International Journal of Food Microbiology 251 (2017) 67–72

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterization of bacterial communities of donkey milk by high-throughput sequencing☆

Maria de los Dolores Soto del Rio, Alessandra Dalmasso *, Tiziana Civera, Maria Teresa Bottero

Dipartimento di Scienze Veterinarie, Università di Torino, Largo Braccini 2, 10095 Grugliasco, TO, Italy

ARTICLE INFO

Article history:
Received 28 October 2016
Received in revised form 15 March 2017
Accepted 28 March 2017
Available online 30 March 2017

Keywords:
Donkey milk
Bacterial communities
High-throughput sequencing

ABSTRACT

The interest in donkey milk (DM) is growing because of its functional properties and nutritional value, especially for children with allergies and food intolerances. However, most of the available reports of DM microbiota are based on culture-dependent methods to investigate food safety issues and the presence of lactic acid bacteria (LAB).

The aim of this study was to determine the composition of DM bacterial communities using a high-throughput sequencing (HTS) approach.

Bulk milk samples from Italian donkey dairy farms from two consecutive years were analysed using the MiSeq Illumina platform. All sample reads were classified into five phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia*. The most prevalent genera—*Pseudomonas*, *Ralstonia*, *Acinetobacter*, *Cupriavidus*, *Citrobacter* and *Sphingobacterium*—were Gram-negative bacteria.

The core microbiota was composed of genera that comprise commonly associated milk bacteria, LAB and species normally found in soil, water and plants. Reads assigned to LAB genera—*Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Lactobacillus*, and *Carnobacterium*—corresponded on average to 2.55% of the total reads per sample. Among these, the distribution of reads assigned to coccus- and bacillus-shaped LAB was variable between and within the farms, confirming their presence and suggesting a complex population of these bacteria in DM.

The present study represents a general snapshot of the DM microbial population, underlining its variability and motivating further studies for the exploitation of the technological potential of bacteria naturally present in DM.

1. Introduction

Donkey milk (DM) has recently received growing interest since it has been reported to be an adequate replacement for children with cow milk protein allergy, mainly due to its tolerability, nutritional contents and good taste (Monti et al., 2012). In fact, studies have demonstrated a number of qualities that make DM more favourable than cow milk: better digestibility (Tidona et al., 2011), lower allergenicity (Vincenzetti et al., 2008) and a set of unique nutritional and physicochemical characteristics (Guo et al., 2007).

Following the growing demand for DM, several new dairy farms have opened in the last few years. Italian donkey dairies are generally small, with 20 to 25 milking jennies and one or two stallions; their overall average daily production is approximately 2000 l, for a total of 700,000 l per year (Milonis and Polidori, 2011). The production is mainly used for direct human consumption, while a smaller part is destined for the cosmetics and food industries. Pasteurized donkey milk is usually sold directly from the farms. However, considering its target consumers and nutritional properties, it can be sold raw, with 3 days of shelf life (similar to raw bovine milk) (Giacometti et al., 2016).

The composition of DM is closer to human milk than to cow milk and has been fully described (Salimei and Fantuz, 2012). It contains high levels of lactose and essential amino acids (Guo et al., 2007) as well as low concentrations of β-lactoglobulin and casein—the most common allergens in cow milk (Vincenzetti et al., 2008). One of the main characteristics of DM is its high concentration of lysozyme: from 1300 to 4000 mg/l, compared to 0.09 mg/l in cow milk and 40–200 mg/l in human milk (Carminati et al., 2014; Chiavari et al., 2005; Vincenzetti et al., 2008). This enzyme has bactericidal properties; it hydrolyses the murein of bacterial cell walls, causing lysis of sensitive bacteria (Chiavari et al., 2005). Currently, there is no confirmed hypothesis as to why DM is so rich in lysozyme, but it seems to positively affect the animals, defending against infections in both the mammary gland and the

☆ The authors declare no conflict of interest.All authors participated in both research and manuscript preparation. All authors have approved the final version of this article.The research has been supported by a grant of the University of Turin (ex 60% 2013).

* Corresponding author.
E-mail addresses: mariadelosdolores.sotodelrio@unito.it (M.D. Soto del Rio), alessandra.dalmasso@unito.it (A. Dalmasso), tiziana.civera@unito.it (T. Civera), mariateresa.bottero@unito.it (M.T. Bottero).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.03.023
0168-1605/

foal. In addition to lysozyme, DM lactoferrin concentration is twice as high as in bovine milk (Malacarne et al., 2002), and other components have been described, such as immunoglobulins, free fatty acids and members of the lactoperoxidase peroxide system (Zhang et al., 2008), that might act synergistically against specific bacteria (Šarić et al., 2012).

Traditional microbiological tests and biomolecular culture-dependent methods have been used to study the bacterial population of DM, mainly focusing on hygienic conditions and/or the presence of lactic acid bacteria (LAB) (Cavallarin et al., 2015; Pilla et al., 2010; Zhang et al., 2008; Šarić et al., 2012). Moreover, in the last few years, culture-independent methods, based on the direct analysis of DNA without a culturing step, have also been used to characterize the milk of different species (Quigley et al., 2013). PCR-denaturing gradient gel electrophoresis (PCR-DGGE), for example, has been successfully applied to the study of the microbiota of milk and dairy products (Delgado et al., 2013). However, limitations in the resolution still need to be overcome, especially for the analyses of matrices with diverse microbial communities (Ogier et al., 2004). Recently, rapid developments of high-throughput sequencing (HTS) methods have allowed a deeper and more precise evaluation of the milk microbiota from different animals, including cattle, goat, sheep, buffalo and humans (Quigley et al., 2013).

Notwithstanding the extensive literature on DM, no high-throughput analysis of its bacterial population has yet been performed, despite ever-increasing interest from both technological and commercial points of view. For this reason, the present study aimed to contribute to the knowledge of DM by characterizing its microbiota using an HTS approach.

2. Materials and methods

2.1. Milk sampling and DNA extraction

Five donkey dairy farms (A, B, C, D, E) in the northwest part of Italy were sampled during the spring (March) of 2013 (samples A.2013, B.2013, C.2013, D.2013, E.2013) and 2014 (samples A.2014, B.2014, C.2014, D.2014, E.2014); in the second year, an additional farm was included (F; sample F.2014). These are small dairies, with a few milking jennies, family-run and with a limited production (around 1 l per day, per animal); the general characteristics of the surveyed farms are summarized in Table S1. The biochemical characterization, the shelf life and the safety of the samples have been reported in a previous work (Cavallarin et al., 2015).

Bulk milk samples from healthy jennies, collected in sterile tubes, were transported to the laboratory immediately after sampling in cool conditions and stored at −20 °C until DNA extraction. Samples were treated as reported elsewhere (Dalmasso et al., 2011), and DNA was extracted from 3 ml of milk following the manufacturer protocol of the Dneasy Blood & Tissue kit (Qiagen) and quantified with a Nanodrop 2000 (Thermo Fisher Scientific). To minimize the bias associated with single extractions, triple extractions of each sample were done in parallel and mixed in a final pool.

2.2. High-throughput sequencing

Illumina libraries were prepared following the protocol described by Dalmasso et al. (2016) with the NEXTflex 16S V4 Amplicon-Seq Kit (Bioo Scientific, Austin, USA). Briefly, the bacterial V4 region of the 16S ribosomal gene was amplified from 50 ng of DNA for each sample. The universal primers 515F and 806R tailed with Illumina barcoded adapters were used with the following touchdown PCR conditions: an initial 9 cycles (15 s at 95 °C, 15 s at 68 °C, 30 s at 72 °C) and then another 23 cycles (15 s at 95 °C, 15 s at 58 °C, 30 s at 72 °C). The PCR products were purified using Agencourt XP Ampure Beads (Beckman Coulter). The quality of the final products was assessed with a Bioanalyzer 2100 (Agilent Technologies).

The samples were quantified with Qubit (Invitrogen) and pooled in equal proportions for their paired-end sequencing with Illumina MiSeq for 312 cycles (150 cycles for each paired read and 12 cycles for the barcode sequence) at IGA Technology Services (Udine, Italy). To prevent focusing and phasing problems due to the sequencing of "low diversity" libraries, 30% PhiX genome was spiked in the pooled library.

2.3. Bioinformatics and data analyses

Sequence reads were trimmed with the collection command line tools of FASTX-Toolkits (http://hannonlab.cshl.edu/fastx_toolkit/) so that the quality score for each read was above 20 with >50 base pairs. The PRINSEQ standalone lite version (Schmieder and Edwards, 2011) was used to check and prepare the data set for the downstream analyses.

Data were then analysed with the QIIME software, version 1.9.0 (Caporaso et al., 2012). Using the uclust method (Edgar, 2010), sequences >97% identical were considered to correspond to the same operational taxonomic unit (OTU). Representative sequences were submitted to the RDPII classifier (Wang et al., 2007) to obtain the taxonomy assignment and relative abundance of each OTU using the Greengenes 16S rDNA database v13.8 (McDonald et al., 2012).

Alpha diversity was evaluated with QIIME to obtain the rarefaction curves. A rarefaction curve shows the variation in the number of OTUs identified at a given percentage of identity as a function of the number of sequence reads obtained per sample. Ideally, an optimal coverage is identified by the plateau of the curve, which indicates that increasing the number of reads does not change the number of OTUs that can be determined.

Moreover, Good's coverage (a sampling completeness indicator that indicates what percent of the total species is represented in the sample), Chao1 and ACE (richness estimators that calculate an approximate number of species in the samples using different methods), and Shannon and Simpson indices (estimators of the samples' diversity taking into account the approximated number of species and how evenly they are distributed) were determined.

Beta diversity was evaluated with the UniFrac method. Weighted UniFrac distance matrices and OTU tables were used to plot the principal coordinate analysis (PCoA) and to perform Adonis and Anosim statistical tests with the compare_category.py script of QIIME to evaluate differences between the farms, their practices and their characteristics.

The core microbiota of the samples was obtained with the compute_core_microbiome.py script in QIIME; OTUs present with >0.001% of the reads of each sample, in at least 9 samples, were included. The pseudo-heatmap was plotted with the gplots package in the R environment (http://www.r-project.org) using the OTUs table generated by QIIME.

Table 1
Numbers of sequences analysed, observed OTUs, coverage and diversity estimators for all the studied samples.

Sample	Reads	Good's coverage	Observed OTUs	Chao 1	ACE	Shannon	Simpson
A.2013	294,557	0.994	5078	6875.19	6833.26	7.09	0.96
A.2014	188,349	0.993	3760	5616.95	5513.89	6.15	0.92
B.2013	203,091	0.993	4008	5410.35	5345.92	6.52	0.94
B.2014	223,728	0.993	4338	6002.83	6138.72	5.46	0.81
C.2013	279,374	0.993	5745	7880.04	7818.74	7.37	0.97
C.2014	850,529	0.998	7686	9465.44	9477.87	6.60	0.92
D.2013	172,717	0.996	2316	2965.35	2964.90	5.65	0.90
D.2014	220,559	0.997	2019	2928.77	2853.42	2.90	0.46
E.2013	254,323	0.994	3839	5474.76	5453.59	5.73	0.87
E.2014	501,861	0.997	5012	6989.73	7026.50	5.99	0.92
F.2014	554,203	0.997	5759	7702.27	7826.34	5.84	0.89