Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal

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A B S T R A C T

The aim of this work was to characterize *Enterococcus* spp. isolated from *Alheira*, *Chouriça de Vinhais* and *Salpicão de Vinhais,* fermented meat products produced in the North of Portugal, concerning their potential pathogenicity. One hundred and eighty two isolates (76 identified as *Enterococcus faecalis*, 44 as *Enterococcus faecium*, 1 as *Enterococcus casseliflavus* and 61 as *Enterococcus* spp.) were studied. Twenty six percent of isolates were gelatinase producers. None of the isolates produced lipase nor DNase activities. Hemolytic activity using sheep and human blood from two types (A and O) was assessed. One isolate was β-hemolytic in human blood. Results obtained in sheep blood were quite different from those obtained in human blood. Biofilm production in batch and in fed-batch mode was evaluated. In batch mode, only 28.0% and 3.9% of isolates were classified as moderate and strong biofilm producers, respectively, and in fed-batch mode, 35.7% and 63.2% of isolates were classified as moderate and strong biofilm producers, respectively. The presence of 13 virulence genes (*efaAfs*, *efaAfm*, *esp*, *agg, cylM*, *cylB*, *cylA*, $cylL_L$ $cylL_s$ and *gelE*) were investigated by PCR. The majority of enterococcal isolates showed the presence of one or more virulence factors, the most frequent genotype being $efaAfs^+$ $gelE^+$ agg^+ (41.5%). *E. faecalis* isolates harbored multiple virulence traits, while *E. faecium* isolates were generally free of virulence determinants. Phenotypic and genotypic evidence of potential virulence factors were identified in *Enterococcus* spp. isolates, which is a reason of concern.

1. Introduction

Enterococci have as their main habitat the gastrointestinal tract of humans and warm-blooded animals (Murray, 1990), but they also occur in soil, surface waters and on plants and vegetables, due to their ability to grow and survive under severe environmental conditions (Franz, Holzapfel, & Stiles, 1999; Giraffa, 2002; Murray, 1990).

Many fermented meat products contain enterococci and a lot of benefits have been attributed to them, such as their contribution to ripening and aroma development, probiotic properties and the production of antimicrobial substances (Giraffa, 2002). However, several studies have shown that enterococci possess virulence determinants, such as enterococcal surface protein gene (*esp*), aggregation substances (agg), cell wall adhesins efaAfm and efaAfs of *Enterococcus faecium* and *Enterococcus faecalis*, respectively, gelatinase (gelE) and cytolysin (cyl) (Eaton & Gasson, 2001; Franz et al., 2001; Mannu et al., 2003; Semedo, Santos, Lopes, et al., 2003; Cariolato, Andrighetto, & Lombardi, 2008; Valenzuela et al., 2008). The presence of such virulence factors, intrinsic and acquired antibiotic resistance and their association with human disease can explain their potential pathogenic activity (Jonhson, 1994; Franz et al., 1999; Giraffa, 2002).

Products such *Alheira*, *Salpicão* and *Chouriça*, which represent an important economic resource to the North region of Portugal, have high counts of *Enterococcus* spp.: *Alheira* contain enterococci in numbers ranging from 10^4–10^8 CFU/g (Ferreira et al., 2006) and *Salpicão* and *Chouriça* in numbers ranging from 10^4–10^7 CFU/g (Ferreira et al., 2007). These data prompted the present study, having as its main objective the characterization of isolates of *Enterococcus* spp. concerning their potential pathogenicity.

2. Material and methods

2.1. Origin of isolates

From 1060 enterococci isolated from traditional fermented meat products (Ferreira et al., 2006, 2007), 182 were selected after phenotypic characterization and RAPD–PCR typing (Barbosa, Ferreira, & Teixeira, 2009). Samples were collected from various stages of production (in the processing plants) and final products (supermarkets and *Feira do Fumeiro)* between September 2003 and 2005, from *Alheira* (143), *Salpicão de Vinhais* (14) and *Chouriça de Vinhais* (25) manufactured by 16 producers.

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2.2. Growth and storage conditions

Isolates were grown on Brain Heart Infusion Agar (BHIA) (Lab M, Lancashire, United Kingdom) at 37 °C for 24 h and stored at −80 °C in Brain Heart Infusion Broth (BHIB) containing 30% (v/v) of glycerol (Sigma, Steinheim, Germany), and sub-cultured twice before use in assays.

2.3. Virulence factors

2.3.1. Production of hydrolytic enzymes

All experiments were performed in duplicate and *Staphylococcus aureus* ATCC 25213 was used as a positive control.

2.3.1.1. Gelatinase test. Gelatinase activity was assessed according to Tiago et al. (2004).

2.3.1.2. Lipase test. Lipase activity was assessed according to Tiago et al. (2004). A positive reaction was indicated by a clear halo around the colonies.

2.3.1.3. DNase test. DNase activity was tested as described by Ben Omar et al. (2004) by using the medium DNase agar (Pronadisa, Madrid, Spain) with 0.05 g/l of methyl green (Sigma). A clear halo around the colonies was indicative of a positive result.

2.3.2. Hemolytic activity

Production of hemolysin was determined by streaking enterococcal isolates on sheep and human blood agar plates (Psoni et al., 2006). Sheep blood agar was prepared using Azide Blood agar base (ABAB) (Difco, Sparks, Maryland, USA) with 5% defibrinated sheep blood (Liofilchem, Roseto degli Abruzzi-TE, Italy). Human blood agar was prepared using ABAB with 5% of human blood from types A and O (both from voluntary donors). *E. faecalis* F2 (from a collection of Tracy Eaton, Division of Food Safety Sciences, Institute of Food Research, Norwich, United Kingdom) and *E. faecalis* DS16 (from a collection of C. B. Clewell, Department of Oral Biology, School of Dentistry, University of Michigan, Ann Arbor, USA) were used as β-hemolysis controls. The presence or absence of zones of clearing around the colonies was interpreted as β-hemolysis (positive hemolytic activity) or γ-hemolysis (negative hemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as α-hemolysis and taken as negative for the assessment of hemolytic activity (Semedo, Santos, Martins, et al., 2003).

2.3.3. Biofilm assay

Quantification of biofilm production in batch assay was carried out using the modified protocol of Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000). All the plates were incubated aerobically for 72 h at 37 °C in a shaker incubator at 120 rpm (New Brunswick scientific, Edison, NJ, USA). For the fed-batch assay, 100 μl of the medium was discarded every 24 h and filled with 100 μl of fresh culture medium, according to Cerca, Pier, Vilanova, Oliveira, and Azeredo (2004). The observation of biofilms was done according to Stepanović et al. (2000) by measurement of the optical density (OD) at 630 nm by using an enzyme-linked immunosorbent assay reader (Microplate reader, Bio-Rad, Richmond, CA).

All experiments were done six times for each strain and the results averaged. Optical density (OD) values from the wells that had not been inoculated with bacteria were used as negative controls and two positive controls were also used: *E. faecalis* P1 and *E. faecalis* F2 (from a collection of Tracy Eaton). The cut-off value (ODc) for determining a biofilm producer and the classification of the strains as: non-biofilm producers ($OD \leqslant ODc$), weak biofilm producers ($ODc < OD \leqslant 2 \times ODc$), moderate biofilm producers ($2 \times ODc < OD \leqslant 4 \times ODc$) and strong biofilm producers ($4 \times ODc > OD$) was performed as described by Stepanović et al. (2000).

2.3.3.1. Statistical analysis. To test significant differences between the batch modes considered (batch and fed-batch) and within replicates, the Kruskal–Wallis test was applied (Walpole & Myers, 1993). All the calculations involved were performed using Microsoft® Office Excel 2003 (Copyright© 1985–2003 Microsoft Corporation).

2.4. Virulence genes

2.4.1. Primers and bacterial strains

The primers used for the amplification of genes *esp*, *agg*, *gelE*, *efaAfs* and *efaAfm*, *cylA*, *cylB* and *cylM* were described by Eaton and Gasson (2001) and the remaining primers of *cyl* operon: $cylL_L$ and $cylL_S$ were developed by Semedo, Santos, Martins, et al. (2003). All the primers were purchased from MWG Biotech AG. The positive controls used were: *E. faecalis* DS 16 ($cylL_L^+$, $cylL_S^+$) (from a collection of C. B. Clewell), *E. faecalis* F2 ($cylA^+$, $cylB^+$, $cylM^+$, $efaAfs^+$), *E. faecium* F10 ($efaAfm^+$), *E. faecalis* P1 (agg^+, $gelE^+$) and *E. faecalis* P36 (esp^+) (from a collection of Tracy Eaton).

2.4.2. DNA extraction and PCR conditions

Total DNA was extracted according to Pitcher, Saunders, and Owen (1989) using the guanidium thiocyanate method and PCR amplifications were performed in a ThermoCycler (Bio-Rad) in 0.2 ml reaction tubes each with 25 μl of mixtures using 0.5 mM of each primer, 0.1 mM of deoxynucleoside triphosphates (dNTP's, ABGene, Surrey, United Kingdom), 1× of PCR Buffer (MBI Fermentas, Mundolsheim, France), 2.5 mM of $MgCl_2$ (MBI Fermentas), 2 U of Taq polymerase (MBI Fermentas) and 100 ng/μl of enterococcal DNA. Amplification reactions were performed under the following conditions: initial cycle of 94 °C for 1 min; 35 cycles of 94 °C for 1 min, annealing temperature of 55 °C for 1 min, 72 °C for 2 min; a final extension step of 72 °C for 7 min and thereafter cooled to 4 °C. For each PCR reaction a negative control (sample without template) and a positive control (sample with DNA from each strain according to the studied gene) were included.

2.4.3. Gel electrophoresis

All amplification products were combined with 3 μl of loading buffer (Bio-Rad) and 15 μl of these mixtures were applied to a submerged horizontal agarose gel (Seakem® LE Agarose, Rockland, ME USA, 0.8% (w/v)) in 1x TAE Buffer (40 mM tris, 20 mM acetic acid and 1 mM EDTA, pH 8.3; Bio-Rad) containing 0.5 μg/ml of ethidium bromide. Electrophoretic separation was at 90 V for 2 h and, on each gel, a molecular weight marker (100 bp PCR ladder, Bio-Rad) was included at two positions. The gels were photographed on an UV transilluminator (GelDoc2000, Bio-Rad) and image analysis was accomplished using Quantity One® software (Bio-Rad).

3. Results and discussion

3.1. Virulence factors

3.1.1. Production of hydrolytic enzymes

3.1.1.1. Gelatinase test. The results obtained in this study – 26% of the total isolates were gelatinase producers – are in agreement with previous studies (Franz et al., 2001; Semedo, Santos, Lopes, et al., 2003). Among the positive strains 92% belong to the species *E. faecalis* and 2% and 6% to *E. faecium* and other species, respectively. A low incidence of gelatinase activity among non-*faecalis* isolates was previously described (Franz et al., 2001; Semedo,

Santos, Lopes, et al., 2003). A few studies have found gelatinase producers among dairy and meat enterococci (Eaton & Gasson, 2001; Franz et al., 2001; Semedo, Santos, Lopes, et al., 2003). This enzyme is considered a virulence factor since the ability to hydrolyze collagens and certain bioactive peptides suggests its participation in the initiation and propagation of inflammatory processes involving *E. faecalis* (Waters, Antiporta, Murray, & Dunny, 2003).

3.1.1.2. Lipase and DNase tests. The capacity to produce lipase and DNase activity were not found in any of the isolates investigated. Although in other food isolates, lipase and DNase producers were previously found, their importance as virulence factors in enterococci seems to be reduced (Semedo, Santos, Lopes, et al., 2003).

3.1.2. Hemolytic activity

In this study, two kinds of blood were used: sheep and human blood (types A and O). The strains used as controls were *E. faecalis* F2 and *E. faecalis* DS16, which harbored the plasmid pAD1. This is a pheromone-responsive plasmid where the cytolysin determinant resides. Cytolysin is a bacterial toxin expressed by organisms that display hemolytic activity (Gilmore et al., 1994). Among the isolates investigated no differences were observed between the two types of human blood used, A and O. This could mean that the hemolytic activity is not dependent on the human blood type (at least, for types A and O). From 182 food isolates, only 1 (0.5%) was β-hemolytic and the other 181 were non-hemolytic (147 (80.8%) γ and 34 (18.7%) α-hemolytic) for human blood. When these results were compared with those for sheep blood, no equivalence was found, since no isolates were β-hemolytic and, from 182 non-hemolytic isolates, 30 (16.5%) were α-hemolytic. Nevertheless, absence of hemolytic activity in enterococci does not necessarily mean that these bacteria are not virulent (Franz et al., 1999). Similarly, Semedo, Santos, Martins, et al. (2003) found different results when using blood from different sources (sheep and horse). To evaluate hemolysin production several researchers used human or even horse blood, instead of sheep blood (Creti et al., 2004; Poeta, Costa, Klibi, Rodrigues, & Torres, 2006). The β-hemolytic strain was determined to be a member of the species *E. faecalis* and similar results were found by Eaton and Gasson (2001).

3.1.3. Biofilm assay

Results showed that enterococci could form biofilms on polystyrene surfaces. The strains were classified as non-biofilm producers ($OD \leqslant 0.080$), weak biofilm producers ($0.080 < OD \leqslant 0.160$), moderate biofilm producers ($0.160 < OD \leqslant 0.320$) and strong biofilm producers ($0.320 > OD$) (Stepanović et al., 2000), for each assay: batch and fed-batch.

According to the results of the Kruskal–Wallis test no significant differences ($P > 0.01$) were observed between independent replicates. However, significant differences in biofilm production were observed between batch and fed-batch modes and between isolates ($P < 0.01$).

Fig. 1 shows the results for both batch and fed-batch assays. For batch mode a low percentage of biofilm producers were detected. Only 28.0% and 3.9% of food isolates were classified as moderate and strong biofilm producers, respectively. According to Cerca et al. (2004), the amount of biofilm produced could be influenced by changes in the availability of nutrients over time. Therefore, the production of biofilm was evaluated also in fed-batch mode. As observed in Fig. 1, the percentage of isolates which were classified as moderate or strong producers was higher when the fed-batch mode was used; 35.7% and 63.2% of isolates were classified as moderate or strong biofilm producers, respectively.

Results concerning biofilm formation by enterococcal food isolates were not found in the literature. However, a few reports were published about biofilm production by *E. faecalis* on root canals (Dunavant, Regan, Glickman, Solomon, & Honeyman, 2006; George, Kishen, & Song, 2005) and, very recently, on orthopedic implant infections (Arciola et al., 2008). No data was found about biofilm production by *E. faecium*. In the present study in batch assay, 13.6% of *E. faecalis* isolates were classified as strong biofilm producers, while only 2.6% were non-biofilm producers. No *E. faecium* isolate were classified as strong biofilm producers and 25% were non-biofilm producers. In fed-batch assay, 76.3% and 43.2% of *E. faecalis* and *E. faecium*, respectively, were strong biofilm producers. Only 1.3% of the *E. faecalis* isolates were classified as weak biofilm producers.

It is generally accepted that organisms in biofilms are more resistant to biocides (such as antibiotics) than in their planktonic form. This is basically due to the development of resistance phenotypes upon attachment to surfaces and within the biofilm (Brown & Gilbert, 1993). In food processing environments, biofilm formation by enterococci, which could carry virulence factors and even resistances to several antibiotics, is a matter of concern. Producers need to clean and sanitize their instruments and materials in order to prevent this adherence and also the product contamination with virulent strains during food processing.

3.2. Virulence genes

To cause infection, enterococci must possess virulence factors which allow the infecting strains to colonize and invade host tissue

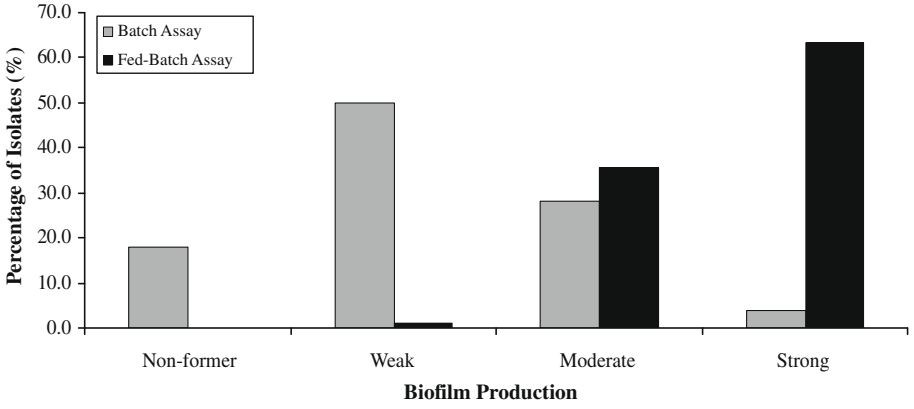

Fig. 1. Classification of enterococci isolates according to their biofilm production capacity in both batch and fed-batch modes.

and translocate through epithelial cells and evade the host's immune response (Jonhson, 1994). The occurrence of the surface adhesin genes (*efaAfs*, *efaAfm* and *esp*), the aggregation protein gene (*agg*), the cytolysin genes (*cylM*, *cylB*, *cylA*, *cylL$_L$* *cylL$_s$*) and extracellular metallo-endopeptidase gene (*gelE*) by species among the isolates, is presented in Table 1.

The *efaAfs* or *efaAfm* genes were only found in *E. faecalis* and *E. faecium* isolates, respectively, by Martín et al. (2006). However, Semedo, Santos, Lopes, et al. (2003) detected negative amplifications for the gene *efaAfs* in *E. faecalis* strains and also found *E. faecalis* and other species with gene *efaAfm*. In the present study, the gene *efaAfs* was detected in 45% of the isolates. All *E. faecalis* amplified the 705 bp fragment of the cell wall adhesin *efaAfs*, but it was also amplified by 1 (2.2%) *E. faecium* isolate and 5 (8.1%) enterococci isolates of other species. In addition, the gene *efaAfm* was detected in 27% of isolates. Just one *E. faecium* isolate did not amplify the 735 bp fragment of the cell wall adhesin gene *efaAfm* while 2 (2.6%) *E. faecalis* isolates and 4 (6.5%) *Enterococcus* spp. isolates amplified this fragment (Table 1). Production of the adhesin-like *E. faecalis* and *E. faecium* endocarditis antigens (EfaAfs and EfaAfm, respectively) are considered to be potential virulence determinants and expression of the EfaAfs was previously identified by Lowe, Lambert, and Smith (1995) using serum from a patient with *E. faecalis* endocarditis. The role of *efaAfm* is not yet known.

The *esp* gene was detected in only 3 (16.5%) *E. faecalis* isolates. Coque, Patterson, Steckelberg, and Murray (1995) and Shankar, Baghdayan, Huycke, Lindahl, and Gilmore (1999) found the *esp* gene exclusively in *E. faecalis* strains. The production of Esp is proposed to promote the primary attachment to biotic and abiotic surfaces and to be involved in hiding the protein from the immune system (Toledo-Arana et al., 2001).

In the present study, the *agg* gene was detected in 53 (69.7%) *E. faecalis* isolates and also in 2 other isolates (1 *E. faecium* and 1 *Enterococcus* spp.). The gene *agg* has been described only in *E. faecalis* strains by several researchers (Eaton & Gasson, 2001; Franz et al., 2001; Mannu et al., 2003; Martín et al., 2006). However, Semedo, Santos, Lopes, et al. (2003), in agreement with this study, detected this gene in food and clinical isolates, belonging to species other than *E. faecalis*. Agg is a surface protein which is located on pheromone-responsive *E. faecalis* plasmids. It is produced in response to pheromones secreted by potential recipient *E. faecalis* cells and causes the aggregation of donor and recipient cells, thus facilitating the transfer of plasmids that may carry virulence traits and antibiotic resistance genes (Dunny, 1990). Agg may also increase enterococcal adherence to intestinal and renal epithelial cells (Kreft, Marre, Schramm, & Wirth, 1992).

The *cyl* genes were detected in 1.6% of isolates. Eaton and Gasson (2001) described a higher percentage of *cyl* genes among clinical strains compared with food strains. All isolates, which carried *cyl* genes, carried the five *cyl* genes and were from the same specie, *E. faecalis*. Poeta et al. (2006) observed a low number of isolates presenting the five *cyl* genes and in most of the previous works some isolates with one to four cytolysin genes were found (Eaton & Gasson, 2001; Poeta et al., 2006; Semedo, Santos, Martins, et al., 2003). Production of cytolysin appears to be a major risk factor associated with pathogenic enterococci: a fivefold increased risk of death of patients within 3 weeks of bacteraemia caused by β-hemolytic enterococci, compared with bacteraemia caused by non-β-hemolytic strains was reported by Huycke, Spiegel, and Gilmore (1991).

The gene for gelatinase (*gelE*) is located in an operon together with a gene (*fsr*) encoding a serine protease (Qin, Singh, Weinstock, & Murray, 2000). Roberts, Singh, Okhuysen, and Murray (2004) studied *E. faecalis* isolates from human infections and showed that neither *fsr* nor *gelE* production were probably required to cause infection. However, these findings obviously did not indicate whether *fsr* or *gelE* affect the severity of the disease (Roberts et al., 2004). In the present study, only the presence of *gelE* gene was investigated. The 419 bp fragment of *gelE* gene was amplified for 29.7% of isolates (51 *E faecalis*, 1 *E. faecium* and 2 *Enterococcus* spp.). Lopes et al. (2005) reported the detection of *gelE* in species other than *E. faecalis* or *E. faecium*.

In this study *E. faecalis* and *E. faecium* showed significant differences in the incidence of the virulence determinants investigated. All the *E. faecalis* possessed virulence determinants (1, 2, 3, 4, 8 and 9 virulence determinants were found in 10, 26, 35, 2, 2 and 1 isolates, respectively). Apart from 2 isolates, showing 2 and 3 virulence genes each, and with the exception of *efaAfm*, no virulence determinants were detected in *E. faecium* isolates.

The more frequent genotype was *efaAfs*$^+$ *gelE*$^+$ *agg*$^+$ (41.5%). It is relevant to note that one isolate which carried all the virulence genes, was also β-hemolytic, when tested for hemolysis in human blood. The interaction of such a strain with susceptible hosts (*e.g.* immunocompromised patients, pregnant women) is certainly a cause of concern.

3.3. Comparison between phenotypic and molecular screening

Both phenotypic and genotypic assays are important to evaluate the virulence potential of enterococci. So, the comparison between the genetic determinants found and their expression was studied.

3.3.1. Presence of gelE gene and gelatinase production

Although similar percentages were observed for isolates which carry *gelE* gene (29.7%) and were gelatinase producers (26.4%), it is important to highlight that only 23% of the isolates were positive for both genotypic and phenotypic characters. Phenotypic testing revealed apparent "silent" genes when the presence of the determinant using PCR was observed but was not phenotypically expressed. This has been previously reported (Creti et al., 2004; Eaton & Gasson, 2001). It is also important to note that 6 (3%) isolates were gelatinase producers, but nevertheless did not show *gelE* gene.

3.3.2. Cytolysin production and β-hemolytic behavior

For the isolate that showed hemolytic activity the whole operon, *cylL$_L$L$_S$MBA*$^+$, was detected. Cytolysin, as expressed by various isolates of *E. faecalis*, is most frequently a plasmid-encoded toxin, but it may also be chromosomally encoded (Ike & Clewell, 1992). As mentioned before, Gilmore et al. (1994) showed that production

Table 1
Occurrence of virulence determinants among enterococcal isolates.

	N[a]	Virulence determinants									
		efaAfs	efaAfm	esp	agg	cylM	cylB	cylA	cylL$_L$	cylL$_S$	gelE
E. faecalis	76	76 (100.0)	2 (2.6)	3 (3.9)	53 (69.7)	3 (3.9)	3 (3.9)	3 (3.9)	3 (3.9)	3 (3.9)	51 (67.1)
E. faecium	44	1 (2.2)	43 (97.7)	0 (0.0)	1 (2.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.2)
Enterococcus spp.	62	5 (8.1)	4 (6.5)	0 (0.0)	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.2)

[a] Number of tested isolates; values in parenthesis indicate percentage.

Table 2
Comparison between the presence of the genes of the *cyl* operon and the detection of hemolysin in enterococci.

Enterococci (no.)	Presence of genes of the *cyl* operon	Number of isolates	Type of hemolysis detected in isolates with different *cyl* operon genotype		
			β-Hemolysis	α-Hemolysis	γ-Hemolysis
E. faecalis (76)	$cylL_LL_SMBA^+$	3	1	–	2
	cyl genes undetected	73	–	1	72

Table 3
Comparison of biofilm production with $gelE^+$ and esp^+ genes in batch and fed-batch assays.

	Batch assay	Fed-batch assay
Non former (n)	33	0
$gelE^+$	2 (6.1)	0 (0.0)
esp^+	0 (0.0)	0 (0.0)
Weak (n)	91	2
$gelE^+$	31 (34.1)	1 (50.0)
esp^+	3 (3.3)	0 (0.0)
Moderate (n)	51	65
$gelE^+$	20 (39.2)	11 (16.9)
esp^+	0 0.0)	1 (1.5)
Strong (n)	7	115
$gelE^+$	1 (14.3)	42 (36.5)
esp^+	0 (0.0)	2 (1.7)

Values in parenthesis indicate percentage.

of cytolytic activity by *E. faecalis* requires expression of five reading frames: *cylM*, *cylB*, *cylA*, $cylL_L$ and $cylL_s$.

For two non-hemolytic isolates the whole operon $cylL_LL_SMBA^+$ was also detected (Table 2). Eaton and Gasson (2001) also found two *E. faecalis* strains, which appeared to have no hemolytic activity, but carried *cyl* genes, and reported them as apparent "silent" genes.

Comparing the presence of hemolytic activity with other virulence genes, it is interesting to note that for isolates that showed α-hemolysis, only one virulence gene was present, in comparison with these that showed β-hemolysis, which had five virulence genes (besides *cyl* genes).

3.3.3. Biofilm production and correlation with gelE and esp genes

In Table 3, a low percentage of isolates which carried the *gelE* and *esp* genes was evident, although they were moderate, and strong biofilm producers, for both batch and fed-batch assays. In batch assay, although 34.1% and 6.1% of $gelE^+$ isolates were weak and non-biofilm producers, respectively, 50.0% of isolates, $gelE^+$ and esp^+, were non-biofilm producers. It is important to stress that the majority of isolates carrying the $gelE^+$ and esp^+ genes were *E. faecalis.* Only one *E. faecium* and two *Enterococcus* spp. were $gelE^+$. Although some reports revealed that biofilm production by *E. faecalis* requires the presence of certain genes, such as *gelE* (Hancock & Perego, 2004) and *esp* (Toledo-Arana et al., 2001), other authors have shown that $gelE^+$ and esp^+ genes were not required for biofilm formation by several *E. faecalis* isolates (Mohamed & Murray, 2005; Tendolkar, Baghdayan, Gilmore, & Shankar, 2004).

Although more studies are required to generate more information about those *Enterococcus* spp. isolates, phenotypic and genotypic evidence of potential virulence factors were identified and, based on these results, we cannot exclude the possibility of some food isolates being responsible for enterococcal infections, particularly among high-risk consumers.

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