Safety assessment and molecular genetic profiling by pulsed-field gel electrophoresis (PFGE) and PCR-based techniques of *Enterococcus faecium* strains of food origin

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ABSTRACT

Enterococcus faecium is authorized as animal probiotic in the European Union, but this microorganism has emerged as an important cause of nosocomial infections in humans. We investigated the safety of 14 potential probiotic *E. faecium* strains with antimicrobial activity, previously isolated from food, following the guidance proposed by EFSA. None of the enterococci harboured the genes encoding the enterococcal surface protein (*esp*), putative glycoside hydrolase (hyl_{Efm}), and mobile insertion sequence IS*16* element. All strains were susceptible to ampicillin. The genetic relatedness of these enterococci was determined by pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC-PCR) and restriction analysis of amplified *16S rDNA* (ARDRA). PFGE analysis of *Sma*I patterns evidenced four subgroups, whereas RAPD and ERIC-PCR analysis gave nine and eight different subgroups, respectively. ERIC-PCR yielded the highest diversity, followed by RAPD and PFGE, while ARDRA achieved the lowest diversity. In conclusion, we demonstrated the absence of well-known enterococcal virulence markers in a collection of *E. faecium* strains from food, which renders them safe to be used in the food industry or as probiotics in animal production, and that ERIC-PCR is a reliable tool to be used for molecular genetic profiling of potential probiotic enterococci.

Keywords: *Enterococcus faecium*; food origin; safety assessment; genetic relatedness.

1. Introduction

Enterococcus faecium is a natural commensal of the human and animal gastrointestinal tract and has been frequently isolated from dairy products, fermented sausages, vegetables, fish, water and soils (Araújo et al., 2011; Cintas, Casaus, Havårstein, Hernández, & Nes, 1997; Giraffa, 2002; Gomes et al., 2008; Ogier & Serror, 2008). However, *E. faecium* has emerged as an important cause of nosocomial infections due to their ability to acquire resistance to antibiotics, most importantly to penicilin/ampicillin, aminoglycosides (high-level resistance) and glycopeptides, and to accumulate virulence factors (Arias & Murray, 2012; Klare et al., 2005; Leavis et al., 2007; Rice et al., 2003; Werner et al., 2008). Despite this fact, some strains are currently used in the food industry and authorized as animal probiotics. According to EFSA, a case-by-case approach, including (i) the strain identification using molecular methods, (ii) the lack of ampicillin resistance, and (iii) the lack of the three genetic elements associated with the hospital enterococcal strains encoding the enterococcal surface protein (*esp*), putative glycoside hydrolase (hyl_{Efm}) (initially described as a hyaluronidase), and mobile insertion sequence IS*16* element, must be adopted to demonstrate the safety of *E. faecium* strains (EFSA, 2012).

In addition to exclude high-risk hospital-adapted *E. faecium* clones, the strain typing has been considered to assess the safety of potential probiotic using molecular methods (Vankerckhoven et al., 2008). In the last years, the use of reliable genotyping methods for microbial identification at strain level has supposed an important progress in several areas, including food microbiology and technology (Jurkovič et al., 2007; Martín-Platero, Valdivia, Maqueda, & Martínez-Bueno, 2009), epidemiological and clinical microbiology (Klare et al., 2005; Leavis et al., 2003; Vankerckhoven et al., 2004), and microbial ecology (Michel et al., 2007). Pulsed-field gel electrophoresis (PFGE) and

PCR-based typing methods (*e.g.*, random amplified polymorphic DNA [RAPD], enterobacterial repetitive intergenic consensus [ERIC-PCR] and restriction analysis of amplified *16S rDNA* [ARDRA]) have been used to study the genetic diversity of *E. faecium* strains isolated from different origins (Jurkovič et al., 2007; Weiss, Domig, Kneifel, & Mayer, 2010).

In the last years, our group has isolated and characterized a number of *E. faecium* strains, including some bacteriocin-producing strains, from different origins such as dairy and meat products (Casaus et al., 1997; Cintas et al., 1997; Cintas et al., 1995), wild and hunting animals (Almeida et al., 2011; Martín et al., 2006; Sánchez et al., 2007), and fish, seafood and fish products (Almeida et al., 2011; Araújo et al., 2015; Muñoz-Atienza et al., 2013). Due to their interesting biotechnological properties, some of these strains, including 14 enterococci, are potential candidates for their application in the food industry or as probiotics in animal production. In this work, we present (i) the safety assessment of the 14 *E. faecium* strains following the EFSA guidance (EFSA, 2012), including the evaluation of virulence markers (*esp*, hyl_{Efm} and IS*16*) and ampicillin susceptibility, to exclude enterococcal strains belonging to the high-risk hospital adapted clones, and (ii) the comparative analysis of four genotyping techniques (PFGE, RAPD, ERIC-PCR and ARDRA) as tools to determine the genetic relatedness of these enterococci.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Fourteen *E. faecium* strains with antimicrobial activity, previously isolated from food and taxonomically identified were used in this study (Table 1). These strains were

grown aerobically in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Ltd., Basingstoke, United Kingdom) at 37 ºC.

2.2. PCR detection of virulence markers

Detection of virulence markers (*esp*, hyl_{Efm} and IS*16*) in the 14 *E. faecium* strains was performed by PCR following the EFSA guidance (EFSA, 2012). The specific oligonucleotide primers and cycling conditions are shown in Table 2. The oligonucleotide primers were obtained from Sigma-Genosys Ltd. (Cambridge, United Kingdom). PCR-amplifications were performed from total genomic DNA from *E. faecium* strains obtained by the Wizard DNA Purification Kit (Promega, Madrid, Spain) in 25 µL reaction mixtures with 5 to 50 ng of purified DNA, 0.7 µM of each primer and MyTaq Mix 1× (Bioline, GmbH, Germany) using an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The positive control strains for detection of markers associated with the hospital strains were the following: *E. faecalis* P36 for *esp* (Eaton & Gasson, 2001), *E. faecium* C68 for hyl_{Efm} (Vankerckhoven et al., 2004), and *E. faecium* P2-5 (clonal complex 17 [CC17] adapted to the hospital environment) for IS*16*. PCR products were analyzed by electrophoresis on 1.5 % (w/v) agarose (Pronadisa, Madrid, Spain) gels stained with GelRed (Biotium, California, USA) at 90 V for 50 min, and visualized with the Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain). The molecular size marker used was HyperLadder 50bp (Bioline GmbH, Germany).

2.3. Determination of ampicillin susceptibility

The susceptibility of the 14 *E. faecium* strains was evaluated using a broth microdilution test (Huys et al., 2011). Individual colonies were suspended in a sterile

glass tube containing 5 mL saline solution (0.85% NaCl; Merck KGaA, Darmstadt, Germany) to a turbidity of 1 in the McFarland scale (approx. 3×10^8 CFU/mL), and further diluted to 6×10^5 CFU/mL in Iso-sensitest (IST) broth (Oxoid) (Klare et al. 2005). Fifty microlitres of the diluted enterococcal suspensions was added to each microplate well previously loaded with 50 µL of a serial ampicillin (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) dilution, resulting in a final bacterial concentration of 3×10^5 CFU/mL and a final antibiotic concentration of 0.125 to 16 mg/L. After incubation (37 ºC, 18 h), the minimum inhibitory concentration (MIC) was established as the lowest antibiotic concentration that inhibited bacterial growth, and interpreted according to the breakpoint identified by EFSA (2012). *E. faecalis* CECT795 (Colección Española de Cultivos Tipo, Valencia, Spain) was used as quality control.

2.4. PFGE

PFGE analysis was performed using the methodology described by Descheemaeker et al. (1997) and Kelly et al. (2010), and unless otherwise stated, all the reagents were obtained from Sigma-Aldrich Corp. PFGE macrorrestriction of purified genomic DNA was carried out at 30 ºC for 24 h in a restriction buffer containing 15 U of *SmaI* (Takara, Shiga, Japan). The chromosomal restriction fragments were separated by PFGE in a CHEF DR-II PFGE apparatus (Bio-Rad, Hercules, CA, USA) containing a 1% (w/v) Pulsed Field Certified Agarose (Bio-Rad) gel. The electrophoresis was performed in an electrophoresis chamber equilibrated at 14 ºC at a constant voltage of 6 V/cm^2 with a ramped pulse time of 1–35 s for 23 h. Lambda Ladder PFG Marker (New England Biolabs, Ipswich, Massachusetts, USA) was used as molecular size standard. The gel

was stained with ethidium bromide and visualized with the Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain)).

2.5. RAPD

RAPD was performed using the specific oligonucleotide primer and cycling conditions shown in Table 2. PCR-amplifications were performed in 25 μL reaction mixtures with 5 to 50 ng of purified DNA, 0.7 μM of primer, 0.2 mM of each dNTP, buffer 1×, 3 mM $MgCl_2$ and 0.75 U of Platinum Taq DNA polymerase (Invitrogen, Madrid, Spain). PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose (Pronadisa, Madrid, Spain) gels stained with GelRed (Biotium, California, USA) at 90 V for 90 min and visualized with the Gel Doc 1000 documentation system (Bio-Rad). The molecular size marker used was 1Kb Plus DNA ladder (Invitrogen).

2.6. ERIC-PCR

ERIC-PCR amplifications were performed using the specific oligonucleotide primers and cycling conditions shown in Table 2. PCR-amplifications and PCR-product visualization were carried out as described above.

2.7. ARDRA

PCR amplifications were performed using the specific oligonucleotide primers and cycling conditions shown in Table 2. PCR-amplifications were carried out in 50 μL reaction mixtures with 5 to 50 ng of purified DNA, 0.7 μM of each primer, 0.2 mM of each dNTP, buffer 1×, 2 mM $MgCl_2$ and 1.5 U of Platinum Taq DNA polymerase (Invitrogen). The expected PCR-product was analyzed by electrophoresis on 1% (w/v) agarose (Pronadisa) gels stained with GelRed (Biotium) at 90 V for 50 min. Digestion

of the amplified product (4 µL) was performed in 20 µL reaction mixtures containing 5 U of the restriction enzyme *Hha*I (New England Biolabs) at 37 ºC for 3 h. The restriction fragments patterns obtained after enzyme inactivation (65 ºC for 20 min) were analyzed by electrophoresis on 1% (w/v) agarose (Pronadisa) gels at 90 V for 90 min using HyperLadder 50bp (Bioline GmbH) as molecular size marker. PCR-product visualization were carried out as described above.

2.8. Data analysis

The resulting patterns obtained from PFGE-*Sma*I digestion, RAPD, ERIC-PCR and ARDRA-*Hha*I digestion were interpreted after constructing dendrograms using the unweighted-pair group method with arithmetic mean (UPGMA) and the similarity based on the Dice's coefficient analyzed with the Phoretix v.5.0 software (Nonlinear Dynamics Ltd., United Kingdom). The discriminatory power of the technique was evaluated using the Simpson´s index of diversity (ID) described by Hunter and Gaston (1988), and calculated from the following equation: $ID = 1 - [l/N (N - l)] \Sigma n_j (n_j - l)$, where N is the total number of strains and n_j is the number of strains belonging to type j. ID values close to 0 or 1 revealed low and high diversity, respectively.

3. Results

3.1. PCR detection of virulence markers and determination of ampicillin susceptibility

None of the *E. faecium* strains of food origin carried any of the virulence markers *esp*, hyl_{Efm} or IS*16* (Table 3). The distribution of MICs for ampicillin is summarized in Table 3.

3.2. PFGE

PFGE patterns of the 14 *E. faecium* strains showed that 13 were clustered into a major group (G1), separated from *E. faecium* T136 (56% of similarity), with bands of, approximately, 48.5–485.0 Kb (Fig. 1). The enterococci from group G1 were further subdivided into three well-defined subgroups (SG1.1 to SG1.3) with a similarity coefficient of 70%. The subgroup SG1.1 was composed by seven *E. faecium* strains (T2, L50, BNM58, SMF8, SMA8, SMA7 and T29), including four enterococci (L50, BNM58, SMF8 and SMA8) that showed identical PFGE patterns. In subgroup SG1.3, five strains (LPP29, TPM76, TPP2, CV2 and CV1) showed 100% of similarity, while subgroup SG1.2 was composed of only one strain (*E. faecium* P13). The resulting index of diversity of PFGE analysis was 0.824.

3.3. RAPD

RAPD patterns of the 14 *E. faecium* strains showed that 13 enterococci were clustered into a main group (G1), separated from *E. faecium* SMA7 (16% of similarity), with DNA fragments ranging from, approximately, 650 to 4,000 bp (Fig. 2). Moreover, the enterococci in G1 were subdivided into eight subgroups (SG1.1 to SG1.8) with a similarity level of 70%. The subgroup SG1.4 was composed of *E. faecium* BNM58 and SMF8 and the subgroup SG1.8 by five strains (LPP29, TPM76, CV2, CV1 and TPP2). The remaining subgroups (SG1.1, SG1.2, SG1.3, SG1.5, SG1.6 and SG1.7) were composed of only one *E. faecium* strain (T2, T29, L50, SMA8, T136 and P13, respectively). The resulting index of diversity of RAPD analysis was 0.934.

3.4. ERIC-PCR

ERIC-PCR patterns of the 14 *E. faecium* strains showed that 13 enterococci were clustered in a major group (G1), separated from *E. faecium* LPP29 (24% of similarity),

with DNA fragments of, approximately, 100–3,000 bp (Fig. 3). In addition, all *E. faecium* strains in group G1 were divided into seven subgroups (SG1.1 to SG1.7) with a similarity coefficient of 70%. The subgroups SG1.3, SG1.5 and SG1.7 were composed of four (L50, BNM58, SMF8 and SMA8), three (P13, T136 and TPP2), and two strains (CV2 and CV1), respectively. The remaining subgroups (SG1.1, SG1.2, SG1.4 and SG1.6) were composed of only one strain (T2, T29, SMA7 and TPM76, respectively). The resulting index of diversity of ERIC-PCR analysis was 1.000.

3.5. ARDRA

ARDRA profiles of the 14 *E. faecium* strains showed the presence of 2 well-defined and highly divergent groups (G1 and G2; 88% of similarity) with DNA fragments ranging from, approximately, 100 to 1,200 bp (Fig. 4). The first group (G1) included six *E. faecium* strains (T2, T29, L50, BNM58, SMF8 and SMA8) with identical profiles, and *E. faecium* SMA7 (94% of similarity); however, the second group (G2) was composed of seven *E. faecium* strains (P13, T136, LPP29, TPM76, TPP2, CV2 and CV1) with a similarity of 100%. The resulting index of diversity of ARDRA analysis was 0.604.

4. Discussion

In the last years, the emergence and dissemination of acquired genetic elements, including genes encoding antibiotic resistance and/or virulence factors, amongst clinical *E. faecium* isolates has become a cause of great concern, as well as the possibility of their transmission amongst strains used in the food and feed industry (Arias & Murray, 2012; EFSA, 2012). In this sense, the detection of three virulence marker markers (*esp*, hyl_{Efm} or IS*16*) by PCR and the determination of the MIC for ampicillin have been

proposed by EFSA (2012) as a method to assess the safety of *E. faecium* and to identify strains potentially linked to nosocomial infections. Therefore, we have assayed the safety of 14 *E. faecium* strains isolated from food in order to identify those safe for use in the food industry or as probiotics in animal nutrition. Our results showed that the tested virulence markers (*esp*, hyl_{Efm} or IS*16*) were not found in any of the 14 enterococci, and all *E. faecium* strains were susceptible to ampicillin according to the breakpoint (≤ 2 mg/L) established by EFSA (EFSA, 2012). In previous studies, the mobile IS*16* element (designated as a mutator type transposase) has been proposed as a specific marker to predict hospital-adapted *E. faecium* strains (Leavis et al., 2007; Werner et al., 2011). The absence of IS*16* element amongst our non-clinical enterococci is in agreement with Werner et al. (2011), who suggested a host range of IS*16*-bearing plasmids limited to hospital-adapted strains. The gene *esp* has been located on a putative pathogenicity island (Leavis et al., 2004), and recently renamed as integrative conjugative element ICE*Efm1* since it can be mobilized and is self-transmissible (Top et al., 2011). Previous studies have associated its presence in *E. faecium* with biofilm formation (Heikens, Bonten, & Willems, 2007), urinary tract infection (Leendertse et al., 2009) and endocarditis (Heikens et al., 2011). On the other hand, hyl_{Efm} is carried by large transferable megaplasmids in hospital-adapted *E. faecium* strains (Freitas et al., 2010). A previous study reported that the acquisition of hyl_{Efm}-plasmid by the non-colonizing *E. faecium* D344SRF from the clinical isolate *E. faecium* C68 promoted its colonization in mice gastrointestinal tract (Rice et al., 2009). In this work, the absence of *esp* and hyl_{Efm} in the 14 enterococci may also be related to their non-clinical origin. The lack of these genes is in concordance with a previous study in which none of the *E. faecium* strains commercially used as probiotics harboured any of these virulence factors (Vankerckhoven et al., 2008). Recently, the presence of *esp* and *hyl* has been

reported more frequently in ampicillin-resistant vancomycin-resistant *E. faecium* (VREF) than in ampicillin-susceptible VREF strains (Vankerckhoven et al., 2004). In fact, the increase of the occurrence of enterococci implicated in hospitals outbreaks has been mainly attributed to the spread of ampicillin-resistant VREF exhibiting *esp* and/or *hyl* (Klare et al., 2005; Vankerckhoven et al., 2008; Werner et al., 2008).

Molecular genotyping techniques have been mainly employed in epidemiological studies to identify the source of the microorganisms responsible for the infection, to recognize their spreading mode, reservoirs and vectors, and to evaluate specific infection control measures and treatments (Singh et al., 2006). Additionally, these techniques have been evaluated to identify probiotic strains from other bacteria in animal feed (Weiss et al., 2010), and to confirm the capacity of implantation, dominance and survival of inoculated bioprotective strains in fermented food (Rubio, Bover-Cid, Martin, Garriga, & Aymerich, 2013). In this study, the relationship amongst the 14 *E. faecium* strains was analyzed by PFGE and three PCR-based methods (RAPD, ERIC-PCR and ARDRA). PFGE has been used as a preferential method for genotyping analysis of *E. faecium* in epidemiological studies (Klare et al., 2005; Vankerckhoven et al., 2004; Werner et al., 2011), but it has been also employed in studies focused on *E. faecium* genetic diversity in food (Jurkovič et al., 2007) and genetic relatedness between clinical isolates and probiotic cultures (Noguchi, Nakaminami, Nakase, & Sasatsu, 2011; Vankerckhoven et al., 2008). Nowadays, several alternative techniques have been successfully applied to the genotyping of enterococci below the species level because they reduce time and costs and only require standard equipment (Werner, Willems, Hildebrandt, Klare, & Witte, 2003). In our work, the PFGE analysis of *Sma*I macrorrestriction patterns evidenced four subgroups, whereas RAPD and ERIC-PCR analysis gave nine and eight different subgroups, respectively. Moreover, ERIC-PCR

295 reached the highest values, followed by RAPD and PFGE, while ARDRA achieved the
296 lowest value. The fact that ERIC-PCR was the most discriminating technique is in
297 agreement with the results of Jurkovič et al. (2007), who reported that ERIC-PCR was
298 more suitable than PFGE for typing of enterococci from cheeses. Martín-Platero et al.
299 (2009) observed that ERIC-PCR and RAPD were useful tools to identify and genotype
300 enterococci isolated from cheeses. Moreover, Weiss et al. (2010) reported that RAPD
301 proved the superior discriminatory power for *E. faecium* strains from animal feed in
302 comparison to other PCR-based methods. The lowest discriminatory power obtained for
303 ARDRA is in agreement with previous works that reported the usefulness of this
304 method to identify bacteria at the species level but not to cluster strains within the same
305 species (Michel et al., 2007; Weiss et al., 2010).

306 In this work, the analysis of the patterns showed a high heterogeneity amongst the
307 enterococci likely due to their different origin, although some relatedness was observed.
308 With regard to this, the PFGE- and ARDRA-patterns of *E. faecium* L50, BNM58,
309 SMF8 and SMA8 showed 100% of similarity, but based on the patterns obtained with
310 RAPD and ERIC-PCR, these strains were less related (67% and 73% of similarity,
311 respectively). The patterns of *E. faecium* LPP29, TPM76, CV2 and CV1 revealed a
312 similarity of 100% when were analyzed by PFGE, RAPD and ARDRA methods, but
313 these strains were poorly related by ERIC-PCR (55% of similarity), with the exception
314 of *E. faecium* CV1 and CV2 that showed a similarity of 75%.

315 In conclusion, we demonstrated, according to the EFSA guidance on the safety
316 assessment of *E. faecium* (EFSA, 2012), the absence of well-known enterococcal
317 virulence markers in a collection of 14 *E. faecium* strains of food origin, which renders
318 them safe to be used in the food industry or as probiotics in animal production.

Moreover, our results indicated the reliability of ERIC-PCR for genotyping enterococci and, very likely, for their specific monitoring.

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Figure captions

Figure 1. Dendrogram of PFGE-*Sma*I digestion patterns showing the genetic relationships amongst the 14 *E. faecium* strains.

Figure 2. Dendrogram of RAPD patterns showing the genetic relationships amongst the 14 *E. faecium* strains.

Figure 3. Dendrogram of ERIC-PCR patterns showing the genetic relationships amongst the 14 *E. faecium* strains.

Figure 4. Dendrogram of ARDRA-*Hha*I digestion patterns showing the genetic relationships amongst the 14 *E. faecium* strains.

Table 1
Origin of the *E. faecium* strains used in this study.

Strain	Origin	Reference
BNM58	Albacora (*Thunnus alalunga*)	Muñoz-Atienza *et al.* (2013)
CV1	European squid (*Loligo vulgaris*)	Muñoz-Atienza *et al.* (2013)
CV2	European squid (*L. vulgaris*)	Muñoz-Atienza *et al.* (2013)
LPP29	European seabass (*Dicentrarchus labrax*)	Muñoz-Atienza *et al.* (2013)
SMA7	Cold-smoked Atlantic salmon (*Salmo salar*)	Muñoz-Atienza *et al.* (2013)
SMA8	Cold-smoked Atlantic salmon (*S. salar*)	Muñoz-Atienza *et al.* (2013)
SMF8	Atlantic salmon (*S. salar*)	Muñoz-Atienza *et al.* (2013)
TPM76	Rainbow trout (*Oncorhynchus mykiss*)	Muñoz-Atienza *et al.* (2013)
TPP2	Rainbow trout (*O. mykiss*)	Muñoz-Atienza *et al.* (2013)
T2	Mullet (*Liza ramada*)	Almeida *et al*. (2011)
T29	Mullet (*L. ramada*)	Almeida *et al*. (2011)
L50	Spanish dry-fermented sausage	Cintas *et al*. (1995)
P13	Spanish dry-fermented sausage	Cintas *et al*. (1997)
T136	Spanish dry-fermented sausage	Casaus *et al*. (1997)

Table 2

Primers and PCR conditions used in this study.

Genes or PCR-based genotyping methods (Primer reference)	Primer	Sequence 5´-3´	PCR conditions		PCR product length (bp)	PCR conditions reference
esp	14F	AGATTTCATCTTTGATTCTTGG	95 ºC 1 min	1 cycle	Approx., 500	Leavis *et al*. (2003)
(Leavis *et al*., 2003)	12R	AATTGATTCTTTAGCATCTGG	95 ºC 15 s			
			52 ºC 15 s	35 cycles		
			72 ºC 10 s			
			72 ºC 4 min	1 cycle		
hyl_{Efm}	Forward	GAGTAGAGGAATATCTTAGC	95 ºC 1 min	1 cycle	661	Rice *et al*. (2003)
(Rice *et al*., 2003)	Reverse	AGGCTCCAATTCTGT	95 ºC 15 s			
			48 ºC 15 s	35 cycles		
			72 ºC 10 s			
			72 ºC 4 min	1 cycle		
IS*16*	IS16-F	CATGTTCCACGAACCAGAG	95 ºC 1 min	1 cycle	547	Werner *et al*. (2011)
(Werner *et al*., 2011)	IS16-R	TCAAAAAGTGGGCTTGGC	95 ºC 15 s			
			53 ºC 15 s	35 cycles		
			72 ºC 10 s			
			72 ºC 4 min	1 cycle		
RAPD	M13	GAGGGTGGCGGTTCT	94 ºC 5 min	1 cycle	Multiple bands	Hosseini *et al*. (2009)
(Huey & Hall,1989)			94 ºC 1 min			
			45 ºC 1 min	33 cycles		
			72 ºC 1 min			
			72 ºC 15 min	1 cycle		
ERIC-PCR	ERIC-1R	ATGTAAGCTCCTGGGGATTCAC	94 ºC 3 min	1 cycle	Multiple bands	Ventura & Zink (2002)
(Versalovic *et al*., 1991)	ERIC-2	AAGTAAGTGACTGGGGTGAGCG	94 ºC 30 s			
			48 ºC 1 min	35 cycles		
			72 ºC 5 min			
			72 ºC 7 min	1 cycle		
ARDRA (*16S rDNA*)	P1	AGAGTTTGATCMTGGCTC	94 ºC 5 min	1 cycle	5,000	Michel *et al*. (2007)
(Michel *et al*., 2007)	P2	ACATCGAGGTGCCAAAC	94 ºC 30 s			
			57 ºC 1 min	30 cycles		
			72 ºC 6 min			
			72 ºC 7 min	1 cycle		

Table 3

Virulence markers and ampicillin susceptibility of *E. faecium* strains.

E. faecium strains	Virulence markers	Ampicillin MIC (mg/L)[a]
BNM58	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	1
CV1	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.25
CV2	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.25
LPP29	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.25
SMA7	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.5
SMA8	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.5
SMF8	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.5
TPM76	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.25
TPP2	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.25
T2	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	$\leq$ 0.125
T29	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.5
L50	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.5
P13	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	$\leq$ 0.125
T136	*esp*$^-$, *hyl*$_{Efm}^-$, IS*16*$^-$	0.5

[a]MICs determined by a microdilution test. The ampicillin dilution range was 0.125–16 mg/L. Strains showing a MIC $\leq$ 2 mg/L were considered as susceptible (EFSA, 2012).

Figure 1

Fig. 1

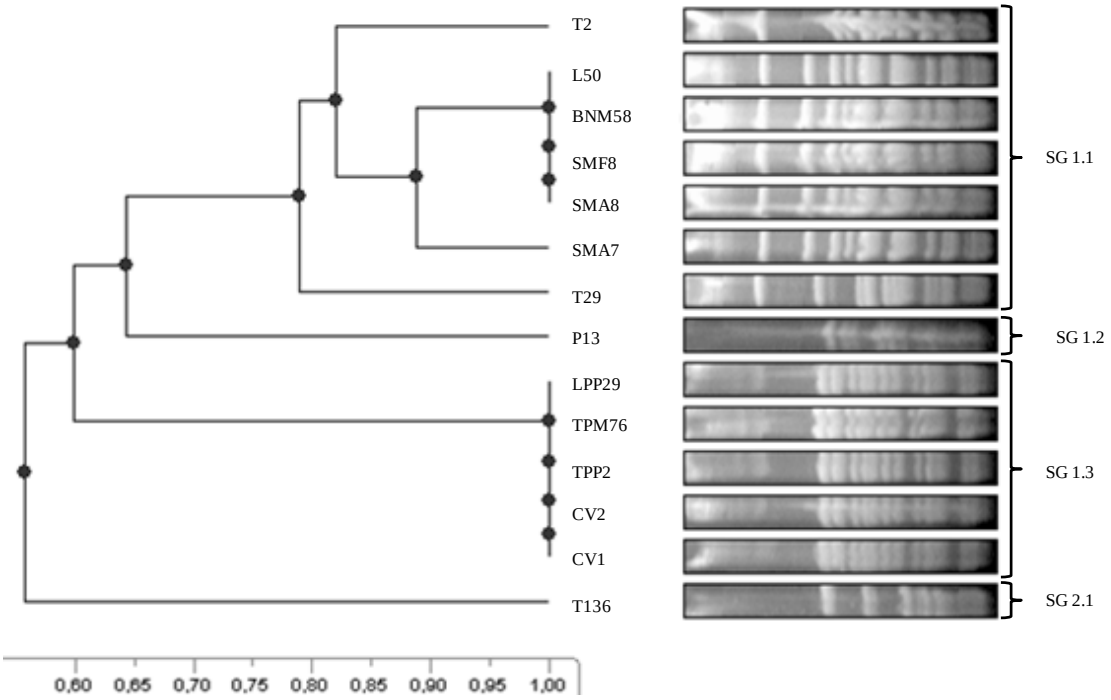

Figure 2

Fig. 2

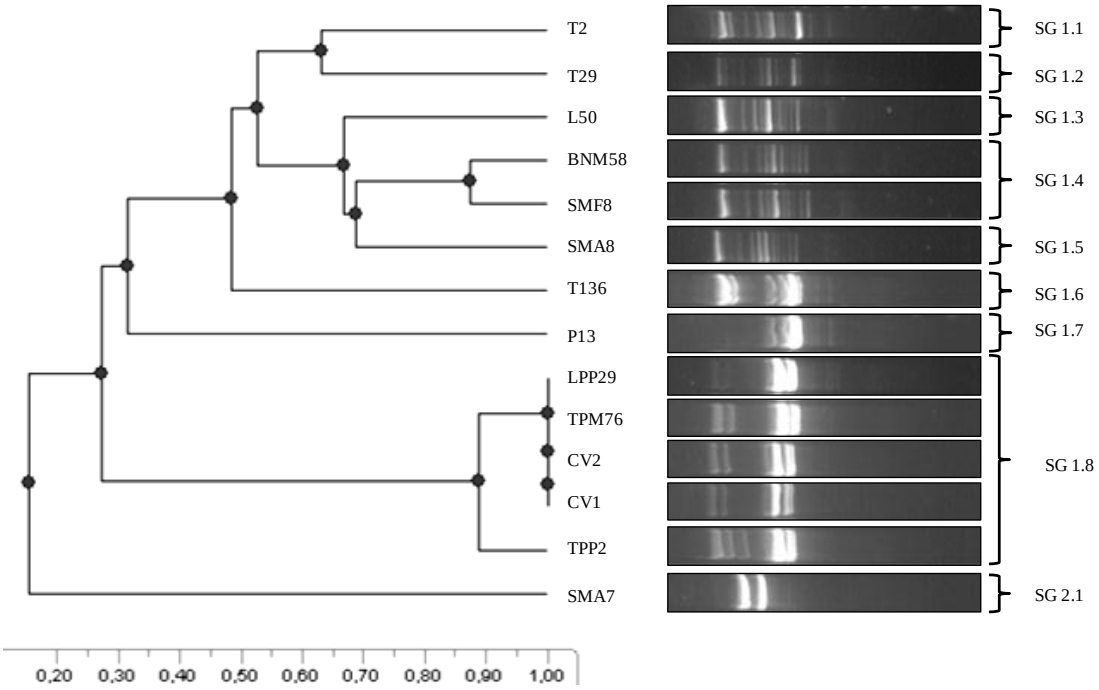

Figure 3

Fig. 3

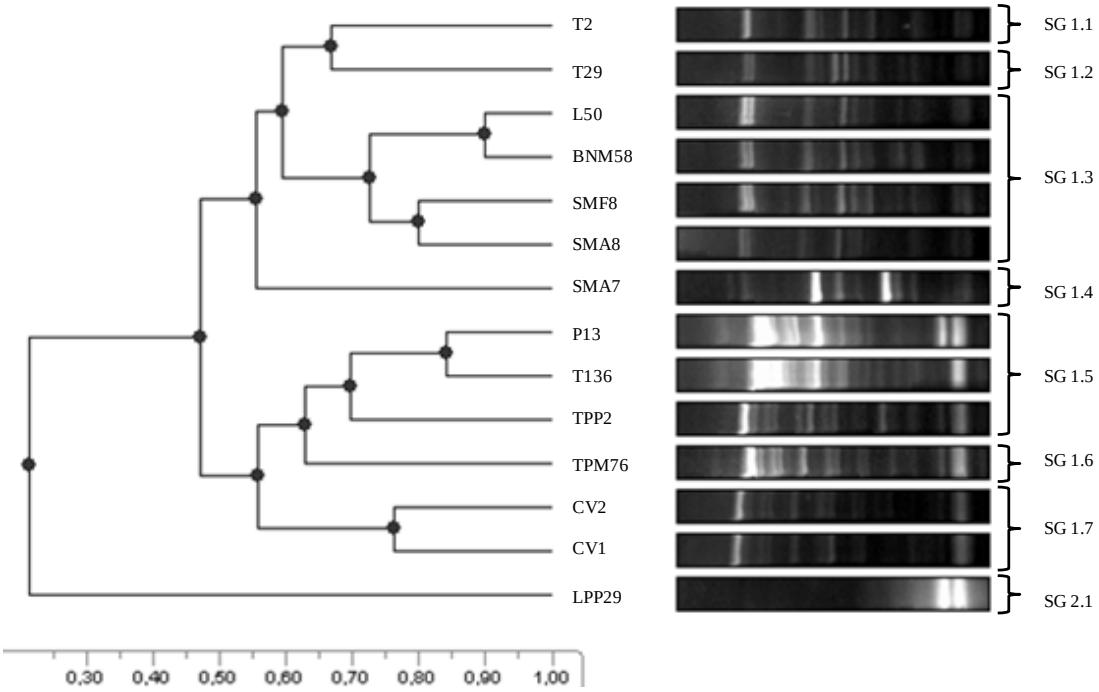

Figure 4

Fig. 4