

ABSTRACT

Milks from non traditional animal species (i.e., donkey, camel, and buffalo) are recently gaining momentum mainly due to the fact that they are considered suitable to supplement the needs of special population groups (i.e., infants, the elderly). Research on donkey milk has dramatically increased over the past few years; therefore, this study aims to critically summarize the current research, characterizing the microbiota diversity of donkey milk and finally offering an insight into its unique functional properties, namely, antimicrobial, antioxidant and ACE-inhibitory activities. The purpose of this project was to provide general information about donkey milk produced in Cyprus by characterizing and identifying the microbiota diversity of raw donkey milk, for potential application into the production of a nutraceutical fermented donkey drink. The project consists of four main parts.

There has been a trend recently to isolate wild-type strains from natural sources for use as starter cultures in food fermentation. Therefore, the first part of the project has concentrated on the study of the diversity, technological and the safety aspects of LAB isolated from raw donkey milk, in order to determine their potential to be used as starters/adjuncts cultures in dairy products. Gram-positive, catalase negative bacteria (257) were isolated using selective microbiological media from eleven raw milk samples, collected over seven months from a donkey farm in Cyprus. All isolates were identified by phenotypic and molecular methods. Organisms identified with partial 16S rDNA sequence analysis were classified within the genus of *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Enterococcus*. Following identification, the predominant LABs were assessed for technological properties; acidification capacity, proteolytic, lipolytic and autolytic activities and production of exopolysaccharides (EPS) and diacetyl. Furthermore, in order to assess their suitability as starter/adjunct cultures, their susceptibility to antibiotics, the absence of virulence factors, the lack of haemolytic activity and production of biogenic amines were also investigated. The safety profile of the isolates revealed that their great majority were susceptible to clinically important antibiotics (i.e. vancomycin) and production of biogenic amines (i.e. tyramine) while the presence of some virulence genes occurred in a few isolates.

For the second part of the project a total of 77 isolates isolated from donkey milk, selected upon their technological and safety properties were screened for their antimicrobial activity against several spoilage and foodborne pathogenic bacteria. Amongst them, 3 *E. faecium* strains showed antimicrobial activity against specific *L. monocytogenes*, *S. aureus* and *B. cereus* strains. Mass spectrometry analysis demonstrated that all *enterococci* used in this

study produced peptides with masses consistent with those for enterocins A and B which was also confirmed by PCR amplification. The cell free supernatant of the identified bacteriocin-producing enterococci were equally active over a wide range of pH and heat treatments making them excellent candidates for potential applications in bio-preservation. Furthermore, bacteriocins produced by these strains were tested for their capability to control post-processing contamination and growth of *L. monocytogenes* in experimentally contaminated fresh whey cheese produced in Cyprus during refrigerated storage. A strain of *E. faecium* was considered bactericidal while the other two were classified as bacteriostatic.

As there is increasing demand for probiotics, one of the questions investigated in this study was; can probiotic candidates be isolated from raw donkey milk? 77 isolates were tested for their survival at low pH. Then, the isolates that showed the highest survival rates (9) were selected for further characterization; i.e. resistance to bile salts, adhesion (BATH test), autoaggregation, coaggregation and bile salt hydrolysis.

The last part of the project was concentrated on the production of a functional fermented donkey milk beverage rich in bioactive peptides, with ACE-inhibitory, antioxidant and antimicrobial activities. LAB isolated previously from raw donkey milk (9) were evaluated for their ability to produce fermented milk rich in ACE-inhibitory, antioxidant and antimicrobial activities. The antioxidant peptide capacity was determined using two antioxidant assays while antihypertensive capacity was evaluated by measuring the capacity to inhibit ACE *in vitro* and the antimicrobial activity by using well diffusion assay against important foodborne pathogens. An important limiting factor of the large-scale diffusion of food carrying potential bioactivities is the bioavailability of the peptides responsible of such bioactivities. The main factors influencing the bioavailability of peptides are the resistance to digestion enzymes and the absorption by the intestinal epithelium. Therefore, the bioavailability of the bioactive peptides were evaluated using an *in vitro* digestion model. All activities were elevated when milk was fermented while a further significant increase was observed after simulated gastrointestinal digestion. The milk that was fermented with *E. faecium* DM33 exhibited the strongest antioxidant activity and the highest antimicrobial activity. The highest ACE-inhibitory activity was observed in milk fermented with *Lb. casei* DM214.

In conclusion, the main contribution of this PhD thesis was to provide new knowledge about the microbiota diversity of LAB population presented in raw donkey milk. This research demonstrates that natural environments are rich in diversity and therefore could be considered

as a valuable source of bacterial strains. Moreover, this project provides evidence that raw donkey milk is an excellent source of wild LAB that are able to grow well and produce fermented donkey milk with ACE-inhibitory, antimicrobial or antioxidant activities. Therefore, results of our study, illustrate that there indigenous strains of LAB showing interesting technological and potential probiotic properties that could potentially be utilized further by the food industry in the fields of food microbiology (i.e. biosafety/bio-preservation) or in dairy technology (i.e. fermented dairy products). Moreover, this project demonstrated that donkey milk fermented with LAB can be considered as a potential functional food.

Keywords: Donkey milk, LAB, Bacteriocins, Probiotics, Bioactive Peptides