Food Control 73 (2017) 81–90

Contents lists available at ScienceDirect

Food Control

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

Raw donkey milk as a source of *Enterococcus* diversity: Assessment of their technological properties and safety characteristics

Maria Aspri [a], Despina Bozoudi [a], Dimitris Tsaltas [a], Colin Hill [b], Photis Papademas [a, *]

[a] Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus
[b] Department of Microbiology, University College Cork, Ireland

ARTICLE INFO

Article history:
Received 19 October 2015
Received in revised form
17 April 2016
Accepted 15 May 2016
Available online 18 May 2016

Keywords:
Donkey milk
Enterococci
Technological properties
Antibiotic resistance
Virulence
Probiotics

ABSTRACT

The diversity, the technological and the safety aspects, and the probiotic potential of enterococci isolated from raw donkey milk were studied, 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 techniques and further tested for their technological and probiotic properties. 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. The results obtained in this study showed that Enterococci in raw donkey milk should not pose a risk to human health, while they possess interesting technological characteristics which could be exploited as potential starter/adjunct cultures for food fermentations.

1. Introduction

Lactic acid bacteria (LAB) are gram positive, catalase negative bacteria, that occur naturally as indigenous microbiota of raw milk. Among LAB, enterococci are natural inhabitants of the gastrointestinal tract of humans and animals and are also present in vegetables, raw meat, milk and cheeses (Giraffa, 2002). They are commonly found in high levels in a variety of cheeses produced in Italy, Spain and Greece (Feta, Cebreino, Water-buffalo mozzarella, Hispanico and Vecano) from raw or pasteurized ewes'and goats' milk (Psoni, Kotzamanides, Andrighetto, Lombardi, Tzanetakis, & Litopoulou-Tzanetaki, 2006). Their presence during ripening of cheeses plays an important role in the development of flavor, aroma and taste through their metabolic and technological traits such as proteolytic and lipolytic activity or the production of metabolites like diacetyl due to citrate breakdown (Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Moreover, apart from their technological properties, enterococcal strains are used as biopreservatives in order to extend the shelf life of cheese and meat products through the production of bacteriocins. Enterocins are frequently active against several Gram positive food spoilage bacteria and food-borne pathogens, such as *Listeria monocytogenes, Staphylococcus aureus* and *Clostridium botulinum* (Franz, Van Belkum, Holzapfel, Abriouel, & Gálvez, 2007; Giraffa, 1995). The last few decades, an increased interest has been raised, dealing with the use of enterococci with desirable technological and metabolic properties as starter cultures, co-cultures or probiotics due to their ability to survive and compete in the gastrointestinal track (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011; Martín-Platero, Valdivia, Maqueda, & Martínez-Bueno, 2009). The selection of starter cultures for food fermentation is a multidisciplinary approach in order to select those microorganisms that are potentially the most suitable for industrial applications which must be free of virulence factors and antibiotic resistance (Vogel, Ehrmann, & Gänzle, 2002), amongst other characteristics. Therefore, besides their beneficial role in fermented foods, the presence of enterococci in food is of considerable concern for the food industry and consumers. Indeed, enterococci are often associated with hospital acquired infections such as bacteraemia, endocarditis urinary tract and pelvic infections (Franz, Holzaphel & Stiles, 1999). One of the reasons for the rise of nosocomial infections related to enterococci might be their ability to develop resistance against a wide variety of

* Corresponding author.
E-mail address: photis.papademas@cut.ac.cy (P. Papademas).

http://dx.doi.org/10.1016/j.foodcont.2016.05.022
0956-7135/

antibiotics, especially to the glycopeptide antibiotic vancomycin (Franz et al., 2011). Furthermore, several studies have recently shown that they may harbor virulence factors such as the ability to produce aggregation substances (agg), gelatinase and haemolysin (Martín-Platero et al., 2009).

The nutritional and therapeutic properties of donkey's milk are known since ancient times but nowadays research focuses on its nutrient composition, potential health effects and immunomodulation activity (Trinchese et al., 2015). For instance, most of the recent studies are focusing on the protein and peptide characterization of donkey milk after in vitro human digestion, in order to investigate the bioactivity of these peptides such as antioxidant, antimicrobial and ACE-inhibitory activity (Piovesana et al., 2015). Therefore, taking into account that modern food trends are turning towards alternative products which are more responsive to new market demands, donkey milk represents a novel possibility for food technology science due to its promising properties. Donkey milk resembles human milk in many ways and is claimed to have special therapeutic properties since is recommended as a substitute for cow's milk protein allergy (CMPA) (Polidori & Vincenzetti, 2013).

Although the chemical composition and nutritional properties of donkey's milk have been widely investigated, information regarding the natural microbiota of this milk is limited. However, there are some studies on donkey's milk microbiota but they are mainly focused on the detection of pathogenic bacteria (Cavallarin et al., 2015; Conte, Foti, Malvisi, Giacopello, & Piccinini, 2012; Pilla, Dapra, Zecconi, & Piccinini, 2010; Salimei, Fantuz, Coppola, Chiofalo, Polidori, & Varisco, 2004; Sarno, Santoro, Di Palo, & Costanzo, 2012). LAB of donkey milk are not fully characterized, and only few studies refer to the isolation and identification of single strains (Murua, Todorov, Vieira, Martinez, Cencič, & Franco, 2013; Nazzaro, Anastasio, Fratianni, & Orlando, 2008; Sa, Krishnaa, Pavithrab, Hemalathab, & Ingalea, 2011). To our knowledge, the presence of enterococci in raw donkey milk has been reported only by few authors (Carminati, Tidona, Fornasari, Rossetti, Meucci, & Giraffa, 2014; Zhang, Zhao, Jiang, Dong, & Ren, 2008), but no subsequent studies regarding their technological, probiotic properties and safety characteristics were described.

Taking this into account, this study constitutes the first report about the biodiversity, safety evaluation and characterization of technological and probiotic properties of different strains belonging to genus *Enterococcus* spp. isolated from raw donkey milk produced in Cyprus. Selected strains with desirable characteristics could be used as starters or adjunct cultures for the production of a fermented donkey milk with bio-functional properties.

2. Materials and methods

2.1. Collection of milk samples

Milk samples were collected from the "Golden Donkeys Farm" in Larnaca area, Cyprus. In particular, sampling was conducted from March to October 2013 and a total of eleven samples were collected by manually milking 8 jennies. The milk samples were collected in sterilized 250 mL containers, placed in cool-boxes and immediately transported to the laboratory at 4–6 °C and maintained at this temperature for analysis within 12 h.

2.2. Enumeration of microorganisms

The samples were evaluated for total aerobic bacteria, Enterobacteriaceae, LAB, yeasts and molds and *Staphylococci,* by the standard pour plate method after serial dilutions in saline solution (0.85% w/v). Table 1 shows the growth media, incubation time, temperature and method used for each evaluated group of microorganisms. *Salmonella* spp. and *Listeria monoytogenes* analysis was performed by using the methods suggested by ISO, 2002 and ISO, 1996 respectively.

2.3. Isolation of lactic acid bacteria

The colonies between 30 and 300 on each petri dish were counted as total LAB. Representative LAB strains were isolated from MRS, acidified MRS and M17 agars, according to different morphological characteristics (i.e size, shape and/or color). Purity of the isolates was checked by streaking on MRS and M17 agar, respectively, followed by microscopic examination. Stock cultures of identified strains of LAB were stored in MRS or M17 broth using 40% (v/v) glycerol as cryoprotective agent at −80 °C.

2.4. Biochemical, physiological and phenotypic strain characterization

The purified isolates were examined by cell morphology applying Gram staining, catalase and oxidase tests, as recommended in Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley, & Williams, 1994). Gram-positive, catalase-negative rods and cocci were presumptively identified as LAB. Further classification was done according to the biochemical criteria described by (Harrigan & McCance, 1976), such as growth at various temperatures (15 °C, 30 °C, 37 °C and 45 °C), salt content (2, 4, 6.5, 8% w/v NaCl), pH (4.4, 6, 8 and 9.6) and litmus milk reduction.

2.5. Molecular identification of LAB strains

The identification of the isolates at genus and species level was confirmed by applying molecular techniques. Bacterial DNA from each strain was obtained by lysis of cells at 96 °C for 10 min with IGEPAL CA-630 (Sigma-Aldrich). 16S F: 5′TGCCTAATACATGCA′3 and R: 5′CTTGTTACGACTTCA′3 (Eurofins, MWG Operon, Ebesberg, Germany) were used to amplify the 16S rRNA gene fragments of LAB isolates, using a thermal cycler (PTC-200 Peltier, MJ Research, Inc., Watertown, MA, USA). PCR reactions contained template DNA, 1X KOD PCR reaction buffer, 200 μM of each dNTP, 2.5μM $MgCl_2$, 0.5μM of each primer, 1 μl KOD Hot Start DNA polymerase (Novagen), and distilled water was added to a final volume of 50 μl according to the manufacture instructions.

The PCR conditions were an initial denaturing step for 2 min at 95 °C followed by 34 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 10 s, and elongation at 70 °C for 30s, followed by a final extension step of 7 min at 72 °C. Amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel, in 1X TAE buffer, stained with Midori green (Anachem, UK) and visualized under UV light. A 100 bp DNA ladder (Invitrogen) was used as a marker. The PCR products were purified using a commercial Purification kit (Purelink PCR, Invitrogen, USA) following the manufacturer's instruction. The purified PCR products were sent to Source Bioscience (Dublin, Ireland) for Sanger sequencing. Sequences were then compared to those in the GenBank database using the BLAST algorithm (http://www.ncbi.nih.gov). The identities of the isolates were determined on the basis of the highest matching (similarity >97%).

2.6. Technological properties

2.6.1. Acidification activity

Acidifying activity of the strains was determined according to the International Dairy Federation (IDF) standard 306 (IDF, 1995), as well as a pH meter (Hanna Instruments, Padova, Italy). Tubes