Preliminary results on antifungal activity of donkey milk

IOLANDA ALTOMONTE[1], SIMONA NARDONI[1], FRANCESCA MANCIANTI[1,2], STEFANIA PERRUCCI[1,2], ROSARIO LICITRA[1], FEDERICA SALARI[1], MINA MARTINI[1,2]

[1] Department of Veterinary Science, University of Pisa, Italy
[2] Interdepartmental Research Center Nutrafood "Nutraceuticals and Food for Health", University of Pisa, Italy

SUMMARY

Donkey milk is being increasingly studied for its use in the human medicine and its antimicrobial activity has been reported in the literature. However, there have been only few studies on milk's inhibiting activity against fungi. Donkey milk is currently used in the personal healthcare industry, thus evaluating its antifungal activity against some dermatophytes is of interest in relation to the natural prevention or control of dermatophyte infections. This preliminary study evaluated the *in vitro* antifungal activity of donkey milk. Four fresh pasteurized bulk milk samples were collected from a donkey farm. Pasteurized donkey bulk milk samples were analysed in relation to gross composition, individual mineral content (Ca, P, Mg, K, Na, and Zn mg/L), fatty acid profile, and lysozyme activity. The milk samples were tested against isolates of *Microsporum canis*, *Microsporum gypseum,* and *Trichophyton mentagrophytes*. Sensitivity testing was assessed by a microdilution test, starting by a milk concentration of 90%. To calculate minimal inhibitory concentration values, 80%, 70%, 60% 50%, 40%, 30%, 20%, and 10% dilutions were tested. Donkey milk was proven to inhibit mycotic growth. In particular, *M. canis* and *T. mentagrophytes* failed to grow in 60% donkey milk and *M. gypseum* appeared to be sensitive to 70%. The antidermatophyte effect could be related to the milk content of some fatty acids with reported antifungal activities. In the analysed donkey milk, C10:0 on average constituted 70% of the short chain fatty acids and the milk fat showed a relatively high content of linoleic and alpha linolenic acid (15.34 and 4.87 g/100 g of the total fatty acids respectively). We also found a high lysozyme activity (1402.50 U/mL of milk). In conclusion, donkey milk showed an overall *in vitro* antidermatophytic effect. On the basis of the promising results obtained in this preliminary report, further studies are needed to evaluate the *in vivo* use of donkey milk against dermatophytosis.

KEY WORDS

Donkey milk; fatty acids; antifungal activity; dermatophytes.

INTRODUCTION

Milk contains an overall pool of biologically-active molecules which have been shown to have a positive clinical impact[1].
Donkey milk is being increasingly studied for its use in the human diet[2]. Donkey milk has shown *in vitro* anti inflammatory, antiproliferative[3] and antitumour actions[4]. In addition to its nutritional value, these properties would make donkey milk a means to improve human health. The antimicrobial activity of donkey milk was also reported[5]. Gram positive bacteria appeared to be more susceptible to lysozyme from milk, however different results have also been found against gram negative bacteria, in particular *Escherichia coli* and *Salmonella enteritidis*[6,7], depending on the milk composition.
There have been only few studies on milk's inhibiting activity against fungi. To the best of our knowledge, the antifungal activity of donkey milk has been evaluated only against the endosaprophyte yeast *Candida albicans*, and the antropophilic dermatophytes, *Trichophyton mentagrophytes* and *Trichophyton rubrum*[5].

Corresponding Author:
Federica Salari (federica.salari@unipi.it).

Dermatophytes are a group of keratinophilic and keratinolytic molds, which are physiologically and taxonomically related, and are responsible for ringworm both in human and animals. Zoophilic dermatophytes spend their life on the animals' keratin and some species can metabolize human keratin, causing zoonotic diseases. Cats and lagomorphs are reservoir hosts of *Microsporum canis* and *T. mentagrophytes*, respectively. Other dermatophytes called geophilic, such as *Microsporum gypseum*, are also able to colonize and digest keratin on hair-baits in the soil. All the three fungal species are responsible for human and animal ringworm and can colonize the host skin or annexa.
Since donkey milk is also an essential component of many cosmetic products, the understanding of its antifungal activity against some dermatophytes is of interest, this could provide a natural way to prevent and/or control such human infections.
The aim of this preliminary study was to evaluate the *in vitro* antifungal activity of pasteurized donkey milk.

MATERIALS AND METHODS

Four fresh pasteurized bulk milk samples were collected in September 2018 (once a week) from a farm rearing about 160 Amiatina donkeys. The farm produced pasteurized milk for

human consumption in accordance with the requirements of Regulation (EC) No 853/2004. The samples were immediately cooled to 4°C and transferred to the laboratory. The chemical composition of each sample was analysed in triplicate. After drying and incineration, dry matter and ash were determined by the gravimetric determination of residues, using methods of the Association of Official Analytical Chemists[8]. Lactose was determined by infrared analysis (MilkoScan; Italian Foss Electric, Padova, Italy). Protein was calculated as total nitrogen (N) (determined by Kjeldahl method) multiplied by 6.38. Individual mineral content (Ca, P, Mg, K, Na, and Zn mg/L) was determined in all samples by atomic absorption spectroscopy and UV-visible spectroscopy according to Horwitz[9] and Murthy and Rhea[10]. Milk fat extraction was performed following the Röse-Gottlieb method[11].

Fatty acid methyl esters (FAME) were prepared using methanolic sodium methoxide according to Christie[12] and analysed by gas chromatography, as described by Martini *et al.*[13]. The lysozyme (LZ) activity was evaluated in each milk sample using a commercial fluorimetric method on a microplate (EnzChek Lysozyme Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). The test uses a suspension of *Micrococcus lysodeikticus* labelled with fluorescein. This microorganism is sensitive to the lithic activity of LZ which leads to a variation in the intensity of the fluorescence measured at ~485/530 nm (excitation/emission). Milk was diluted and no defatting methods were used. The results were compared with an LZ standard curve and expressed in U mL^{-1}.

Three feline clinical isolates of *M. canis* and *M. gypseum*, respectively, and three rabbit isolates of *T. mentagrophytes* were selected for *in vitro* testing. Before the assay, the molds were maintained on Malt Extract Agar.

Sensitivity was assessed by a microdilution test, performed following the methods described by CLSI for molds[14], starting by a milk concentration 90%. To calculate a minimal inhibitory concentration (MIC) value, dilutions of 80%, 70%, 60% 50%, 40%, 30%, 20%, and 10% were obtained. Negative controls were performed by incubating the dermatophytes in culture medium, and the sensitivity against Terbinafine was evaluated as conventional antimycotic model. All cultures were incubated at 25°C for at least 72 hours, or until a visible growth in control wells was present. All sensitivity tests were performed in quadruplicate.

For the results of the milk composition, means and standard deviations were calculated by JMP (2002).

RESULTS

The chemical composition and lysozyme activity of the analysed donkey milk are reported in Table 1. In the analysed donkey milk, C10:0 constituted on average 70% of the short chain fatty acids and the milk fat showed a relatively high content of linoleic and alpha linolenic acid (15.34 and 4.87 g/100 of the total fatty acids respectively). We also found a high LZ activity (1402.50 U/mL of milk).

All the fungal isolates were sensitive to Terbinafine with MIC values of 0.0156 µg/ml (*M. canis*), 0.16 µg/ml (*M. gypseum*), and 16 µg/ml (*T. mentagrophytes*). Donkey milk was proven to inhibit mycotic growth. In particular, *M. canis* and *T. mentagrophytes* failed to grow with a concentration of 60% donkey milk, and *M. gypseum* appeared to be sensitive to 70%.

DISCUSSION

The results on chemical composition and lysozyme activity were consistent with previous studies[15,16].

To the best of our knowledge, the only published paper dealing with the antifungal activity of donkey milk was the one by Koutb *et al.*[5], who referred a minimal lethal concentration of 32 mg/ml against *T. mentagrophytes* and *T. rubrum*, using a 50-fold concentrated milk, while no growth inhibition was observed, when testing *C. albicans*. The MIC value reported by us *versus T. mentagrophytes* appears to be strongly lower when compared to literature data. For the first time we tested the donkey milk activity against *M. canis* and *M. gypseum*. The content of various fatty acids with proven antifungal actions in the analysed donkey milk (Table 1) supports the

Table 1 - Chemical composition of the pasteurized milk.

Gross composition (g/100 mL)		
	Mean	**SD**
Dry matter	9.30	0.227
Crude Protein	1.54	0.079
Fat	0.33	0.193
Lactose	6.99	0.104
Ash	0.36	0.026
Mineral profile (mg/L)		
	Mean	**SD**
Ca	598.54	185.86
P	406.65	101.49
Mg	86.69	10.530
K	652.81	82.970
Na	171.77	46.095
Zn	3.29	1.017
Fatty acids classes (g/100 g of fat) and content of individual fatty acids with reported antifungal activity		
	Mean	**SD**
SFA[1]	51.69	10.065
MUFA[2]	27.51	8.391
PUFA[3]	20.59	2.977
SCFA[4]	11.90	3.234
MCFA[5]	42.02	6.845
LCFA[6]	46.07	9.459
C10.0	8.39	2.572
C12:0	8.73	3.419
C14:1	0.37	0.130
C17:1	0.34	0.086
C18:2 c9, c12	15.34	4.001
C18:3 c9, 12, 15	4.87	1.454
C18:3 cis-6,9,12	0.08	0.02
	Mean	**SD**
Lysozyme activity (U/mL)	1402.50	286.65

[1] SFA: saturated fatty acids; [2] MUFA: monounsaturated fatty acids; [3] PUFA: polyunsaturated fatty acids; [4] SCFA: short chain fatty acids (≤C10); [5] MCFA: medium chain fatty acids (C11-C17); [6] LCFA: long chain fatty acids (≥C18).

potential involvement in the inhibition of the tested fungal isolates. It has been hypothesized that fatty acids act against fungi by the disruption or disintegration of the plasma membrane[17,18] or by increasing the rate of H+ influx across the plasma membrane, which could interfere with the germination[19].

Bergsson *et al.*[18] and Clément *et al.*[19] highlighted the antifungal activity of capric acid (C10:0). They also found antifungal actions for myristoleic acid (C14:1n-5), and gamma-linolenic acid (C18:3 cis-6,9,12) from bovine milk whey[19]. Similar activities against plant pathogenic fungi have also been found for alpha linolenic (C18:3 c9, 12, 15) and linoleic acids (C18:2 c9, c12)[20]. C10:0 constituted on average the 70% of the short chain fatty acids in the donkey milk analysed, in agreement with previous studies[21]. In addition, donkey milk fat showed a high content of linoleic and alpha linolenic acid. Of the various dairy animal milks, donkey milk has been reported to be one of the richest sources of alpha linolenic acid[2]. With regard to C17:1, which is also found in the donkey milk, an antifungal action has been proposed[17].

Some of the minerals found in donkey milk, such as zinc, are components of metal-based therapeutics and drugs[22]. Of these, Zn (II) complexes and zincum oxide nanoparticles have shown antifungal activities[23,24].

The literature[25] has reported the antibacterial activity of some milk bioactive proteins, such as LZ. Although there have been relatively few studies on the antifungal activity of LZ, some authors have found LZ lytic activity against fungal pathogens for animal and plants[26,27]. Donkey milk also has a high quantity of LZ (about 1-1.5 g L^{-1}), which seems to be involved in its low bacterial count[28]. In agreement with recent investigations [Martini et al., unpublished data], we also found a high activity of LZ in the pasteurized milk.

The antifungal activity of lactoferrin or its peptides, in combination with azole antifungal agents, have also been demonstrated against *C. albicans* and dermatophytes[29]. Furthermore, lactoferrin is a minor component (0.097-0.133 g L^{-1}) of donkey milk and has a poorer thermal resistance compared to LZ[30].

CONCLUSIONS

Donkey milk shows an overall antidermatophyte effect. This could be due to the synergistic action of fatty acids, lysozymes and other milk components.

The results on mycotic growth inhibition obtained in this preliminary report are promising, considering also that cosmetic products containing 60-70% of donkey milk are already present on the market. Further studies are needed in order to evaluate the *in vivo* use of donkey milk against dermatophytosis.

ACKNOWLEDGEMENTS

The authors would like to thank the Complesso Agricolo Forestale Regionale Bandite di Scarlino, Scarlino, Italy.

This work was supported by PRA 2017 (Ateneo Research Project, University of Pisa). Improvement of productive performance of dairy donkey n I52F17000260005.

References

1. Hill D.R., Newburg, D.S. (2015). Clinical applications of bioactive milk components. Nutr Rev, 73:463-476.
2. Martini M., Altomonte I., Licitra R., Salari F. (2018). Nutritional and Nutraceutical Quality of Donkey Milk. J Equine Vet Sci, 65: 33-37.
3. Jirillo, F., Jirillo, E., Magrone, T. (2010). Donkey's and goat's milk consumption and benefits to human health with special reference to the inflammatory status. Current Pharmaceutical Design, 16: 859-863.
4. Mao X., Gu J., Sun Y., Xu S., Zhang X., Yang H., Ren F. (2009). Anti-proliferative and anti-tumour effect of active components in donkey milk on A549 human lung cancer cells. Int Dairy J, 19: 703-708.
5. Koutb M., Khider M., Ali E.H., Hussein N.A. (2016). Antimicrobial Activity of Donkey Milk against Dermatomycotic Fungi and Foodborne Bacteria. International Journal of Biomedical Materials Research, 4: 11-17.
6. Šarić L.Ć., Šarić B.M., Mandić A.I., Torbica A.M., Tomić J.M., Cvetković D.D., Okanović Đ.G. (2012). Antibacterial properties of Domestic Balkan donkeys' milk. Int Dairy J, 25: 142-146.
7. Šarić L.Ć., Šarić B.M., Kravić S.Ţ., Plavšić D.V., Milovanović I.L., Gubić J.M., Nedeljković N.M. (2014). Antibacterial activity of Domestic Balkan donkey milk toward Listeria monocytogenes and Staphylococcus aureus. Food and Feed Research 41: 47-54.
8. AOAC International (1990). Official methods of analysis. 15th edition. AOAC International, Arlington, VA.
9. Horwitz W. (2000). Official Method of Analysis of AOAC International. 17th edition. AOAC International, Gaithersburg, VA.
10. Murthy G.K., Rhea U. (1967). Determination of major cations in milk by atomic absorption spectrophotometry. J Dairy Sci, 50: 313-317.
11. AOAC International. (1995). Official Methods of Analysis. 16th edition. Vol. II. AOAC International, Arlington, VA.
12. Christie W.W. (1982). A simple procedure of rapid transmethylation of glycerolipids and cholesteryl esters. J Lipid Res, 23: 1072-1075.
13. Martini M., Altomonte I., Licitra R., Salari F. (2018). Short communication: Technological and seasonal variations of vitamin D and other nutritional components in donkey milk. J Dairy Sci, 101: 8721-8725.
14. CLSI. (2008). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard 2nd edition. Clinical and Laboratory Standards Institute, Wayne, PA, M38-A2.
15. Martini M., Altomonte I., Salari F., Caroli A.M. (2014). Short communication: Monitoring nutritional quality of Amiata donkey milk: Effects of lactation and productive season. J Dairy Sci 97: 6819-6822.
16. Ragona G., Corrias F., Benedetti M., Paladini I., Salari, F., Altomonte I., Martini M. (2016). Amiata donkey milk chain: animal health evaluation and milk quality. Ital J Food Saf, 5: 5951.
17. Avis T.J., Bélanger R.R. (2001). Specificity and mode of action of the antifungal fatty acid cis-9 heptadecenoic acid produced by Pseudozyma flocculosa. Appl Environ Microbiol, 67: 956-960.
18. Bergsson G., Arnfinnsson J., Steingrimsson O., Thormar H. (2001). In vitro killing of Candida albicans by fatty acids and monoglycerides. Antimicrob Agents Chemother, 45: 3209-3212.
19. Clément M., Tremblay J., Lange M., Thibodeau J., Belhumeur P. 2008. Purification and Identification of Bovine Cheese Whey Fatty Acids Exhibiting In Vitro Antifungal Activity. J. Dairy Sci., 91: 2535-2544.
20. Walters D., Raynor L., Mitchell A., Walker R., Walker K. (2004). Antifungal Activities of Four Fatty Acids against Plant Pathogenic Fungi. Mycopathologia, 157: 87-90.
21. Martini M., Salari F., Altomonte I., Ragona G., Piazza A., Gori R., Casati D., Brajon G. (2018). Effects of pasteurization and storage conditions on donkey milk nutritional and hygienic characteristics. J Dairy Res, 85: 445-448.
22. Chohan Z.H. (2009). Metal-based antibacterial and antifungal sulfonamides: synthesis, characterization, and biological properties. Transit Metal Chem, 34: 153-161.
23. Karimiyan A., Najafzadeh H., Ghorbanpour M., Hekmati-Moghaddam S.H. (2015). Antifungal Effect of Magnesium Oxide, Zinc Oxide, Silicon Oxide and Copper Oxide Nanoparticles Against Candida albicans. Zahedan. J. Res. Med. Sci., 17: 2179.
24. Diaz J.R.A., Baldo M.F., Echeverría G., Baldoni H., Vullo D., Soria D.B., Supuran C.T., Camí G.E. (2016). A substituted sulfonamide and its Co (II), Cu (II), and Zn (II) complexes as potential antifungal agents. J Enzyme Inhib Med Chem, 31: 51-62.
25. Brumini D., Criscione A., Bordonaro S., Vegarud G.E., Marletta D. (2016). Whey proteins and their antimicrobial properties in donkey milk: a brief review. Dairy Sci Technol, 96: 11-14.
26. Düring K., Porsch P., Mahn A., Brinkmann O., Gieffers W. (1999). The non-enzymatic microbicidal activity of lysozymes. FEBS Lett, 449: 93-100.
27. Samaranayake Y.H., Samaranayake L.P., Pow E.H., Beena V.T., Yeung K.W. (2001). Antifungal effects of lysozyme and lactoferrin against genetically similar, sequential Candida albicans isolates from a human immunodeficiency virus-infected southern Chinese cohort. J Clin Microbiol, 39: 3296-3302.
28. Altomonte I., Salari F., Licitra R., Martini M. (2019). Donkey and human milk: Insights into their compositional similarities. Int Dairy J, 89: 111-118.
29. Clare D.A., Swaisgood H.E. (2000). Bioactive Milk Peptides: A Prospectus. J Dairy Sci, 83: 1187-1195.
30. Ozturkoglu-Budak S. (2018). Effect of different treatments on the stability of lysozyme, lactoferrin and b-lactoglobulin in donkey's milk. Int J Dairy Technol, 71: 36-45.