a). Furthermore, these studies show that consumers prefer this product for its moisturising and rebalancing properties for membranes of skin cells (Cosentino *et al.* 2015a). As demonstrated by Polidori and Vincenzetti (2010) and Cosentino *et al.* (2012), these properties are principally due to the high amount of lysozyme and to the antioxidant activity of fatty acids contained in donkey milk. Donkeys may graze on a high number of plant species, highlighting their characteristics as autochthonous animals that adapt to marginal lands. Donkeys are particularly bred in Southern Italy, where *O. ficus-indica* is more widespread than in other areas of Italy. The objective of our research was to assess the chemical composition and antioxidant profile of donkey milk produced from animals fed with forage based on opuntia cladodes. Therefore, we hypothesised that such an alternative diet could increase the antioxidant activity of the milk.

MATERIALS AND METHODS

Cactus pear cladodes sampling

The research was conducted during the summer season in 2015 using ten trees grown in a commercial orchard located in Roccapalumba, in Sicily (37° 48' 00" N 13° 38' 00" E). Thirty young cladodes were sampled and analysed when freshly harvested during July, with average day/night temperatures of 25/28 °C. All sampled cladodes were weighed, and a tissue sample was taken. Spines and glochids were taken off through excision of a circular (1 cm diameter, 2 mm thickness) portion of epidermis tissue around the areola. The sample was a portion 4-cm-wide cut across all the longitudinal and transverse sections of the cladodes. Tissue samples were further cut into small pieces of 4 × 4 cm and dried in an oven at 105 °C for 48 h, to calculate the dry weight. Ash content was obtained after 24 h at 550 °C. The chemical and physical analyses on collected cladodes showed a size of 25.6 ± 3.5 cm; fresh weight 650 ± 50 g; dry matter 12 ± 0.9% (w/w); ash 25 ± 0.8% (w/w) of dry weight; and carbohydrates 62 ± 5.7 g.

Animals and feeding regimes

Eighteen pluriparous donkeys, of Martina Franca breed, foaled in July 2015, weighing 300 ± 10 kg, were investigated starting from the 2nd postfoaling month. The study was carried out in a rearing farm situated near San Martino

d'Agri, Basilicata (40°14'00"N 16°03'00"E) at an altitude of 750 m. The lactating donkeys, which were kept in box stalls with paddocks without access to pasture, were divided into two groups: standard feeding (SF) and alternative feeding (AF) based on *O. ficus-indica* (L.) cladodes addition. Each group was fed with isoproteic diets based on mixed hay and compound feed, with the following composition: safflower meal (25%), wheat middling (24%), soybean meal (20%), wheat bran (10%), distillers' grains (5%), corn gluten flour (3%), rice bran (3%), calcium carbonate (2.3%), maize germ meal (2.0%), sodium bicarbonate (2%), sodium chloride (1.20%), vitamin mineral supplement (1%), beet pulp pressed (1%) and molasses cane (0.5%).

The standard feeding was based on mixed hay (74.5%) and compound feed (25.5%), while the alternative feeding was based on mixed hay (67.33%), compound feed (17.82%) and *O. ficus-indica* (L.) Miller young cladodes (14.85%).

To evaluate important differences between the estimated and observed dry matter intake during the test, the administered dry matter was increased by 3%. The following equations were adopted for estimating the digestible energy (DE) content of feeds administered (National Research Council of the National Academies 2007):

For forage: $DE \times (\text{Mcal/kg}) = 4.22 - 0.11 \times (\% \text{ADF}) + 0.0332 \times (\% \text{CP}) + 0.00112 \times (\% \text{ADF}^2)$.

For energy feeds and protein supplements: $DE \times (\text{Mcal/kg}) = 4.07 - 0.055 \times (\% \text{ADF})$.

*ADF = acid detergent fibre and *CP = crude protein.

Milk sampling and chemical composition analysis

All the milk in the first month of lactation was available to the foal. Milk samples were collected for 15 days, starting from the 2nd postfoaling month. During the trial, milk samples were collected every 3 days from each group by mechanical milking (40 kPa vacuum level, 60 pulse per min), at 11.00 am. From 08.00 am to the end of milking, the foals were separated from the mares, but were kept in the adjacent box, maintaining visual and acoustic contact. Samples were stored in 500 mL polyethylene bottles, refrigerated immediately at 4 °C and transported in thermal boxes to a laboratory for analytical determinations. The following parameters were measured: protein, fat and lactose content by Milkoscan FT 6000 according to International Dairy Federation standard (ISO 2013), dry matter and ash content (AOAC 1990).

DPPH and ABTS radical scavenging assay the milk samples

Antioxidant activity was measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assays. The DPPH assay was performed according to the method of Brand-Williams *et al.* (1995) with some modifications (Cosentino

et al. 2015b). The stock solution was prepared by dissolving 24 mg DPPH in 100 mL methanol and then storing at -20°C until needed. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 ± 0.02 units at 517 nm using the spectrophotometer. For the ABTS assay, the procedure followed the method of Arnao (2001) with some modifications (Cosentino *et al.* 2015b); stock solutions included 7.4 mM ABTS^{•+} and 2.6 mM potassium persulfate solutions. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was diluted by mixing 1 mL ABTS^{•+} solution with 60 mL methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using the spectrophotometer. Fresh ABTS^{•+} solution was prepared for each assay.

The radical scavenging activity of the donkey milk against DPPH radical was determined as described by Russo *et al.* (2012); the absorbance was measured after 30 min at 517 nm against a blank solution. The antioxidant activity was also evaluated by the percentage of inhibition in the ABTS test. Both tests were carried out in triplicate on each sample.

Statistical analysis

Data were processed by two-way ANOVA and Tukey's test to evaluate the significant differences ($P \leq 0.05$).

RESULTS AND DISCUSSION

Both diets administered during the test were made to ensure the same contribution of protein and fat. In the AF group, the neutral detergent fibre (NDF) content was slightly lower (-2.2%) compared to the SF group, however, this aspect did not have a significant impact on the total digestibility of the administered ration because the total ADF content in both diets was very similar. The larger amount of fibre (crude fibre (CF) and NDF) in the AF group compared to the SF group was offset by a higher contribution of non-structural carbohydrates (or non fibrous carbohydrate, NFC), which are highly digestible (soluble fibre and sugar). This increased contribution was also used to balance the lower level of starch in the first compared to the second diet (Table 1).

Chemical composition was unaffected by the dietary treatments, and no significant differences were observed between the two experimental groups. For the SF group and AF group, respectively, the following data were calculated by mean of values for each sampling: dry matter (g/100 g) 8.23 vs 8.26; protein content (g/100 g) 1.25 vs 1.26; fat content (g/100 g) 0.22 vs 0.23; lactose content (g/100 g) 6.33 vs 3.32; ash (g/100 g) 0.43 vs 0.44. Results are reported in Table 2. The low dry matter content of the donkey milk was similar to that observed in the literature by

Table 1 Nutritional composition (%) of the two different diets^a: SF = standard feeding; AF = alternative feeding with cladodes of *Opuntia ficus-indica*.

Ingredients	Dry matter (%)	
	AF	SF
Crude Protein	1	16.8
Ether Extract	2.5	2.5
Crude Fibre	27.6	25.9
Ash	10.4	10.5
Starch	4.2	5.3
NDF	51.0	53.2
ADF	37.2	38.2
NFC	19.5	16.2
Digestible Energy (kcal/kg)	2000	2098

NDF, neutral detergent fibre; ADF, acid detergent fibre; NFC, nonfibrous carbohydrates;

^aAll measurements were done in triplicate.

other authors for equid milk (Markiewicz-Kęszycka *et al.* 2015; Santos and Silvestre 2008). Ivanković *et al.* (2009) observed in Croat breeds values of dry matter that ranged from 8.61 to 9.13 per cent. The protein content was lower than that of native Sicilian and Croat breeds in summer, 18.1 g/L and 14.3 g/L, respectively (Cosentino *et al.* 2012; Giosuè *et al.* 2008), but decreased by 20–25% over days 28–150 of lactation (Guo *et al.* 2007). The average fat content of the donkey milk was much lower than that of other mammals; in fact, values observed from other authors ranged from 0.01 to 1.8 g/100 g. The results also confirm the low lipid content of equid milk, either hand or machine milked (Giosuè *et al.* 2008; Malissiova *et al.* 2016; Piccione *et al.* 2008). The lactose content of the donkey milk was found to be unaffected by the diets (Paolino *et al.* 2012). The ash content was constant throughout the experimental period.

In this work, we performed two assays to verify the antioxidant scavenging activity of milk produced by lactating donkeys fed with SF or AF. As showed by Frankel and Meyer (2000), the antioxidant capacity of food is affected by several features: the composition of the test system, the substrate and the mode of inducing oxidation. For this reason, it is necessary to employ several methods to measure total antioxidant capacity. In this case, we choose the ABTS and DPPH tests, the most popular methods of evaluating TAC (total antioxidant capacity) levels in milk. The results obtained are shown in Figure 1 (ABTS test results) and Figure 2 (DPPH test results).

In the SF group, we noticed a weak increase of radical scavenging activity (RSA)% value. However, in both tests, the values of RSA% in SF did not differ significantly in all periods of observation. The RSA% value of AF on day 0 did not differ significantly from the values of SF in both test, but

Table 2 Chemical composition of donkey milk with standard feeding (SF) and alternative feeding (AF) during 15 days of treatment ^a (mean ± SE).													
Parameters	Sampling days												
	0		3rd		6th		9th		12th		15th		
	SF	AF	SF	AF	SF	AF	SF	AF	SF	AF	SF	AF	AF
Dry matter (%)	8.27 ± 0.30	8.35 ± 0.30	8.25 ± 0.35	8.31 ± 0.31	8.26 ± 0.35	8.21 ± 0.30	8.21 ± 0.30	8.22 ± 0.40	8.23 ± 0.38	8.18 ± 0.40	8.16 ± 0.35	8.26 ± 0.38	
Protein (%)	1.30 ± 0.10	1.29 ± 0.08	1.28 ± 0.05	1.30 ± 0.06	1.26 ± 0.05	1.28 ± 0.04	1.25 ± 0.06	1.24 ± 0.06	1.22 ± 0.06	1.25 ± 0.05	1.20 ± 0.05	1.22 ± 0.07	
Fat (%)	0.24 ± 0.10	0.27 ± 0.07	0.21 ± 0.09	0.22 ± 0.10	0.19 ± 0.09	0.25 ± 0.08	0.23 ± 0.10	0.21 ± 0.11	0.24 ± 0.07	0.23 ± 0.10	0.22 ± 0.08	0.20 ± 0.07	
Lactose (%)	6.28 ± 0.35	6.30 ± 0.33	6.32 ± 0.37	6.31 ± 0.33	6.37 ± 0.37	6.25 ± 0.32	6.33 ± 0.43	6.35 ± 0.45	6.35 ± 0.41	6.32 ± 0.43	6.33 ± 0.42	6.40 ± 0.45	
Ash (%)	0.45 ± 0.05	0.49 ± 0.04	0.44 ± 0.04	0.48 ± 0.04	0.44 ± 0.04	0.43 ± 0.03	0.40 ± 0.05	0.42 ± 0.05	0.42 ± 0.05	0.38 ± 0.05	0.41 ± 0.05	0.44 ± 0.05	

^a All measurements were done in triplicate.

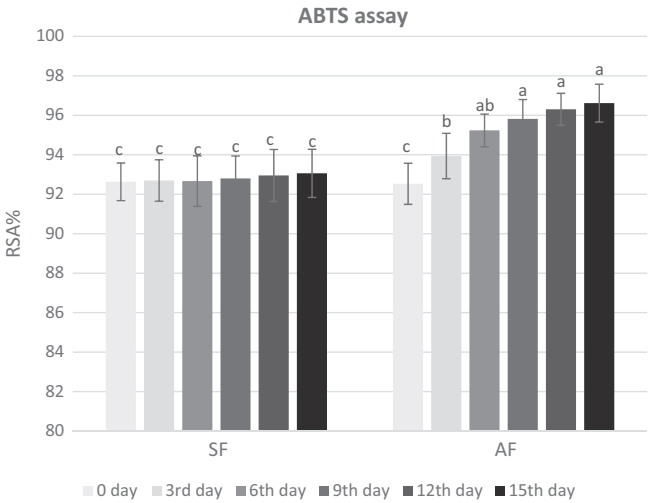


Figure 1 Radical scavenging activity (%) of donkey milk (measured by ABTS test) from animals fed two different diets: standard feeding (SF) and alternative feeding (AF) based on *Opuntia ficus-indica* during 15 days of treatment. The ABTS assay was performed 12 h later on the first administration (day 0) and after 3, 6, 9, 12, 15 days*. (Different letters indicate significant differences at $P \leq 0.05$ and a Tukey's test between different treatments at same day after feeding date: 3, 6, 9, 12, 15 days). *All measurements were done in triplicate.

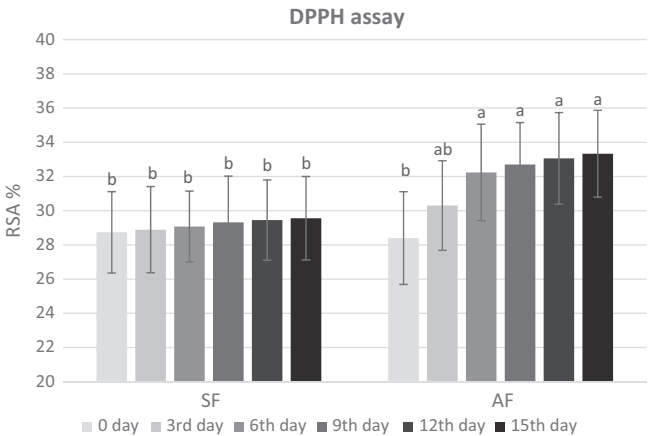


Figure 2 Radical scavenging activity (%) of donkey milk (measured by DPPH test) from animals fed two different diets: standard feeding (SF) and alternative feeding (AF) based on *Opuntia ficus-indica* during 15 days of treatment. The DPPH assay was performed 12 h later on the first administration (day 0) and after 3, 6, 9, 12, 15 days*. (Different letters indicate significant differences at $P \leq 0.05$ and a Tukey's test between different treatments at same day after feeding date: 3, 6, 9, 12, 15 days). *All measurements were done in triplicate.

it considerably increases during the observed period. This increase could be seen, especially from the ABTS tests where RSA% values pass from 95.5 to 96.6. It means that the AF affected the antioxidant capacity of the milk.

In both assays, the most important increase in RSA% was recorded between day 0 and 6. In the last days, the antioxidant capacity underwent a slight increase, but the differences were not significant. The ABTS test recorded a difference between the values of milk sampled on 6th day and 9th day, while the DPPH test showed equal values from the 6th until the end of observed period. In fact, it is known that for milk samples, the TAC determined by the ABTS test was significantly higher than the values produced by the DPPH test (Martysiak-Żurowska and Wenta 2012). Therefore, the ABTS test was able to detect a difference between the data from the 6th and 9th day, in contrast to the DPPH test. Moreover, the RSA% difference between day 0 and 3rd day was more significant for the DPPH test ($P < 0.01$) than the ABTS test ($P < 0.05$).

In summary, the TAC was constant in the SF group, while it increased in the AF group. The RSA% growth was evident in the first week, and then it stopped, but the values remained high.

CONCLUSION

Preliminary results showed an increase in the antioxidant activity of donkey milk of groups given an alternative feed containing *O. ficus-indica*. These results provide an important added value because the real increase in the antioxidant activity and also the lack of difference highlighted in terms of the milk's chemical composition leads us to conclude that the cactus pear is a good constituent of the animals' diet. This is an important finding because *O. ficus-indica* needs a low levels of water to grow and for this reason could be used for animal feeding in parts of the world where water is scarce, but also throughout the rest of the world because it allows a low water footprint with equal production in term of milk quantity and quality. More work is necessary to assess this particular trend and also to test the nutraceutical value of donkey milk from alternative feeding based on the cladodes of cactus pear. In the future, declining water resources and global desertification may even increase *Opuntia* spp. importance as an effective food production system including both fruits and vegetable parts.

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