

UNVEILING THE ATYPICAL MICROBIOLOGY OF RAW DONKEY MILK BY MALDI-TOF MS

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Objective. Raw donkey milk is considered an excellent substitute for breast milk in infant feeding and clinical nutrition. In Europe donkey milk is mostly produced in small-scale farms with diversified production and its microbial ecology is complex and not yet been fully explored. Traditional microbiology, based on standard culture media, often fails to detect rare or slow-growing species. This study aims to apply an in-depth microbiological characterisation of raw donkey milk focusing on atypical colonies present on traditional culture-based media in order to map microbial diversity.

Methods. A total of 16 samples of raw donkey's milk, purchased from two different farms authorized for production and sell of raw milk in two regions of northern Italy between 2024 and 2025, were analysed using a combination of different culture conditions. After an enrichment phase (in Buffered Peptone Water and Brain Heart Infusion), traditional culture media (Baird-Parker, MacConkey, MacConkey Sorbitol and Violet Red Bile Glucose Agars) were used, in combination with various incubation temperatures (25, 30, 37 °C), growth atmospheres (aerobic and microaerophilic) and extended incubation periods (up to 5 days) for the isolation of coliforms and staphylococci coagulase positive. The isolated colonies showing specific phenotypic characteristics with atypical morphologies from the rest of the typical isolates were selected using the "expert colony selection" technique and identified by MALDI-TOF MS.

Results. A total of 72 bacterial strains belonging to 20 different genera were isolated. The most abundant genera are Staphylococcus (15%) and Pseudomonas (14%) followed by

Enterococcus (8%) and Klebsiella (8%), and <5% Escherichia, Bacillus, Enterobacter, Acinetobacter, Aeromonas, Citrobacter, Agrobacterium, Leclercia, Lelliottia, Lysinibacillus, Morganella, Providencia, Rahnella, Raoultella, Rothia, Serratia. Unknown identification results were observed in 10% of isolates. The presence of lactic acid bacteria belonging to the genus Enterococcus, alongside a minority microbial fraction, such as the genus Lysinibacillus is reported. The species Leclercia adecarboxylata and Morganella morganii had never previously been described in raw donkey's milk. The presence of potentially foodborne pathogens, namely Staphylococcus aureus and Bacillus cereus, has to be highlighted.

Conclusions. Our findings are in line with literature culture-dependent milk bacteriome reporting: i) a relevant lactic acid bacteria community, such as Streptococcus spp. and Enterococcus spp.; ii) spoilage-associated spore-forming genera, such as Bacillus spp.; iii) psychrotolerant and/or psychrophilic taxa (Pseudomonas, Serratia, and Acinetobacter); iv) opportunistic pathogens, such as Rothia, Ralstonia, Citrobacter, Providencia and Rahnella; v) absence or rare classical pathogens. The presence of S. aureus is under the enumeration criteria laid down in Intesa Stato Regioni of 25 gennaio 2007 for raw milk production and sale confirming the milk safety, but the contamination by B.cereus has to be mentioned considering both its spoilage capability and its potential to cause human diseases. Finally, the pathogenic or ambivalent nature of Lysinibacillus fusiformis has yet to be fully characterised. The results enrich current knowledge of the microbial biodiversity associated with this matrix and highlight the potential presence of indigenous strains with technological and probiotic applications.