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Incidence of Virulence Factors and Antibiotic Resistance among Enterococci Isolated from Food

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The incidence of virulence factors among 48 *Enterococcus faecium* and 47 *Enterococcus faecalis* strains from foods and their antibiotic susceptibility were investigated. No strain was resistant to all antibiotics, and for some strains, multiple resistances were observed. Of *E. faecium* strains, 10.4% were positive for one or more virulence determinants, compared to 78.7% of *E. faecalis* strains. Strains exhibiting virulence traits were not necessarily positive for all traits; thus, the incidence of virulence factors may be considered to be strain specific.

Enterococci constitute a major component of the microflora of artisanal cheeses produced in southern Europe (10) and are considered to play an important role in ripening and aroma development (1, 5, 28). This has led to the suggestion that enterococci be included in starter culture preparations for the manufacture of certain Mediterranean cheeses (1, 5, 23). However, enterococci are also major nosocomial pathogens causing a variety of infections (19, 20). *Enterococcus faecalis* strains clearly dominate among enterococci isolated from human infections, while *Enterococcus faecium* strains are associated with the majority of the remainder (15).

A specific cause for concern and contributing factor to pathogenesis of enterococci is their resistance to a wide variety of antibiotics (18, 20). However, antibiotic resistance as such cannot explain the virulence of enterococci. Although enterococci possess subtle virulence traits (25), considerable progress has recently been made in determining these. For example, studies have shown that phenotypes such as β-hemolysin/bacteriocin (also called cytolysin) and aggregation substance (AS), which are encoded by *E. faecalis* pheromone-responsive plasmids, are related to pathogenicity and enhance the virulence of enterococci in animal models (2, 12, 14, 15, 26). AS is an adhesin which mediates the formation of cell clumps that allow the highly efficient transfer of the sex pheromone plasmid on which AS is encoded (4). An interesting variation in the generally similar AS (about 90% homology) encoded by sex pheromone plasmids such as pAD1, pCF10, and pPD1 is encoded by *asa373* on plasmid pAM373. This AS is also involved in a clumping response, but it has little homology to the "classical" AS and therefore represents a rather unique type of adhesin (22). Other virulence factors include the adhesin called enterococcal surface protein (Esp) and gelatinase (Gel), which is an extracellular metalloendopeptidase (26).

This study aimed to determine the incidence of hemolysin; classical AS; and the aggregation substances Asa373, Gel, and Esp and antibiotic susceptibility among enterococci isolated mostly from cheeses. Studies formed part of the European Union (EU) project FAIR-CT97–3078, "Enterococci in Food Fermentations: Functional and Safety Aspects." As *E. faecalis* and *E. faecium* are predominantly associated with human infection and as these species were also predominant among strains isolated from cheese in the EU study, our study was concerned only with strains of these two species.

Enterococci were obtained from six partners associated with the EU project and were identified to species level. In this study, safety aspects were studied for 48 *E. faecium* and 47 *E. faecalis* strains that were preselected as possible starter cultures for cheese manufacture on the basis of functional properties, such as proteolytic and lipolytic activities, and production of volatile compounds. Information on these strains is available in the catalogue of enterococci of the FAIR-E collection (29). Two of the *E. faecium* strains used in this study (E 24 and E 25) are commercial probiotic strains. All enterococci were grown in MRS broth (Merck, Darmstadt, Germany) or Todd-Hewitt broth (THB; Difco, Heidelberg, Germany) at 37°C. *Escherichia coli* strains were grown on a rotary shaker at 250 rpm in Luria-Bertani broth at 37°C with ampicillin (150 μg/ml) or kanamycin (50 μg/ml) added where appropriate.

Production of Gel was tested on Todd-Hewitt agar containing 30 g of gelatin/liter, as described by Coque et al. (6). Production of β-hemolysin was indicated by the formation of clear zones surrounding the colonies on blood agar plates. Blood agar plates were prepared using Columbia blood agar base (Merck) with 5% defibrinated human blood (containing all four blood types). Production of AS by enterococci was studied in the clumping assay in the presence of sex pheromone. Sex pheromone was obtained by growing the pheromone producer *E. faecalis* JH2-2 (Table 1) in THB at 37°C for 18 h. Pheromone-containing supernatant obtained after centrifugation at 10,823 × *g* was sterilized by autoclaving for 15 min, diluted 1:5 in sterile THB, and used in clumping assays in microtiter plates. Two-hundred-microliter volumes were inoculated (0.5%) with the test *Enterococcus* strain, incubated at 37°C, and visually examined for cell clumping after 2, 4, 8, and

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TABLE 1. Bacterial reference strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic	Reference
Bacterial strains		
E. faecalis JH2-2	Rif^r, Fus^r, *gel*	21
E. faecalis OG1X	Plasmid free, Str^r, *gel*	22
E. faecalis OG1X:pAD1	Carries sex pheromone plasmid pAD1 (60 kb)	9
E. faecalis OG1X:pAM373	Carries sex pheromone plasmid pAM373 (37 kb)	22
E. faecalis MMH594	*esp*$^+$	25
Plasmids		
pQESD3.7	*asa1*$^+$	22
pOK12asa373	*asa373*$^+$	21

24 h. The plasmidless strain, *E. faecalis* OG1X, was used as a negative control, while two variants of *E. faecalis* OG1X containing either plasmid pAD1 or pAM373 (9, 22) were used as positive controls (Table 1). In order to account for constitutive clumping in the absence of AS, all enterococci were also tested in THB without sex pheromone. Strains that were positive in the clumping assay or that clumped constitutively were tested for the AS genes *asa1* and *asa373* using gene probes. Total genomic DNA was isolated according to the methods of Pitcher et al. (24), cut with the restriction enzymes *Hin*dIII and *Eco*RI, subjected to electrophoresis on 0.7% agarose gels, and transferred onto nylon membranes (Hybond N+; Amersham Pharmacia Biotech, Freiburg, Germany). Plasmid pQESD3.7 containing the *asa1* gene (21) and plasmid pOK12asa373 containing the *asa373* gene (22) were cut with *Pst*I and *Bsr*GI/*Hpa*II restriction enzymes, respectively. The fragments corresponding to the AS genes were eluted from the gel, labeled, and used as probes together with the enhanced chemiluminescence direct nucleic acid labeling and detection kit (Amersham Pharmacia), according to the manufacturer's instructions.

PCR amplification was used to detect the enterococcal surface protein gene (*esp*). Primers *esp11* and *esp12* were used to amplify the part of the gene encoding the N-terminal region of Esp according to the methods of Shankar et al. (25). DNA from *E. faecalis* strain MMH594 (Table 1) was used in PCR experiments as a positive control.

E-test (Viva Diagnostika, Cologne, Germany) antibiotic strips were used to determine the susceptibility of the strains to ampicillin, benzylpenicillin, chloramphenicol, tetracycline, erythromycin, ciprofloxacin, streptomycin (high range), gentamicin (high range), and vancomycin (Viva Diagnostika). Commercial Mueller–Hinton II agar plates (Becton Dickinson, Heidelberg, Germany) were seeded with enterococci, and antibiotic strips were placed onto the plates according to the manufacturer's instructions. Plates were incubated at 37°C, and results were read after 24 and 48 h of incubation. The MIC determination was based on the reference agar dilution method that has been previously described (22a).

None of the *E. faecium* strains and 23 (48.9%) of the *E. faecalis* strains produced Gel. Kühnen et al. (17) reported a high (63.7%) incidence of Gel production among clinical enterococci. Similarly, Singh et al. (26) reported Gel production by *E. faecalis* for 54% of endocarditis isolates, 58% of nosocomial clinical isolates, and 62% of nosocomial fecal isolates. The incidence of Gel production among food *E. faecalis* strains in our study seems to be slightly lower than that reported for clinical strains by Kühnen et al. (17) and Singh et al. (26). This value was also only slightly lower than the 56% incidence among nine *E. faecalis* strains isolated from food reported by Eaton and Gasson (8). The high incidence of Gel activity among enterococci in this study may be explained by the fact that most enterococci originated from cheese, a protein-rich source. Thus, production of protease may be a selection mechanism for enterococci growing in cheese, as it may enable them to utilize cheese protein as a source for amino acids. However, the incidence of Gel activity of the enterococci in this study may be expected to be even higher, as Eaton and Gasson (8) showed that Gel genes may be silent and that the phenotype may be negative, even though a Gel gene is present. Similar to the results of Eaton and Gasson (8), none of the 48 *E. faecium* strains in this study showed Gel activity, suggesting that Gel production by this species is not common.

Among both *E. faecium* and *E. faecalis,* some strains produced β-hemolysin (Table 2) with the incidence of this trait being higher for *E. faecalis* strains (21.3%), than for *E. faecium* strains (8.3%). Hemolysin plays an important role in enterococcal virulence, as it may increase the severity of the infection (11, 15). Ike et al. (13) showed that 60% of clinical *E. faecalis* isolates were hemolytic compared to 17% of strains from uninfected sources. The incidence of β-hemolysin in our study was much lower than that reported by Ike et al. (13). In addition, this value was much lower than the 44% incidence for *E. faecalis* strains from foods reported by Eaton and Gasson (8). Interestingly, the hemolysin trait appeared to be linked with AS production (presence of the *asa1*-related gene) for 7 of the 10 Hly$^+$ *E. faecalis* isolates in our study. Production of hemolysin and AS is known to be often (85% of hemolytic strains) linked on pheromone-responsive plasmids. However, some hemolytic strains did not exhibit a clumping response, and their hemolysin genes probably reside on the chromosome (13). Four *E. faecium* strains in this study also showed beta-hemolytic activity, but a clumping response was not detected. In contrast, the *E. faecium* strains from food studied by Eaton and Gasson (8) did not show cytolysin activity. Thus, our results suggest that both *E. faecalis* and *E. faecium* strains isolated from foods may produce β-hemolysin and that this trait is not exclusive to clinical isolates.

Only one (2.1%) of the *E. faecium* strains but 17 (36.2%) of the *E. faecalis* strains produced Esp (Table 2). Shankar et al. (25) showed that 29% of their blood isolates and 42% of endocarditis *E. faecalis* isolates were positive for the *esp* gene, while only 3% of isolates from stool showed this trait. The reason for the high incidence of Esp production among *E. faecalis* strains in this study is not known. Our result correlated

TABLE 2. Incidence of virulence factors among *E. faecalis* and *E. faecium* strains isolated from foods

Virulence factor	*E. faecalis* FAIR-E[a] data		*E. faecium* FAIR-E[a] data	
	Strains used (n = 47)	Incidence (%)	Strains used (n = 48)	Incidence (%)
None of the tested virulence factors	E 71, E 82, E 278, E 279, E 292, E 307, E 315, E 348, E 351, E 385	21.3	E 3, E 6, E 9, E 13, E 14, E 15, E 20, E 24, E 25, E 26, E 34, E 50, E 80, E 83, E 84, E 150t1, E 151, E 154, E 160t1, E 170, E 171, E 198, E 207, E 210, E 212, E 215, E 217, E 218, E 225, E 227, E 243, E 254, E 263, E 266, E 284, E 338, E 345, E 349, E 362, E 365, E 366, E 371, E 383	89.6
Gel	E 35, E 63, E 74, E 88, E 176, E 224, E 235, E 237, E 238, E 255, E 256, E 259, E 260, E 265, E 281, E 298, E 299, E 313, E 324, E 325, E 329, E 378, E 377	48.9	None of strains tested	0
Hemolysin	E 74, E 177, E 226, E 237, E 238, E 321, E 324, E 325, E 329, E 372	21.3	E 196, E 201, E 202, E 206	8.3
Clumping phenotype	E 69, E 74, E 77, E 88, E 224, E 229, E 235, E 236, E 237[b], E 238[b], E 259, E 260[b], E 281, E 302, E 313, E 315[b], E 321, E 324, E 329, E 337, E 342[b], E 363, E 372[b], E 404	51.1	None of strains tested	0
asa1-related gene	E 69, E 74, E 77, E 88, E 224, E 229, E 235, E 236, E 237, E 238, E 259, E 260, E 281, E 302, E 313, E 321, E 324, E 329, E 337, E 342, E 363, E 372, E 404	48.9	ND[c]	ND
asa373-related gene	E 69, E 74, E 77, E 88, E 224, E 235, E 236, E 237, E 313, E 321, E 324, E 329, E 342, E 329, E 342, E 363, E 404	31.9	ND	ND
esp	E 63, E 71, E 74, E 85, E 88, E 224, E 229, E 236, E 237, E 238, E 281, E 321, E 324, E 325, E 329, E 339, E 372	36.2	E 280	2.1
Hemolysin and AS[d]	E 74, E 237, E 238, E 321, E 324, E 329, E 372	14.9	None of strains tested	0
Hemolysin, AS, and *esp*	E 74, E 237, E 238, E 321, E 324, E 329, E 372	14.9	None of strains tested	0

[a] As deposited in the FAIR-E collection kept at BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University of Ghent.
[b] Constitutive clumping phenotype, i.e., clumping phenotype observed also in the absence of sex pheromone.
[c] ND, not determined, as clumping phenotype was negative.
[d] AS, presence of AS, i.e., strains that exhibited the clumping phenotype and showed presence of the *asa1*-related or *asa373*-related gene.

well with that of Eaton and Gasson (8), who showed an incidence of 33% for Esp production among *E. faecalis* isolates from food. Similar to the results of Eaton and Gasson (8), who showed that none of the *E. faecium* strains from food produced Esp, a low incidence among *E. faecium* strains was also found in our study. In addition to its role in adhesion, Esp is also thought to play a role in evasion of the immune response (25). Thus, enterococci possessing this trait would clearly be undesirable for use in foods as a starter culture.

None of the *E. faecium* strains showed a clumping phenotype in the clumping assay, while 18 (38.3%) *E. faecalis* strains exhibited a clumping reaction (Table 2). Because an additional six *E. faecalis* strains clumped constitutively, i.e., also in the absence of pheromone, it was unclear whether clumping was as a result of AS. By probing for the *asa1* and *asa373* genes, it was established that, of the six strains clumping constitutively, five (E 237, E 238, E 260, E 342, and E 372) were positive for the AS gene(s) (Table 2). Thus, 23 (48.9%) and 15 (31.9%) of the *E. faecalis* strains in total were positive for the *asa1* and *asa373* genes, respectively (Table 2). Interestingly, while some strains were positive for only the *asa1* gene, none of the *E. faecalis* strains was positive only for the *asa373* gene, which always occurred when the *asa1* gene was present.

AS is an important virulence factor of enterococci, and over one-third of clinical isolates of *E. faecalis* are known to carry pheromone response plasmids (3). In our study, production of AS was common among *E. faecalis* strains isolated from foods. In contrast, *E. faecium* strains did not appear to produce AS, which was not surprising, as pheromone response plasmids are generally associated with *E. faecalis* (7). Eaton and Gasson (8) showed that 67% of the *E. faecalis* strains isolated from food produced *asa1*-related AS, while none of the *E. faecium* strains were positive for such AS genes. The much higher incidence reported by Eaton and Gasson (8) may be explained by the lower number (nine *E. faecalis* strains isolated from food) which they investigated.

Ten (20.8%) *E. faecium* strains and six (12.8%) *E. faecalis* strains were susceptible to all antibiotics tested. All *E. faecalis* strains and all but one *E. faecium* strain were susceptible to vancomycin (Table 3). The *E. faecium* strains were mostly resistant to ciprofloxacin (56.3%), followed by penicillin (45.8%), erythromycin (27.1%), chloramphenicol (10.4%), tetracycline (6.3%), streptomycin (4.2%), gentamicin (2.1%), and vancomycin (2.1%). None were resistant to ampicillin (Table 3). In contrast, the *E. faecalis* strains were mostly resistant to chloramphenicol (63.8%), followed by streptomycin (46.8%), tetracycline (44.7%), erythromycin (31.9%), ciprofloxacin (27.7%), gentamicin (25.5%), penicillin (12.8%), and ampicil-

TABLE 3. Resistance[a] of *E. faecalis* and *E. faecium* strains isolated from food to selected antibiotics

Antibiotic	Data for resistant *E. faecalis* FAIR-E strains		Data for resistant *E. faecium* FAIR-E strains	
	Strains used (n = 47)	Incidence (%)	Strains used (n = 48)	Incidence (%)
Ampicillin	E 235	2.1	None of strains	0
Penicillin	E 71, E 85, E 226, E 229, E 260, E 278	12.8	E 9, E 15, E 20, E 25, E 34, E 50, E 151, E 154, E 160t1, E 170, E 171, E 196, E 198, E 201, E 207, E 210, E 217, E 263, E 266, E 280, E 365, E 371	45.8
Chloramphenicol	E 63, E 71, E 74, E 85, E 88, E 177, E 224, E 226, E 229, E 235, E 236, E 237, E 238, E 256, E 259, E 260, E 265, E 279, E 281, E 298, E 299, E 307, E 324, E 329, E 337, E 348, E 351, E 372, E 378, E 404	63.8	E 25, E 151, E 207, E 280, E 362	10.4
Tetracycline	E 63, E 74, E 85, E 88, E 176, E 224, E 229, E 235, E 236, E 237, E 238, E 260, E 265, E 315, E 321, E 324, E 325, E 329, E 348, E 372, E 378	44.7	E 25, E 84, E 225	6.3
Erythromycin	E 63, E 69, E 77, E 85, E 88, E 224, E 229, E 238, E 260, E 265, E 302, E 307, E 321, E 329, E 348	31.9	E 3, E 25, E 34, E 84, E 151, E 170, E 198, E 201, E 202, E 210, E 345, E 349, E 383	27.1
Ciprofloxacin	E 85, E 229, E 238, E 255, E 256, E 259, E 260, E 265, E 298, E 307, E324, E 378, E 404	27.7	E 3, E 6, E 13, E 14, E 25, E 34, E 84, E 151, E 154, E 160t1, E 170, E 171, E 169, E 198, E 201, E 202, E 206, E 207, E 212, E 217, E 225, E 243, E 254, E 345, E 362, E 366, E 383	56.3
Streptomycin	E 63, E 85, E 88, E 224, E 229, E 235, E 236, E 237, E 238, E 259, E 265, E 279, E 292, E 298, E 321, E 324, E 325, E 329, E 348, E 372, E 378, E 404	46.8	E 84, E 225	4.2
Gentamicin	E 71, E 85, E 88, E 226, E 259, E 260, E 265, E 321, E 329, E 363, E 372, E 378	25.5	E 160t1	2.1
Vancomycin	None	0	E 84	2.1

[a] Resistance interpreted on the basis of MICs for enterococci that are supplied elsewhere (22a). MICs: ampicillin, ≥16 μg/ml; penicillin, ≥16 μg/ml; chloramphenicol, ≥32 μg/ml; tetracycline, ≥16 μg/ml; erythromycin, ≥8 μg/ml; ciprofloxacin, ≥4 μg/ml; high-level streptomycin, > 1,000 μg/ml; high-level gentamicin, >500 μg/ml; and vancomycin, ≥32 μg/ml.

lin (2.1%), while none were resistant to vancomycin (Table 3). No strains tested were resistant to all the antibiotics used in this study, while multiple resistance to five or more antibiotics was observed (Table 3).

Our results showed that a larger number of *E. faecium* strains than *E. faecalis* strains were resistant to penicillin, which may be explained by *E. faecium* being generally more resistant to penicillin than *E. faecalis* (20). The incidence of ampicillin and vancomycin resistance for both *E. faecium* and *E. faecalis* was low, indicating that most of the strains tested did not acquire resistance determinants for these antibiotics. In a study of European cheeses, Teuber et al. (27) also reported a low (4%) incidence of vancomycin-resistant enterococci. While the incidence of aminoglycoside-resistant enterococci was low among *E. faecium* isolates, it was considerably higher for *E. faecalis*. These results indicate that especially *E. faecalis* strains with acquired, high-level aminoglycoside resistance can occur in traditional cheeses. Teuber et al. (27) reported a much higher incidence of gentamicin-resistant enterococci from cheeses, as 80% of isolates were resistant to this compound. The incidence of tetracycline and erythromycin resistance among *E. faecalis* strains was relatively high at 44.7 and 31.9% of the strains, respectively, and this incidence was greater than for *E. faecium* strains. Similarly high incidences of erythromycin and tetracycline resistance among cheese enterococcal isolates were described by Teuber et al. (27). This may be explained by the fact that erythromycin resistance plasmids and transposons are commonly found among enterococci (20). The incidence of chloramphenicol resistance was higher for *E. faecalis* strains (63.8%) than for *E. faecium* strains (10.4%) in this study, and a high (32%) incidence of chloramphenicol resistance among cheese enterococcal isolates was also reported by Teuber et al. (27).

It is difficult to assess the impact of antibiotic-resistant enterococci from foods on potential human pathogenicity. It is clear that in the hospital environment, antibiotics may influence selection of pathogenic enterococci, which may lead to infections or superinfections (20). Most problematic are strains that have acquired multiple antibiotic resistance, especially resistance to vancomycin and to the synergistic action of β-lactams and aminoglycosides (20), which leaves few therapeutic options. Although the enterococci in this study showed acquired resistance traits to a number of antibiotics, they did generally not show resistance to the clinically relevant antibi-

otic ampicillin or vancomycin, and a low incidence of resistance towards gentamicin was observed especially among the *E. faecium* strains. These results indicated that these food enterococcal strains were mostly still susceptible to clinically relevant antibiotics.

Antibiotic resistance alone cannot explain the virulence of enterococci. In order to become pathogenic, they need to express virulence traits associated with adhesion, translocation, and evasion of immune responses and cause pathological changes (16). Our results have shown that virulence factors such as hemolysin, AS, Gel, and Esp also occur among food enterococcal isolates. In general, the incidence of these virulence traits was lower among *E. faecium* strains than among *E. faecalis* strains and *E. faecium* harbored fewer virulence traits than *E. faecalis*. These results correlate well with those of Eaton and Gasson (8). The reason for this may be that the AS and hemolysin virulence factors, for example, are located on pheromone response plasmids, which generally occur in *E. faecalis* strains only. Only a few (10.4%) *E. faecium* strains but a higher number (78.8%) of *E. faecalis* strains in our study were positive for one of the virulence factors tested. However, enterococci exhibiting virulence traits were not necessarily positive for all traits tested. Thus, it appears that the incidence of such virulence factors is strain specific. Should *Enterococcus* strains be selected as starter cultures, for safety considerations each strain should be tested for the different virulence traits as well as for antibiotic resistance. Yet, the question whether enterococci are safe for use as starter cultures remains difficult to answer. Eaton and Gasson (8) showed that the incidence of virulence factors was highest among clinical enterococcal isolates, followed in decreasing order by food strains and starter strains, suggesting that the food and starter strains have a lower potential for pathogenicity. Clearly, based on the lower incidence of virulence traits among *E. faecium* strains in this study and that by Eaton and Gasson (8), it would be advisable that this species would be used rather than *E. faecalis*. One problem that remains, however, is the possibility of transfer of virulence determinants from a strain which harbors these to a starter strain. Eaton and Gasson (8) showed that such transfer is a possibility, especially when pheromone-responsive plasmids are involved. However, these authors were also unable to transfer a plasmid containing virulence determinants into a food *E. faecium* strain by filter mating (8). The possibility of gene transfer under in vitro and in vivo conditions clearly requires further investigation. Although much progress has been made in determinations of enterococcal virulence factors, some virulence traits may presently remain undiscovered.

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