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NOTE TO THE EDITOR

# Population structure and safety aspects of *Enterococcus* strains isolated from artisanal dry fermented sausages produced in Argentina

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## Summary

Enterococci population from Argentinean artisanal dry fermented sausage was identified and their safety aspects were evaluated. Species-specific PCR was used to distinguish between *Enterococcus faecium* (56%) and *Enterococcus faecalis* (17%). Other isolates (27%) were identified as *Enterococcus durans*, *Enterococcus casseliflavus* and *Enterococcus mundtii* by using 16S RNA gene sequence. RAPD analyses showed different biotypes for *Ent. faecium* and *Ent. faecalis* species. Low incidence of antibiotic resistance and high virulence traits in *Ent. casseliflavus* and *Ent. faecalis* were found; the majority of the *Ent. faecium* strains were shown to be free of virulence factors. The absence of virulence/resistance traits and the anti-Listeria activity of *Ent. faecium* isolates may be exploited to enhance natural preservation thereby guaranteeing organoleptic/safety characteristics of artisanal fermented sausages.

In meat products, enterococci together with lactobacilli contribute to the fermentation process, *Enterococcus faecalis* and particularly *Enterococcus faecium* being the major species found (Hugas *et al.* 2003). However, the presence of enterococci in food is highly controversial (Franz *et al.* 2003), because some strains are recognized as major nosocomial pathogens causing bacteraemia, endocarditis and other infections. This is especially troublesome because of their high level of intrinsic and acquired antibiotic resistance to a wide variety of antibiotics, in particular the emergence of vancomycin-resistant strains (Mazuski 2008). Enterococcal strains harbouring antibiotic resistance and virulence factor genes have been detected in different meat-related foods (Martín *et al.* 2005; Rizzotti *et al.* 2005; McGowan-Spicer *et al.* 2008; Templer *et al.* 2008). Because the consumption of fermented meat products exposes consumers to high enterococci concentration, this study was conducted to identify this population from traditional Argentinean fermented sausages and to evaluate their safety aspects.

Artisanal 'salame' was obtained using local, traditional technologies and sampled as described by Fontana *et al.* (2005). Isolation was carried out on *Enterococcus* selective agar (Slanetz & Bartley, SB; Oxoid) incubating at 42°C for 24 h. After counting, 90 isolates were picked at each sampling time and grown on BHI broth (Brain Heart Infusion; Oxoid). The coccal-shaped colonies, Gram positive, catalase negative, salt tolerant (6·5% NaCl) and able to grow at 10 and 45°C were stored at −80°C with 20% glycerol in BHI for further characterization.

During sausage fermentation, enterococci steadily increased in number from 2·4 to 5·4 log CFU $g^{-1}$ within the first 5 days of fermentation, remaining stable until the end of the ripening period. In sausages, no hurdles are found for enterococci growth, allowing them to coexist with lactobacilli as the dominant population (Martín *et al.* 2005; Ferreira *et al.* 2006; Garcia Fontan *et al.* 2007; Paramithiotis *et al.* 2008). The enterococci persistence during sausage ripening can be attributed to their wide range of growth temperatures as well as their high salt tolerance, contributing to sausage flavour by means of their glycolytic, proteolytic and lipolytic activities (Hugas *et al.* 2003).

For genotypic characterization, total DNA was obtained from a single colony using Microlysis (Labogen, UK)

 

according to the manufacturer protocol. Colony lysis and PCR experiments were performed in a GeneAmp PCR System 9700 (Applied Biosystems) thermocycler, and the products were visualized on agarose gel electrophoresis. Using species-specific primers for *ddl* gene (D-alanine/D-alanine ligase) in a Multiplex PCR assay (Dutka-Malen *et al.* 1995), 56% of the isolates from SB agar were identified as *Ent. faecium* while 17% were *Ent. faecalis* (Fig. S1 supplemental material). The prevalence of these two species in fermented foods has also been reported (Paramithiotis *et al.* 2008; Martín *et al.* 2009). Because most of the isolates obtained at time 0 of the fermentation process showed negative amplification for *ddl* gene (Fig. S1), they were identified as *Enterococcus durans*, *Enterococcus casseliflavus/flavescens* and *Enterococcus mundtii* (accession numbers: AY865650, AY865651, AY946202 respectively) by means of 16S rDNA nucleotide sequence (Fontana *et al.* 2005). Intraspecies diversity of enterococci isolates was evaluated by RAPD-PCR according to the conditions and primers described by Fontana *et al.* (2005). RAPD profiles indicated that primers RAPD2 and M13 were more discriminative than XD9, enabling the differentiation of 10 biotypes for *Ent. faecium* isolates and four for *Ent. faecalis* (Fig. S2a,b respectively, supplemental material), while only one biotype was found for *Ent. durans*, *Ent. casseliflavus* and *Ent. mundtii* isolates (data not shown). These molecular typing techniques have been widely used to characterize enterococci isolated from meat (Martín *et al.* 2005, 2009).

A molecular screening of 12 different virulence determinants was performed to investigate the potential pathogenesis of *Enterococcus* strains. The primer sequences, source and annealing temperature used in PCR reactions are listed (Table S1 supplemental material). Primers for *cad*, *asa373*, *fsrA* and *efaAfs* genes were designed in this study from sequences deposited in EMBL website (EFU91527, *cad*; EFA132039, *asa373*; AF108141, *fsrA*; U03756, *efaAfs*) using Primer 3 Web site. Structural genes for haemolysin/cytolysin (*cyl*MBA) and aggregation substances (*agg*, *asa373*, *prgB*) were not detected in any analysed *Enterococcus* strains. However, *Ent. faecalis* CRL1518, CRL1490 and *Ent. casseliflavus* CRL1488 possessed between 4 and 5 out of 12 virulence determinants investigated (Table 1). These strains isolated in an early stage of the ripening process were not found in subsequent sausage fermentation stages (data not shown). Even when

**Table 1** Patterns of virulence, antibiotic resistance genes and MICs of *Enterococcus* strains from Argentinean fermented sausages

| | Virulence determinants* | | | | | | Antibiotic resistance genes† | | | | MIC ($\mu$g ml$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strains | *gelE* | *esp* | *fsrA* | *ace* | *cad* | *efaA$_{fs}$* | *ermB* | *ermC* | *tetM* | *blaZ* | ERY | TET | AMP | CHL | VANC | GEN |
| *Enterococcus casseliflavus* CRL1488 | + | – | + | + | + | + | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Enterococcus mundtii* CRL1516 | – | – | – | – | – | – | + | – | + | + | >32 | 8 | <8 | <8 | <4 | <500 |
| *Enterococcus faecalis* CRL1518 | + | – | – | + | + | + | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecalis* CRL1490 | + | – | – | + | + | + | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecalis* CRL1495 | – | – | – | – | – | + | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecalis* CRL1691 | – | – | – | – | – | + | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Enterococcus faecium* CRL1491 | + | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1492 | – | + | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1493 | + | – | – | – | – | – | – | + | – | + | 8 | <4 | 8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1633 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1634 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1635 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1636 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1632 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1494 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Ent. faecium* CRL1489 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |
| *Enterococcus durans* CRL1631 | – | – | – | – | – | – | – | – | – | – | <0·5 | <4 | <8 | <8 | <4 | <500 |

ERY, erythromycin; AMP, ampicillin; TET, tetracycline; CHL, chloranphenicol; VANC, vancomycin, GEN, gentamycin.

*Positive control used in PCR: *Ent. faecalis* OG1rFpCF10 for *prgB*, *cad*, *gelE*, *agg* genes; *Ent. faecalis* OG1rF for *ace*, *fsrA*; *Ent. faecalis* ISS-S-118 for *esp*, *cylA*, *efaA$_{fs}$*; *Ent. faecalis* C48F3t for *cylB* and *cylM* genes.

†Positive controls used in PCR: *Staphylococcus epidermidis* DST-ST11, *Staph. epidermidis* DST-ST12 and *Ent. faecium* FAIR-E 132 (Università di Verona, Verona, Italy) for *tet*M, *erm*C and [*aac*(6′)-*Ie*-*aph*(2′′)-*Ia*] respectively; *Streptococcus pyogenes* 190 and *Strep. pyogenes* C61 (Università di Ancona, Ancona, Italy) for *erm*A and *erm*B respectively; *Staphylococcus aureus* ATCC 29213, *Ent. faecalis* A256, MN1 and MN2 (Università Cattolica del Sacro Cuore, Piacenza, Italy) for *blaZ*, *vanA*, *vanB* and *vanC* respectively.

*gelE* and *fsr*A were detected in *Ent. casseliflavus* CRL1488, no halo was observed around the colonies when gelatinase activity was tested on agar (0·8% gelatine and 5% agar) as occurred in the other tested strains. This low incidence of virulence determinants among enterococci population may be attributed to the high number of *Ent. faecium* isolated in this study; this being in agreement with the fact that *Ent. faecium* strains, having food origin, generally harbour less determinants than *Ent. faecalis* strains (Eaton and Gasson 2001; Franz *et al.* 2003). Thus, the high number of virulence factors detected in enterococci from chorizo reported by Martín *et al.* (2005) was correlated to the large rate of *Ent. faecalis* present.

When the antibiotic susceptibility of *Enterococcus* strains was tested, the minimal inhibitory concentration (MIC) of erythromycin, chloranphenicol, vancomycin, tetracycline, gentamycin and ampicillin that prevented enterococci growth was determined by the microdilution technique according to CLSI (2005). Resistance to erythromycin and ampicillin (MIC, 8 $\mu$g ml$^{-1}$) was observed in *Ent. faecium* CRL1493 while *Ent. mundtii* CRL1516 was resistant to erythromycin (MIC > 32 $\mu$g ml$^{-1}$) and moderately resistant to tetracycline (MIC, 8 $\mu$g ml$^{-1}$). The presence of *tet*M and *erm*B genes was confirmed by PCR (Olsvik *et al.* 1995; Sutcliffe *et al.* 1996) in *Ent. mundtii* CRL1516 while *erm*C and *blaZ* (Sutcliffe *et al.* 1996; Tomayko *et al.* 1996) were found in *Ent. faecium* CRL1493 (Table 1). No vancomycin-resistant genes (*vanA*, *vanB* and *vanC*) were detected among the tested enterococci, when a multiplex PCR was used (Clark *et al.* 1998). A low antibiotic resistance among food enterococci, especially *Ent. faecium* strains was also reported by Franz *et al.* (2003). Conversely, an extensive evidence of the wide occurrence of antibiotic-resistant genes and resistant enterococci in the production chain of swine meat commodities was reported (Rizzotti *et al.* 2005). Thus, the role of food enterococci in the spread of antibiotic-resistant genes has been proposed (Cocconcelli *et al.* 2003).

Because of its biotechnological importance (Franz *et al.* 2007), enterococci strains were analysed for antimicrobial substances production using the well diffusion assay (Vignolo *et al.* 1993). All *Ent. faecium* biotypes were analysed, as well as the strain *Ent. mundtii* CRL1516 exhibited anti-Listeria activity, which disappeared after trypsin treatment indicating the proteinaceous nature of the inhibitor (data not shown).

Although the presence of enterococci in food is a matter of controversy, they play an important beneficial role in the production of traditional fermented food products. Our results provide new information on enterococcal ecology as well as the safety characteristics of traditional Argentinean fermented sausages.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Figure S1.** Species-specific PCR for *Enterococcus faecium* and *Enterococcus faecalis* identification. Lane 1, DNA 200 bp ladder; lanes 2–11, time 0 isolates; lanes 12–21, 5-day isolates; lanes 22–29, 14-day isolates; lanes 30 and 31, specific bands of 550 and 951 bp for *Ent. faecium* ATCC 19434 and *Ent. faecalis* ATCC 19433 reference strains respectively.

**Figure S2.** RAPD profiles of different biotypes from *Enterococcus faecium* (a) and *Enterococcus faecalis* (b) isolates using primer RAPD2 and M13.

**Table S1.** Primers sequence, annealing temperature and PCR products size for virulence determinant genes

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