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Summary

During the last years the interest in donkey milk has increased significantly mainly because of its compelling functional elements. Even if the composition and nutritional properties of donkey milk are known, its microbiota is less studied. This short communication aimed to provide a comprehensive characterization of the lactic acid bacteria in raw donkey milk. RAPD-PCR assay combined with 16S rDNA sequencing analysis were used to describe the microbial diversity of several donkey farms in the North West part of Italy. The more frequently detected species were: *Lactobacillus paracasei*, *Lactococcus lactis* and *Carnobacterium maltaromaticum*. Less abundant genera were *Leuconostoc*, *Enterococcus* and *Streptococcus*. The yeast *Kluyveromyces marxianus* was also isolated. The bacterial and biotype distribution notably diverged among the farms. Several of the found species, not previously detected in donkey milk, could have an important probiotic activity and biotechnological potential. This study represents an important insight to the ample diversity of the microorganisms present in the highly selective ecosystem of raw donkey milk.

Key words:

donkey milk, lactic acid bacteria, RAPD-PCR characterization

In recent years donkey milk (DM) has captured scientific attention because of its compelling nutrients and functional elements. The chemical composition of DM is very similar to human milk so it has been used as an adequate replacement for infants with multiple food allergies and intolerances (Mansueto *et al.* 2013). Recently, fermented beverages based on donkey milk were proposed as important sources of probiotics and antioxidants with several health benefits (Perna *et al.* 2015).

Donkey milk presents high levels of lactose and low contents of casein and fat. One of the main particularities of DM is its high lysozyme's content (around 1000-4000 mg/l) compared to cow (0.09 mg/l) and human milk (40–200 mg/l) (Chiavari *et al.* 2005). Lysozyme displays an antibacterial activity against a vast number of Gram-positive bacteria; including lactic acid bacteria (LAB). However some authors suggested that the levels of lactose in DM might contribute to the survival of adapted LAB (Chiavari *et al.* 2005).

The microbiota of DM has been scarcely studied. Most of the available reports focus on its safety and hygienic quality (Chiavari *et al.* 2005; Cavallarin *et al.* 2015). Just lately Carminati *et al.* (2014) investigated the LAB composition from bulk milk samples of a single donkey farm using biomolecular methods.

The aim of this study was to provide an ample characterization of LAB biodiversity in raw donkey milk. We applied a culture-dependent approach, based on randomly amplified polymorphic DNA (RAPD)-PCR combined with 16S rDNA sequencing. A broader survey of the lactic acid bacteria present in DM might contribute to the detection of possible probiotic species with biotechnological potential.

Materials and methods

Milk sampling

Three donkey dairy farms from the Piedmont region, North West part of Italy were sampled during the spring of 2013. In the sampling plan of spring 2014 an additional farm was included. Bulk milk (300 ml) was collected and stored in sterile conditions at 4ºC. Samples were analyzed the day after milking.

Growth and isolation of LAB colonies

Serial dilutions of raw DM were prepared in physiological saline solution and peptone (85:15 v:v) (OXOID LTD, Basingstoke, Hampshire, England). LAB colonies were grown on MRS agar (OXOID LTD) at 30 ºC for 48 hours in anaerobiosis and on M17 agar (OXOID LTD) at 37 ºC for 48 hours. All the analyses were done in technical triplicate.

Well-defined colonies were inoculated in the corresponding liquid medium under the previously mentioned conditions. Isolates were kept at -80 ºC, after the addition of 15% sterile glycerol, until their further analyses.

Identification of isolates by RAPD-PCR and sequencing

RAPD-PCR was performed as described by Andrighetto *et al.* (2002). Profiles were grouped with the Gel Compar II software package (Applied Maths, Kortrjik, Belgium) using the Pearson product moment correlation coefficient and UPGMA cluster analysis. Representative isolates from the different RAPD-PCR clusters and subclusters were identified by sequencing the V1-V3 region of the 16S rDNA. Amplification was performed using primer pairs P1 and P2 (Ruaro *et al* 2013); after purification with ExoSAP-IT kit (GE Healthcare), the PCR products were sequenced with the 3130 Genetic Analyzer (Applied Biosystems). Species attribution was obtained after alignment of DNA sequences with the public database “Ribosomal Database Project”

and with the database available from the National Centre for Biotechnology Information.

Results and Discussion

In the last 20 years donkey milk has been studied mainly in Europe. Most of the available reports were conducted in southern Italy where donkeys' raising is a tradition. The increasing demand from allergic consumers has derived in the opening and spread of donkey dairy farms in the Piedmont region. Cavallarin *et al.* (2015) evaluated the chemical and microbiological quality of DM in this zone, but a deeper characterization of its microorganisms, especially LAB, was still needed. This study describes the lactic acid bacteria present in raw donkey milk, reporting microorganisms with biotechnological potential that were not previously associated with DM.

The general characteristics of the surveyed farms are shown in Table S1. They are all small family-run dairies, located in different geographic areas.

Jennies are milked once a day; obtaining around one liter per day per animal. Raw milk is sold directly for human consumption or for cosmetics production.

We selected a total of 144 colonies from MRS (N=92) and M17 (N=52). Combining the results obtained from RAPD-PCR analysis and 16S rDNA sequencing we found 11 bacterial species and 28 different biotypes (Table 1). LAB species more frequently detected were *Lactobacillus paracasei* (34%), *Lactococcus lactis* (29.9%) and *Carnobacterium maltaromaticum* (9.7%). Less abundant genera were *Leuconostoc*, *Enterococcus* and *Streptococcus*.

Lb. paracasei – the most abundant species in this study – is commonly found in cheese, mainly participating in the ripening process due to its proteolytic and lipolytic activities.

120 Ashokkumar *et al.* (2011) isolated *Lb. paracasei* from DM and tested its bacteriocins
121 for commercial applications in the food processing industry.
122 Different species of the genus *Lactobacillus* can be used as probiotics for the production
123 of donkey milk fermented beverage. Particularly *Lb. rhamnosus* and *Lb. casei* were
124 considered optimum candidates for the production of these beverages (Chiavari *et al.*
125 2005). The authors used strains that were isolated from cheese, so they needed an initial
126 growing phase in DM. Recently Perna *et al.* (2015) investigated the fermentative and
127 antioxidant activity of *Lb. acidophilus* and *Lb. casei* – not isolated from DM –
128 supplemented to donkey milk yoghurt, proposing it as a novel food.
129 Regarding the coccus-shaped bacteria, the prevalent species in this study corresponded
130 to *Lactococcus lactis*, constituting the second most abundant group (Table 1). As far as
131 the authors know there are no previous reports of the isolation of this bacterium in DM;
132 though *Lc. lactis* has been widely described in cow, goat, sheep, buffalo and human
133 milk (Quigley *et al.* 2013).
134 Carminati *et al.* (2014) reported the abundance of cocci in DM. It is interesting to note
135 that the authors found only coccus-shaped LAB; in particular *Enterococcus faecalis*,
136 and *Enterococcus faecium* as the dominant species. In the present study *Enterococcus*
137 spp. represented only the 7.5% of the total isolated colonies. These discrepancies might
138 be the result of our broader sampling.
139 The third most abundant species was *Carnobacterium maltaromaticum* (Table 1), a
140 psychotropic LAB generally recovered from soft cheeses. It was first isolated from
141 milk with a distinct malty or chocolate like flavor. *C. maltaromaticum* is a slow
142 acidifying LAB; some strains are considered protective cultures in fish and meat
143 products because they produce bacteriocins against *L. monocytogenes* (Afzal *et al.*
144 2010).

145 During the isolation process we observed twelve colonies that grew in MRS, with
146 particular morphologies. After their characterization it was determined that the isolates
147 corresponded to the yeast *Kluyveromyces marxianus*. These unpredicted recoveries
148 could be due to the scarce selectivity of culture media. *K. marxianus* is commonly
149 found in dairy products, with a particular contribution in cheese ripening. Recently its
150 potential probiotic features have been studied in isolates from cow milk and whey
151 (Maccaferri *et al.* 2012).
152 Figure 1 shows the bacterial distributions between the farms denoting a high variability
153 of the ecosystem. Profiles of Farm A and B were very similar from one year to another;
154 with a prevalence of *Lb. paracasei* in the former and *Lc. lactis* subsp. *lactis* in the latter.
155 Farm C had a consistent flora of *Lb. paracasei* in 2013 that was completely lost in the
156 second year. Farm D was characterized by a homogeneous profile; still it was the only
157 one that presented a high percentage of *C. maltaromaticum*.
158 Going more into detail the two LAB species that presented a higher number of biotypes
159 were *Lb. paracasei* and *Lc lactis* subsp. *lactis* with 9 and 7 different profiles
160 respectively (Table 1). Analyzing the distribution of the 28 biotypes it could be
161 observed both farm and year specificities (Table 1). Only Farm B and C maintained a
162 single biotype in both years (A6 and G respectively). It is interesting to note how Farm
163 A and B presented a high variability of profiles even if the species distribution was
164 similar in both years (Figure 1).
165 This ample biodiversity is probably due to: i) the recent introduction in the Piedmont
166 region of donkey milk farms, ii) the different geographical environments of the farms
167 iii) the semi-extensive farm type, iv) the barely standardization of the farm practices and
168 v) the bulk milk was probably obtained from different jennies in both samplings -
169 considering the approximately one-year donkeys' gestation period.

Moreover all the biotypes' profiles of *Lb. paracasei* and *Lc. lactis* subsp. *lactis* isolated from donkey milk were compared with isolates from cow cheeses (Grana Padano and Asiago cheeses) previously characterized (data not shown). Donkey biotypes grouped with cow's with a range of 53-76% of similarity for *Lb. paracasei* and 51-64% for *Lc. lactis* subsp. *lactis*. This observation suggests a genetic relatedness among strains from the two different milk species despite the differences in the composition of both milks.

Conclusion

Regardless of the limitations of the culture-dependent methods, this study represents a significant insight to the ample diversity of the lactic acid bacteria present in the highly selective ecosystem of raw donkey milk. Many of the isolated microorganisms – not detected before in DM – belong to species known for their probiotic and biotechnological potential. Promising fermented products have already been obtained even if the bacteria were not isolated from DM. It is clear how a more comprehensive and detailed knowledge of the intrinsic microbiota of donkey milk might enhance the development of new functional foods with important health benefits for susceptible consumers.

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235 **Figure Legends**

236 **Figure 1**: Distribution of the species identified by RAPD-PCR and 16S rDNA

237 sequencing from raw donkey milk.

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239

240 **Table captions**

241 **Table 1.** Lactic acid bacteria and biotypes determined by RAPD-PCR and 16S rDNA

242 sequencing analyses.

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Figure 1

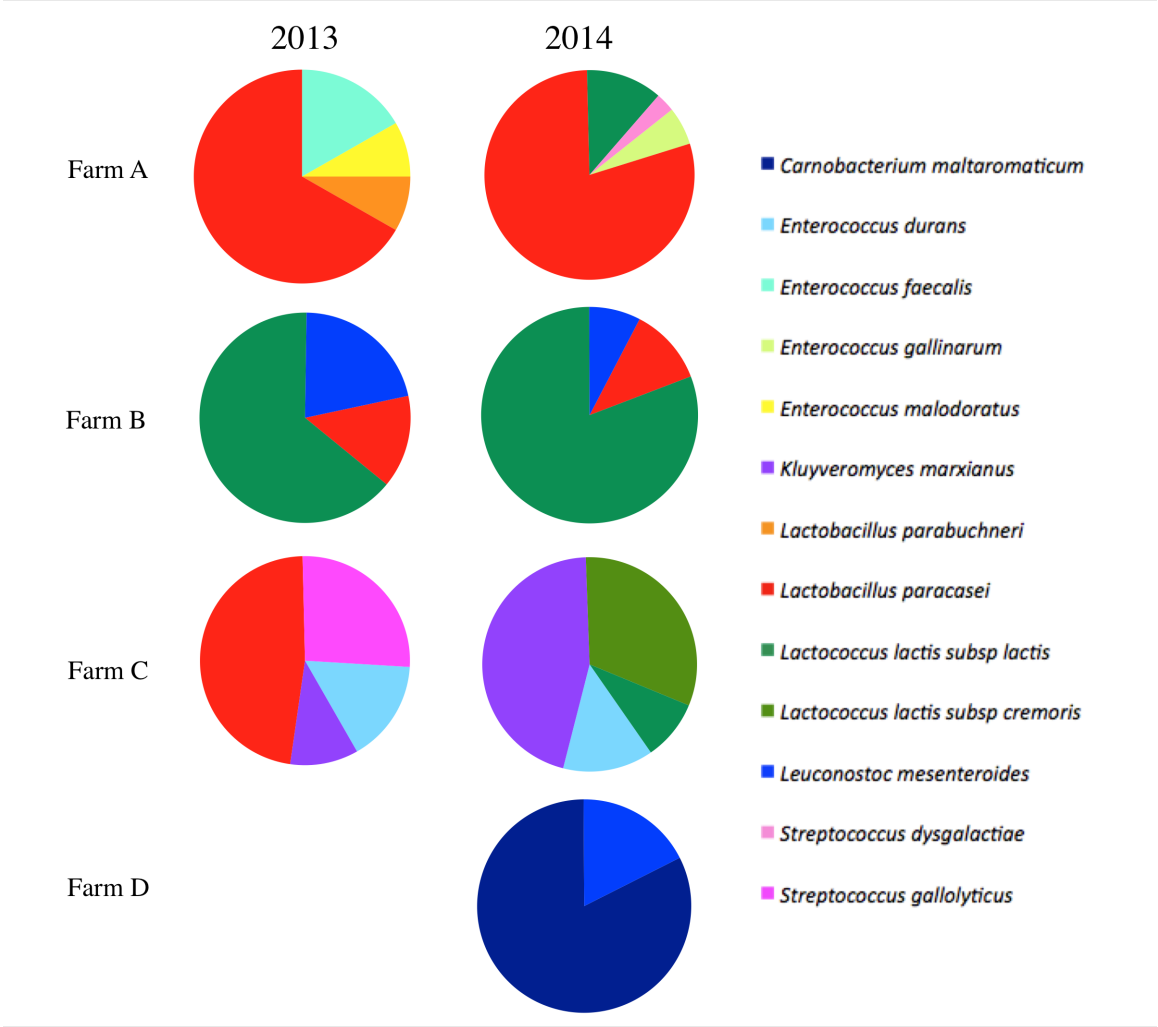
2013
2014
Farm A
Farm B
Farm C
Farm D
Carnobacterium maltaromaticum
Enterococcus durans
Enterococcus faecalis
Enterococcus gallinarum
Enterococcus malodoratus
Kluyveromyces marxianus
Lactobacillus parabuchneri
Lactobacillus paracasei
Lactococcus lactis subsp lactis
Lactococcus lactis subsp cremoris
Leuconostoc mesenteroides
Streptococcus dysgalactiae
Streptococcus gallolyticus

Table 1

	Farm A		**Farm B**		**Farm C**		**Farm D**
	2013	*2014*	*2013*	*2014*	*2013*	*2014*	*2014*
Lactobacillus paracasei	**8** (A1)*	**27** (A2,A3,A4,A5)	**2** (A6)	**3** (A6,A7)	**9** (A8,A9)	0	0
Lactobacillus parabuchneri	**1** (B)	0	0	0	0	0	0
Lactococcus lactis subsp. ***lactis***	0	**4** (C1)	**9** (C2,C3,C4)	**21** (C5,C6)	0	**2** (C7)	0
Lactococcus lactis subsp. ***cremoris***	0	0	0	0	0	**7** (D)	0
Leuconostoc mesenteroides	0	0	**3** (E1)	**2** (E2)	0	0	**3** (E3)
Carnobacterium maltaromaticum	0	0	0	0	0	0	**14** (F)
Enterococcus durans	0	0	0	0	**3** (G)	**3** (G)	0
Enterococcus faecalis	**2** (H)	0	0	0	0	0	0
Enterococcus gallinarum	0	**2** (I)	0	0	0	0	0
Enterococcus malodoratus	**1** (L)	0	0	0	0	0	0
Streptococcus gallolyticus	0	0	0	0	**5** (M)	0	0
Streptococcus dysgalactiae	0	**1** (N)	0	0	0	0	0

*number of colonies (RAPD-PCR biotype)