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Prevalence, acquired antibiotic resistance and bacteriocin production of *Enterococcus* spp. isolated from tunisian fermented food products

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ABSTRACT

In this study, a total of 100 fermented food products including dairy (Lben, Rayeb, Rigouta, and Jben) olive and vegetable products, harvested in Northwestern Tunisia, were investigated for the presence of *Enterococcus* spp. Our results showed high levels of contamination with *Enterococcus* spp., identified according to standard bacteriological, biochemical and phenotypic criteria. 143 isolates were recovered; *E. faecium* (46.15%) was the predominant species, followed by *E. faecalis* (27.27%), *E. casseliflavus* (12.58%), *E. durans* (8.39%) and *E. mundtii* (5.59%). None of the isolates showed acquired resistance againts clinically relevant drugs used for enterococcal infections treatment in human medicins, and no haemolytic activity was demonstrated. Furthermore, over 50% of the isolates within each species exhibited antilisterial bacteriocin production. Further data are needed to enhance understanding of bacteriocin production of enterococci in fermented food products as well as the potential risks to quality and safety, including possible transmission of antibiotic resistant organisms to human consumers.

1. Introduction

Enterococci are Gram-positive, ubiquitous commensals of the gastrointestinal tract in both humans and animals. They are hardy in nature, surviving for long periods in the environment and are able to survive and replicate in a wide range of food matrices. (Aarestrup, Agerso, Gerner-Smidt, Madsen, & Jensen, 2000; Mannu, Paba, Daga, Communian, Zanetti, & Dupré, 2003; Abriouel et al., 2008). The functional characteristics of these bacteria may play an important beneficial role in the various traditional food fermentation processes across the world, contributing to flavor and aroma development or as probiotic adjuncts to improve the microbial balance of the intestine and reduce serum cholesterol levels by excretion of deconjugated bile salts (Franz, Stiles, Schleifer, & Holzapfel, 2003).

Enterococci are also known to produce a number of enterocins, which can inactivate food spoilage microorganisms and pathogenic bacteria such as *Listeria monocytogenes* (Cossart & Toledo-Arana, 2008; Giraffa, 1995; Lundén, Tolvanen, & Korkeala, 2004), contributing to an improved safety profile. *Listeria* spp. are able to survive under extreme physicochemical conditions such as refrigeration temperatures, low pH values and high salt concentrations (Lou & Yousef, 1999), promoting their persistence in fermented foods and on food processing equipment. Enterococci with potent anti-listeria activity have been isolated from a range of fermented food products and have been characterized previously (Aymerich et al., 1996; Casaus et al., 1997). The presence of enterococci in fermented foods is particularly common in products from Mediterranean countries (Franz et al. 2003; Giraffa, 2003). Although this is often regarded as an indicator of unsanitary production methods, they have a long history of safe use (Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006; Ogier & Serror, 2008), and are known to impart beneficial sensory characteristics and improve digestibility. Despite this, interest in the use of enterococci in starter cultures has somewhat diminished due to the emergence of

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these organisms as serious community and nosocomial pathogens, particularly strains exhibiting antimicrobial resistance and harboring virulence factors. (Keyser, 2003; Moreno et al., 2006; Ogier & Serror, 2008; Semedo et al., 2003). Moreover, some enteroccocal strains produce biogenic amines, which is another source of concern in the food industry due to the potential toxicity (Bover-Cid and Franz, Holzapfel, & Stiles, 1999).

Enterococci are intrinsically resistant to many antimicrobial agents, which has led to therapeutic difficulties all over the world (Johnson et al., 2009; Spera & Farber, 1994). Depending on species, they are naturally resistant to cephalosporins, carbapenem, aminoglycosides (at low concentrations), polymixins, lincomycin, clindamycin, and often quinolones, and can acquire resistance to glycopeptides, macrolides, tetracyclines, trimethoprim/sulfamethoxazole, chloramphenicol, and ampicillin (Barbosa, Ferreira, & Teixeira, 2009). In addition these microorganisms present a remarkable genomic flexibility with the ability to incorporate foreign mobile genetic elements carryin, resistance genes to multiple antibiotics through horizontal gene transfer (Hammerum, Lester, & Heuer, 2010).

Studies on the antimicrobial susceptibility patterns of enterococci have affirmed the worldwide emergence of multi-resistant strains, with a high proportion of resistance to vancomycin and the tetracyclines (Hammerum et al., 2010; Werner et al. 2008) and this is a particular problem in countries that do not tightly regulate antibiotic use (Giraffa, Olivari, & Neviani, 2000).).

Typical first-choice antibiotics in the case of a confirmed infection with *Enterococcus* spp. are several β-lactams and aminoglycosides. Second-choice antibiotics include glycopeptides and linezolid (El-Khoury & Fishman, 2003). Over the last few decades, the prevalence of enterococci resistance to β-lactams, glycopeptides, and aminoglycosides (Grayson et al., 1991), as well as to linezolid (Deshpande, Fritsche, Moet, Biedenbach, & Jones, 2007; Scheetz, Knechtel, Malczynski, Postelnick, & Qi, 2008), has been increasing, representing a major cause for concern in the medical community (Koluman, Akan, & Çakiroğlu, 2009). In addition to the pathogenic potential of enterococci the possible transfer of resistance determinants to other pathogenic species, (for example, *Staphylococcus* spp.) (Tenover & McDonald, 2005), is also a risk.

Consumption of fermented food products is a strong ancient tradition in Tunisia and many artisan methods of preparation have been passed down from one generation to another for centuries, and are still enjoyed to this day. The most popular of them are fermented milk products such as 'Lben' and 'Rayeb' and fresh cheeses such as 'Jben' and 'Rigouta' (Abd-El Salam & Benkerroum, 2006). Also, many garden vegetables such as carrots, turnips, peppers, onions, and cauliflower, and fruits such as olives, are commonly preserved by traditional Tunisian techniques. Unfortunately data on the prevalence and antimicrobial resistance of enterococci in such fermented foods is scarce. However, the introduction of enterococci in some fermented food products is likely due to the poor hygiene practices involved with traditional food production and the use of antiquated equipment (Giraffa et al., 2000). We recognize the potential of food products to act as vectors for the transmission of microorganisms, which underpins the importance of monitoring the prevalence of pathogens and antibiotic resistance profiles in the human food chain.

This study aimed to provide the first data on prevalence, acquired antibiotic-resistance and bacteriocin-producing activity of *Enterococcus* spp. isolated from a range of traditional fermented foods from animal and vegetable origin harvested from the Northwestern Tunisia. Such observations are important contributions to understanding the functional characteristics of enterococci in fermented foods and how these can be utilized without a risk to public health.

2. Material and methods

2.1. Sample collection

In order to isolate *Enterococcus* spp., 100 samples of different fermented food products were obtained from retail markets and small farms in several cities of Northwestern Tunisia: 20 samples of each fermented raw milk type (Rayeb and Lben), 20 samples of each fresh cheese (Jben and Rigouta), 10 samples of fermented green olives, and 10 samples of fermented vegetables. A 15 g portion of each food sample was aseptically collected, placed in 135 ml of buffered peptone water (Oxoid, Hampshire, UK), and aseptically transported to the laboratory in sterile polyethylene bags on ice. Samples were homogenized using a Stomacher for 180 s at normal speed and stored at 4 °C. All samples were obtained between January and May 2013 and all microbial analyses were initiated within 24 h of sample collection.

2.2. Microbial enumeration and strain selection

Serial dilutions of fermented food samples were performed in quarter-strength Ringer solution (Oxoid, Hampshire, UK). For the total aerobic viable count, 1 ml of the appropriate dilution was spread plated on PCA agar (Oxoid, Hampshire, UK), and for the enterococci enumeration 0.1 ml was spread on Rothe broth supplemented with 2% agar (Greenberg, Clesceri, & Eaton, 1998; Mallmann & Seligmann, 1950). Plate counts on agar media were carried out in duplicate and read after incubation for 24 h at 30 °C. Microbiological count data were expressed as $\log_{10}$ cfu/mL.Putative colonies of *Enterococcus* spp. (with a whitish color and a maximum diameter of 1 mm) were selected for further characterization and antibiotic susceptibility testing.

2.3. Enterococci; phenotypical and biochemical characteristics

Isolates were Gram stained and tested for catalase and oxidase activity (Sigma Chemical Co., St Louis, MO, USA). Cell morphology was also confirmed by optical microscopy. Additional tests, as described by Smibert and Krieg (1994), were also performed. These included; the ability to grow at 4, 10, and 45 °C on media containing 4%, 6,5% or 10% NaCl, and growth at a range of pH values (3.5 and 9.6). 143 putative *Enterococcus* spp. isolates were characterized as Gram-positive non-sporing cocci, catalase-negative and oxydase negative. These were further tested for carbohydrate utilisation using the API 20 Strep strips (API System, BioMérieux, France), according to the manufacturer's instructions. The API Strep profiles were analysed by the Mini-API system (a compact semi-automated biochemical identification (BioMérieux, France). *Identification* with a probability ≥90% was considered acceptable.

2.4. Antibiotic resistance and haemolytic activity

The susceptibility of the *Enterococcus* spp. strains to a range of relevant clinically most used antibiotics (μg/disc): Amoxicillin (25), Ampicillin (30), Carbenicillin (100), Chloramphenicol (30), Gentamicin (10), Imipenem (10), Kanamycin (30), Ofloxacin (5), Oxacillin (1), Penicillin G (30), Streptomycin (10), Tetracyclin (30), Erytromicin (15), Vancomycin (30), was performed by the disc diffusion method on Muller-Hinton agar according to the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie CA-SFM (2013). Susceptibility testing was also carried out using the ATB Strep system (API system, BioMérieux, France), analysed by the Mini-API system described above. Antibiotic discs were obtained from BioMérieux. Diameters of the zones of inhibition were observed around the antibiotic discs and were taken to