ELSEVIER

International Journal of Food Microbiology 47 (1999) 1–24

International Journal of Food Microbiology

Review

Enterococci at the crossroads of food safety?

Charles M.A.P. Franz[a], Wilhelm H. Holzapfel[b,*], Michael E. Stiles[a]

[a]*Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, T6G 2P5 Canada*
[b]*Institute of Hygiene and Toxicology, BFE, Haid-und-Neustr. 9, D-76131 Karlsruhe, Germany*

Received 23 June 1998; received in revised form 5 January 1999; accepted 30 January 1999

Abstract

Enterococci are gram-positive bacteria and fit within the general definition of lactic acid bacteria. Modern classification techniques resulted in the transfer of some members of the genus *Streptococcus*, notably some of the Lancefield's group D streptococci, to the new genus *Enterococcus*. Enterococci can be used as indicators of faecal contamination. They have been implicated in outbreaks of foodborne illness, and they have been ascribed a beneficial or detrimental role in foods. In processed meats, enterococci may survive heat processing and cause spoilage, though in certain cheeses the growth of enterococci contributes to ripening and development of product flavour. Some enterococci of food origin produce bacteriocins that exert anti-*Listeria* activity. Enterococci are used as probiotics to improve the microbial balance of the intestine, or as a treatment for gastroenteritis in humans and animals. On the other hand, enterococci have become recognised as serious nosocomial pathogens causing bacteraemia, endocarditis, urinary tract and other infections. This is in part explained by the resistance of some of these bacteria to most antibiotics that are currently in use. Resistance is acquired by gene transfer systems, such as conjugative or nonconjugative plasmids or transposons. Virulence of enterococci is not well understood but adhesins, haemolysin, hyaluronidase, aggregation substance and gelatinase are putative virulence factors. It appears that foods could be a source of vancomycin-resistant enterococci. This review addresses the issue of the health risk of foods containing enterococci.

Keywords: *Enterococcus*; Foods; Taxonomy; Sources; Bacteriocins; Probiotics; Antibiotic Resistance; Gene transfer mechanisms; Virulence factors; Colonisation; Pathogenicity

1. Introduction

The enterococci as a group were first described by Thiercelin (1899), and the genus *Enterococcus* was proposed by Thiercelin and Jouhaud (1903) for gram-positive diplococci of intestinal origin. Andrewes and Horder (1906) classified potentially pathogenic bacteria from a patient with endocarditis as *Streptococcus faecalis*. Because of their close resemblance with strains isolated from the human intestine the species epithet 'faecalis' was suggested. Lancefield (1933) developed a serological typing system for streptococci in which those of 'faecal

*Corresponding author. Tel.: +49-721-6625-450; fax: +49-721-6625-111.
E-mail address: wilhelm.holzapfel@bfe.uni-karlsruhe.de (W.H. Holzapfel)

0168-1605/99/$ – see front matter
PII: S0168-1605(99)00007-0

origin' possessed the group D antigen. This correlated with the grouping of Sherman (1937) who proposed a new classification scheme for the genus *Streptococcus* that separated it into four divisions designated: pyogenic, viridans, lactic and enterococcus. The enterococcus group included *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus bovis* and *Streptococcus equinus* as the enterococcal or group D strains. '*Streptococcus durans*' assumed various levels of acceptance, either as a separate species or as a subspecies of *S. faecium* within this group. It was regarded important in food microbiology because it was considered to be of non-faecal origin. Strains of *S. durans* could be differentiated from *S. faecalis* and *S. faecium* by carbohydrate fermentation tests.

The classical taxonomy of the enterococci is vague because there are no phenotypic characteristics that unequivocally distinguish them from other gram-positive, catalase-negative, coccus-shaped bacteria (Devriese et al., 1993). The majority of *Enterococcus* species, however, can be distinguished from other gram-positive, catalase-negative cocci by their ability to grow at 10 and 45°C, in 6.5% sodium chloride, at pH 9.6 and to survive heating at 60°C for 30 min (Hardie and Whiley, 1997; Morrison et al., 1997). Not all *Enterococcus* species possess the group D antigen. The current *Streptococcus* spp., *S. bovis*, *S. suis* and *S. alactolyticus*, as well as pediococci and certain *Leuconostoc* strains also react with Lancefield's group D antiserum. Some strains of lactococci, pediococci, aerococci and leuconostocs grow in the presence of 6.5% sodium chloride, but *E. cecorum*, *E. columbae* and *E. avium* do not (Devriese et al., 1993). Pediococci and some lactococci grow at 45°C, while most lactococci, leuconostocs and some streptococci grow at 10°C, but *E. avium* generally does not (Murray, 1990; Devriese et al., 1993). Moreover, the phylogenetically distinct species or 'species groups' differ to some extent in their cell wall chemistry, physiology, growth and biochemical activity (see Devriese et al., 1993 for review).

Correct species identification is of great importance to both medical and food microbiologists. For example, a clinical isolate would need to be correctly identified for appropriate antibiotic treatment, because susceptibility patterns differ considerably between species (Murray, 1990; Ruoff, 1990; Morrison et al., 1997). Correct species identification is useful for epidemiologic surveillance in hospitals (Murray, 1990). For the food microbiologist, correct identification may be important for selecting a starter strain and labeling of the product to which the starter is added. With the development of more sophisticated starter culture systems and the rapid changes in the taxonomy of lactic acid bacteria (LAB), it is of utmost importance for food microbiologists to be aware of current nomenclature (Stiles and Holzapfel, 1997). As regular inhabitants of the intestine, enterococci may serve as indicators of faecal contamination, and are therefore of particular importance in food and public health microbiology. *E. faecalis* and *E. faecium* have been suspected, but remain unconfirmed, as causative agents of foodborne illness (Dack, 1956; Stiles, 1989). Several strains are used as probiotics and others are involved in a number of food fermentations for the production of certain cheeses and other fermented milk products. They are associated with natural fermentations such as occur in olives and fermented African products (Olasupo et al., 1994; Franz et al., 1996) and enterococci may become the predominant population of in-package, heat-treated meats (Houben, 1982; Bell and De-Lacey, 1984; André Gordon and Ahmad, 1991). *E. faecalis* has assumed major importance in clinical microbiology as one of the leading causes of nosocomial infections, and both *E. faecium* and *E. faecalis* strains have developed resistance to most clinically used antibiotics, including the glycopeptide antibiotics vancomycin and teicoplanin. It is therefore important for food microbiologists to assess the significance of these bacteria in the foods.

2. Phylogeny and taxonomy of enterococci

The genus *Enterococcus* was described by Schleifer and Kilpper-Bälz (1984), who used DNA:DNA and DNA:rRNA hybridisation to demonstrate that *S. faecalis* and *S. faecium* were sufficiently distinct from other streptococci to warrant their transfer to a separate genus. Based on 16S rRNA cataloguing (Ludwig et al., 1985; Williams et al., 1991), DNA:DNA and DNA:rRNA hybridisation (Garvie and Farrow, 1981; Kilpper-Bälz and Schleifer, 1981, 1984; Kilpper-Bälz et al., 1982; Schleifer and Kilpper-Bälz, 1984; Schleifer et al.,

1985) and serological studies with superoxide dismutase antisera (Schleifer et al., 1985), the streptococci *sensu lato* were subdivided into three genera: *Streptococcus sensu stricto*, *Enterococcus* and *Lactococcus* (Devriese et al., 1993).

Enterococci belong to the clostridial subdivision of the gram-positive bacteria, together with the other genera of the LAB: *Aerococcus*, *Carnobacterium*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Devriese et al., 1993; Devriese and Pot, 1995). The bifidobacteria are not phylogenetically related to other members of the LAB, but are closely related to other genera of the actinomyces branch, comprising gram-positive bacteria with high ($>55\%$) mol % G + C in the DNA (Stiles and Holzapfel, 1997). Within the clostridial subdivision the enterococci form a distinct cluster with *Vagococcus*, *Tetragenococcus* and *Carnobacterium* as their closest neighbours (Collins et al., 1989; Aguirre and Collins, 1992; Devriese et al., 1993; Devriese and Pot, 1995). Since 1984, chemotaxonomic and phylogenetic studies have resulted in assignment of 19 species to the genus *Enterococcus*; for reviews see Devriese et al. (1993); Devriese and Pot (1995); Hardie and Whiley (1997); Stiles and Holzapfel (1997).

Based on 16S rRNA sequences, the presence of four 'species groups' within the genus was established, with the 'faecium' group comprising *E. faecium*, *E. durans*, *E. hirae* and *E. mundtii*; the 'avium' group comprising *E. avium*, *E. raffinosus*, *E. malodoratus* and *E. pseudoavium*; the 'gallinarum' group comprising *E. casseliflavus* and *E. gallinarum*; and the possibly related *E. columbae* and *E. cecorum* representing a fourth group (Williams et al., 1991; Devriese and Pot, 1995). All other enterococci described to date, i.e. *E. faecalis*, *E. dispar*, *E. flavescens*, *E. saccharolyticus*, *E. sulfureus* and *E. seriolicida*, form individual lines of descent (Devriese et al., 1993; Devriese and Pot, 1995). Some reclassifications may occur in the near future. For example, SDS–PAGE studies on whole cell proteins could not distinguish between *E. flavescens* and *E. casseliflavus*, indicating that these may be the same species (Devriese and Pot, 1995). *E. solitarius* is phylogenetically more closely related to *Tetragenococcus* than other enterococci (Collins et al., 1990; Williams et al., 1991; Devriese et al., 1993). Based on cultural, biochemical and protein profiling studies (Domenech et al., 1993) and on high (70–100%) DNA:DNA homology (Eldar et al., 1996) it was shown that *E. seriolicida* and *Lactococcus garvieae* belong to a single species, and it was suggested that *E. seriolicida* strains should be reclassified as *L. garvieae* (Teixeira et al., 1996). All strains are β-haemolytic (Domenech et al., 1993). Recently several *Enterococcus* strains isolated from the caecum of donkeys which exhibited phenotypic traits not consistent with any currently known *Enterococcus* species were described. These enterococci were proposed as a new species, i.e. *Enterococcus asini*, and by means of 16S rRNA sequencing their closest relatives were determined to be *E. avium*, *E. faecium* and *E. pseudoavium* (De Vaux et al., 1998)

With the exception of *E. faecium* and *E. faecalis*, the enterococci are rarely reported to be involved in human pathogenesis (Jett et al., 1994; Devriese and Pot, 1995). Moreover, for some *Enterococcus* species there is no evidence at present for a role in human disease (Devriese and Pot, 1995). However, in some countries association of strains of *E. faecalis* and *E. faecium* with human disease has reached proportions of serious concern (Jett et al., 1994; Low et al., 1994; Leclercq, 1997; Morrison et al., 1997). Because *E. faecalis* and *E. faecium* are of importance in medicine and in foods, this review deals mainly with these two species.

3. Environmental sources and food contamination

Enterococci constitute a large proportion of the autochtonous bacteria associated with the mammalian gastrointestinal tract. *E. faecalis* is often the predominating *Enterococcus* spp. in the human bowel, although in some individuals and in some countries, *E. faecium* outnumbers *E. faecalis* (Ruoff, 1990; Devriese and Pot, 1995). Numbers of *E. faecalis* in human faeces range from 10^5 to 10^7 CFU/g compared with 10^4 to 10^5 CFU/g for *E. faecium* (Noble, 1978; Chenoweth and Schaberg, 1990). *E. faecalis*, but not *E. faecium*, has been isolated from the faeces of neonates (Noble, 1978; Murray, 1990).

Although *E. faecalis*, *E. faecium* and *E. durans* are frequently isolated from human faeces, they are

much less prevalent in livestock such as pigs, cattle and sheep (Leclerc et al., 1996). In a study by Devriese et al. (1992), *E. faecalis* was isolated from faeces of pre-ruminant calves and ruminating young cattle and dairy cows, and *E. faecium* from pre-ruminant calves, but not from ruminating young cattle or dairy cows. *S. bovis* was the predominant group D organism isolated from faeces of dairy cows. *E. faecalis*, *E. faecium*, *E. hirae* and *E. cecorum* were the enterococci most frequently isolated from pig intestines, while *E. faecium* predominated in faecal samples (Devriese et al., 1994; Leclerc et al., 1996). The intestinal microflora of young poultry contained principally *E. faecalis* and *E. faecium*, but *E. cecorum* predominated in the intestine of chickens over 12 weeks old (Devriese et al., 1991). Holzapfel and Steyn (unpublished results) noted a predominance of *E. faecalis* and *E. faecium* in the lower intestinal tract of the ostrich. Enterococci are not only associated with warm-blooded animals, but they also occur in soil, surface waters and on plants and vegetables (Mundt, 1961, 1963; Niemi et al., 1993; Jay, 1996; Leclerc et al., 1996). *E. faecium* is also among the predominant microorganisms in raw milk (Devriese and Pot, 1995), which has important implications for the dairy industry.

4. Enterococci in the food

4.1. Meats

The presence of enterococci in the gastrointestinal tract of animals leads to a high potential for contamination of meat at the time of slaughter. In a study of enterococci from raw meat products, *E. faecalis* was the predominant isolate from beef and pork cuts (Stiles et al., 1978). *E. faecium* was also frequently isolated from bologna, a processed meat sausage containing pork (Stiles et al., 1978). Pig carcasses from three different slaughtering plants contained mean log counts of 10^4 to 10^8 enterococci per 100 cm^2 of carcass surface throughout processing, and *E. faecium* and *E. faecalis* were the most predominant *Enterococcus* spp. isolated (Knudtson and Hartman, 1993a). *E. faecalis* predominated the gram-positive coccal species isolated from chicken samples collected at poultry abattoirs (Turtura and Lorenzelli, 1994). The fermented meat products salami and Landjäger were found to contain enterococci at numbers ranging from 100 to 2.6×10^5 CFU/g (Teuber et al., 1996).

Enterococci are among the most thermotolerant of the non-sporulating bacteria (Sanz Perez et al., 1982; Magnus et al., 1986, 1988). Because of this, they can become a spoilage problem in cooked, processed meats. Processed meats are typically salted or cured, and either raw or cooked (Tompkin, 1986). Cooking of processed meats raises the core temperature of products to at least 60°C and frequently above 70°C (Carr and Marchello, 1986). After surviving heat processing, both *E. faecalis* and *E. faecium* have been implicated in spoilage of cured meat products such as pasteurised canned hams and chub-packed luncheon meats (Bell and Gill, 1982; Houben, 1982; Bell and DeLacey, 1984; Magnus et al., 1986). This is especially true where recontamination with competing bacteria is prevented, i.e. when products are heated after packaging in cans or in impermeable plastic films (Bell and DeLacey, 1984). The heat resistance of enterococci in these products is influenced by components such as salt, nitrite and meat tissue (Houben, 1982; Bell and DeLacey, 1984; Magnus et al., 1986, 1988). To prevent spoilage of the processed meats by enterococci, it was suggested that initial contamination by these microorganisms should be kept to a minimum, and that adequate heat processing should be based on D-values of the most heat-resistant enterococci isolated from raw materials (Magnus et al., 1986; André Gordon and Ahmad, 1991).

In spoilage of vacuum-packaged processed meats, members of the genera *Lactobacillus* and *Leuconostoc* usually predominate, but they are often accompanied by varying proportions of enterococci and pediococci (Reuter, 1981; Holzapfel and Gerber, 1986; Von Holy et al., 1991). These products are usually surface contaminated with LAB after heat treatment and before packaging (Dykes et al., 1991). This may explain why other LAB and not enterococci usually predominate in the spoilage of these products. A secondary, in-package heat process of vacuum-packaged Vienna sausages has been suggested to decrease the initial microbial load (Franz and von Holy, 1996a). In these products, spoilage was noticeably delayed and, when it occurred, pediococci predominated (Franz and von Holy, 1996a,b). The fact that pediococci and not enterococ-

ci predominated in the 'secondary-heated', vacuum-packaged Vienna sausages may be attributed to the low incidence of enterococci in this product (Franz and von Holy, 1996b) and the relatively high resistance of pediococci to environmental factors. Higher levels of enterococci in processed meats may result from the practice known as 'reworking', whereby meat from faulty products (e.g. in which the packaging material broke during heat treatment) is added to the raw materials for further processing. If the faulty product contained heat-resistant enterococci, these may multiply during reworking and survive a second heat processing step in greater numbers. In an extreme case we observed *Enterococcus* associated spoilage at a meat processing plant in Alberta, Canada, resulting from rework (Stiles, unpublished data). Because of this high heat resistance and survival under adverse environmental conditions, the enterococci have frequently been suggested as indicators of sanitary quality of food (Knudtson and Hartman, 1992; Devriese et al., 1995; Jay, 1996).

Great potential exists for contamination of meat products with enterococci from intestinal or environmental sources. Effective control of meat contamination by enterococci, which is well within reach, may become more important in future with increasing recognition of these bacteria as opportunistic human pathogens. In contrast, the growth of certain strains of enterococci in cheeses may be highly desirable, and is described below.

4.2. Cheese manufacture

Enterococci occur and grow in a variety of cheeses, especially artisanal cheeses produced in southern Europe (Portugal, Spain, Italy and Greece) from raw or pasteurised goat, ewe's, water-buffalo or bovine milk. High levels of contaminating enterococci usually result from poor hygienic practices during cheese manufacture (Thompson and Marth, 1986; Litopoulou-Tzanetaki, 1990; López-Díaz et al., 1995) and lead to deterioration of sensory properties in some cheeses (Thompson and Marth, 1986; López-Díaz et al., 1995), but they play a major role in ripening and aroma development in other cheeses (Ordoñez et al., 1978; Trovatelli and Schiesser, 1987; Coppola et al., 1988; Litopoulou-Tzanetaki, 1990; Torri Tarelli et al., 1994; Macedo et al., 1995; Centeno et al., 1996). Levels of enterococci in different cheese curds range from 10^4 to 10^6 CFU/g, and in the fully ripened cheeses from 10^5 to 10^7 CFU/g (Table 1). Numbers vary with cheese type and production season and ranged from 10^4 to 10^6 CFU/g for Emmental cheese during a 15 year survey, and from 10^4 to 10^7 CFU/g for Appenzeller cheese (Teuber et al., 1996). *E. faecium* and *E. faecalis* were the dominant enterococcal isolates in these cheeses. Varying levels in different cheeses result from the extent of milk contamination and survival in the dairy environment (dependent on seasonal temperature), as well as survival and growth under the particular conditions of cheese manufacture and ripening (Litopoulou-Tzanetaki, 1990; Litopoulou-Tzanetaki and Tzanetakis, 1992; Macedo et al., 1995). In some cheeses, e.g., Cebreiro, Kefalotyri, Manchego, Picante da Beira Baixa, and Teleme (see Table 1), enterococci are also the predominant microorganisms in the fully ripened product. In other traditional cheeses, LAB such as *Lactobacillus plantarum*, *Weissella* (previously *Leuconostoc*) *paramesenteroides*, *Leuconostoc lactis* or *Leuconostoc paracasei* predominate in the ripened product. However, the enterococci usually represent an important part of the bacterial flora of the ripened cheeses (Table 1).

The dominance or persistence of enterococci in some cheeses during ripening can be attributed to their wide range of growth temperatures, their high tolerance of heat, salt and acid (Ordoñez et al., 1978; Litopoulou-Tzanetaki, 1990; Wessels et al., 1990; Litopoulou-Tzanetaki and Tzanetakis, 1992; Freitas et al., 1995) and their production of proteolytic enzymes involved in casein degradation (Trovatelli and Schiesser, 1987; Wessels et al., 1990). Salt concentration increases during cheese ripening. This is an important selection factor for growth of salt tolerant enterococci, *L. plantarum* and *W. paramesenteroides* during the late stages of cheese ripening (Ordoñez et al., 1978; Litopoulou-Tzanetaki, 1990; Freitas et al., 1995). Enterococci show higher proteolytic activity than other LAB and this is considered important for cheese ripening (Ordoñez et al., 1978; Trovatelli and Schiesser, 1987; Centeno et al., 1996). The beneficial effect of enterococci in cheese making has also been attributed to hydrolysis of milk fat by esterases (Tsakalidou et al., 1993). In addition, enterococci produce typical flavour components such as acetaldehyde, acetoin

Table 1
Numbers and predominance of *Enterococcus* spp. in cheeses from Mediterranean countries

Cheese	Country of origin	Milk source	Enterococci in curd (log CFU/g)	Enterococci at end of ripening (CFU/g)	Predominant bacteria in end product (% of isolates)	Reference
White-brined cheese	Greece	Raw goat milk or mixed goat and ewes' milk	4.0	6.7	*L. plantarum* (47%)[b] *E. faecium* (12%) *L. paracasei* subsp. *paracasei* (10%) *E. faecalis* (9%)	Litopoulou-Tzanetaki and Tzanetakis (1992)
Kefalotyri cheese	Greece	Ewes' milk, cow milk or mixed ewes' and goat milk	4.9	5.8	*E. faecium* (35.6%) *L. plantarum* (18.4%) *L. casei* subsp. *casei* (15.8%) *E. durans* (9.2%) Pediococci (9.2%)	Litopoulou-Tzanetaki (1990)
Teleme cheese	Greece	Pasteurised ewes' milk	n.r.[a]	n.r.	Lactobacilli Leuconostocs Enterococci	Tzanetakis and Litopoulou-Tzanetaki (1992)
La Serena ewe's milk cheese	Spain	Raw ewes' milk	6.2	7.2	Lactobacilli Leuconostocs Enterococci	Del Pozo et al. (1988)
Manchego cheese	Spain	Raw ewes' milk	n.r.	n.r.	Enterococci	Ordoñez et al. (1978)
Cebreiro	Spain	Raw cow milk	n.r.	6.5	*E. faecalis* (30.1%) *E. faecalis* (var *liquifaciens*) (11.9%) *Lact. lactis* (19.0%) *W.* (*Leuc.*) *paramesenteroides* (7.9%) *Leuc mesenteroides* subsp. *mesenteroides* (6.3%) *E. faecium* (4.8%)	Centeno et al. (1996)
Serra cheese	Portugal	Raw ewes' milk	n.r.	n.r.	*Leuc. lactis*, *Lact. lactis*, *Leuc. mesenteroides* subsp. *mesenteroides*/*dextranicum* *E. faecium*	Macedo et al. (1995)
Picante da Beira Baixa cheese	Portugal	Mixture of raw goat and ewes' milk	n.r.	n.r.	*E. faecium*, *E. faecalis*, *E. durans*, *L. plantarum*, *L. paracasei*	Freitas et al. (1995)

[a] n.r. = not reported.
[b] *L.* = *Lactobacillus*; *E.* = *Enterococcus*; *Lact.* = *Lactococcus*; *Leuc.* = *Leuconostc*; *W.* = *Weissella*.

and diacetyl (Trovatelli and Schiesser, 1987, Centeno et al., 1996). This beneficial role of enterococci in development of cheese aroma has led to inclusion of enterococcal strains in certain starter cultures. For example, enterococci were suggested for use as a starter in production of Cebreiro cheese (Centeno et al., 1996). Similarly, *E. durans* was shown to be important for aroma development in Feta cheese when used in a starter together with other LAB (Litopoulou-Tzanetaki et al., 1993). For Mozzarella cheese made from raw water-buffalo milk, a strain of *E. faecalis* was selected together with other LAB for use in a starter culture preparation (Coppola et al., 1988; Parente et al., 1989). A food company sought clearance from the British 'Advisory Committee on Novel Foods and Processes' (ACNFP) for the use of *E. faecium* strain K77D as a starter culture in fermented dairy products (ACNFP, 1996), and the committee decided that the culture was acceptable for such use. Clearly, the enterococci play an important role in the manufacture of cheeses typical of some regions, and their use has a major impact on this part of the dairy industry.

5. Enterococcal bacteriocins: the enterocins

Bacteriocins are microbially produced, membrane-active peptides with antimicrobial activity usually against closely related strains (Klaenhammer, 1993). Bacteriocin production has been reported for gram-positive and gram-negative bacteria. Among the LAB, strains representative of all genera have been reported to produce bacteriocins (Jack et al., 1995).

Because LAB strains are 'generally recognised as safe' (GRAS) in food production (Schillinger et al., 1996), use of either their bacteriocins or the bacteriocin-producing LAB starter cultures for food preservation has received much interest (Holzapfel et al., 1995; Schillinger et al., 1996). For this reason, a tremendous amount of research has been focussed on the genetics, production, mode of action, immunity and secretion mechanisms of LAB bacteriocins; c.f. Klaenhammer (1993); DeVuyst and Vandamme (1994); Jack et al. (1995); Nes et al. (1996), for reviews.

Bacteriocins were divided into three major classes (Klaenhammer, 1993): Class I bacteriocins are ribosomally-synthesised lantibiotics that undergo extensive post-translational modification to produce an active peptide. Lantibiotics contain the unusual amino acids lanthionine and β-methyllanthionine. Nisin is the most extensively studied lantibiotic. It is produced by *Lactococcus lactis* subsp. *lactis* and is used world-wide to preserve foods such as processed cheeses and canned foods (Delves-Broughton, 1990). Class II bacteriocins are small, heat stable nonlantibiotics that are also ribosomally synthesised but do not undergo post-translational modification, except for cleavage of a leader peptide. The classification of class II bacteriocins was modified by Nes et al. (1996). Class IIa comprises the pediocin-like bacteriocins with a generally strong antilisterial effect, class IIb includes the two component bacteriocins, and class IIc consists of the *sec*-dependent secreted bacteriocins. Class III bacteriocins are typically large, heat-labile proteins. A fourth class of bacteriocins contains protein complexed with lipid or carbohydrate moieties. These are not well characterised and it is not clear whether the additional nonprotein chemical moieties are essential for activity (Klaenhammer, 1993; Nes et al., 1996).

Strains of enterococci, including *E. faecium* and *E. faecalis*, are known to produce bacteriocins (Table 2). These are called enterocins and they generally belong to class II. Examples of the best characterised enterocins are enterocin A, a class IIa, pediocin-like bacteriocin (Aymerich et al., 1996), and enterocin B (Casaus et al., 1997), a bacteriocin that is not pediocin-like, but it is similar to the class IIa bacteriocins because of its chemical characteristics, heat stability and anti-*Listeria* activity (Table 2). Enterocin P belongs to class IIc because secretion occurs by the *sec*-pathway (Cintas et al., 1997). Enterocins L50A and L50B are novel bacteriocins (Cintas et al., 1998). Each has antimicrobial activity

Table 2
Well-characterised bacteriocins produced by *E. faecium* and *E. faecalis*

Bacteriocin	Producer organism	Location of genes for production	No. of amino acids		Molecular mass (Da)	Post-translational modification	Reference
			Prepeptide	Mature peptide			
Enterocin A	*E. faecium*	Chromosome	65	47	4829	Cleavage of leader peptide on export, possible disulphide bridge formation	Aymerich et al. (1996)
Enterocin B	*E. faecium*	Chromosome	71	53	5463	Cleavage of leader peptide on export, disulphide bridge formation	Casaus et al. (1997),
Enterocin P	*E. faecium*	Chromosome	71	43	4493	Cleavage of signal peptide on export, possible disulphide bridge formation	Cintas et al. (1997)
Enterocin 50	*E. faecium*	Plasmid pCIZ1	n.a.[b]	44	5190	No modification	Cintas et al. (1998)
Enterocin 50	*E. faecium*	Plasmid pCIZ1	n.a.	43	5178	No modification	Cintas et al. (1998)
Bacteriocin 31	*E. faecalis*	Pheromone responsive plasmid pYI17	67	43	n.r.[a]	Cleavage of signal peptide on export, possible disulphide bridge formation	Tomita et al. (1996)
AS-48	*E. faecalis*	Pheromone responsive plasmid pMB2	105	70	n.r.	Cleavage of leader peptide and head-tail peptide bond formation	Martínez-Bueno et al. (1994)
Cytolysin	*E. faecalis*	Pheromone responsive plasmid pAD1				Modification to form lanthionine containing pre-cursors, removal of a	Booth et al. (1996)
$CylL_L$ peptide			($CylL_L$) 68	38	3437	leader sequence on export and	
$CylL_S$ peptide			($CylL_S$) 63	21	2031	proteolytic activation	

[a] n.r. = not reported.
[b] n.a. = not applicable.

on its own, but together they show synergistic activity (Table 2). They are not post-translationally modified and do not require the presence of a leader or signal peptide for secretion (Cintas et al., 1998). They share homology with the staphylococcal haemolysins, yet they do not show haemolytic activity.

Bacteriocins of some strains of *E. faecalis* are interesting because they are encoded on pheromone-responsive, conjugative plasmids, for example AS-48, cytolysin and bacteriocin 31 (Martínez-Bueno et al., 1994; Booth et al., 1996; Tomita et al., 1996) (Table 2). The antimicrobial peptide AS-48 produced by *E. faecalis* S-48 is similar to bacteriocins because it functions by permeabilising the cytoplasmic membrane of susceptible bacteria (Martínez-Bueno et al., 1994). Unlike most other bacteriocins, this peptide is a cyclic molecule that results from post-translational modification of the primary product during secretion (Martínez-Bueno et al., 1994). In addition, strains of *E. faecalis* produce haemolysin/bacteriocins, which are two component bacteriocins with both haemolytic and bacteriocin activity. These bacteriocins are called cytolysins and contain lanthionine and β-methyllanthionine residues (Booth et al., 1996), suggesting that they are class I bacteriocins. Bacteriocin 31 produced by *E. faecalis* YI717, similar to enterocin P, is secreted by the *sec*-pathway (Tomita et al., 1996). The enterococci produce a wider variety of antimicrobial peptides than has been described for strains of most other LAB genera. Although bacteriocin production has been reported for many other strains of *E. faecalis*, most of these have either not been purified or they are insufficiently characterised for classification as cytolysins or class I, II or III bacteriocins (Arihara et al., 1991; Villani et al., 1993; Maisnier-Patin et al., 1996; Simonetta et al., 1997). Using a semi-nested PCR assay for the AS-48 structural gene, Joosten et al. (1997) showed that production of bacteriocins very similar or identical to AS-48 is common in *E. faecalis* and *E. faecium*.

The enterocins are generally active against other enterococci as well as strains of *Listeria monocytogenes* (Giraffa, 1995). The anti-*Listeria* activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (Devriese and Pot, 1995). Some enterocins are active against other LAB as well as *Clostridium* spp., including *C. botulinum*, *C. perfringens* and *C. tyrobutyricum* (Torri Tarelli et al., 1994; Franz et al., 1996). Bacteriocinogenic enterococci have been isolated from a variety of sources, including fermented meat (Aymerich et al., 1996; Casaus et al., 1997), dairy products (Olasupo et al., 1994; Torri Tarelli et al., 1994; Vlaemynck et al., 1994; Farías et al., 1996), and vegetables (McKay, 1990; Villani et al., 1993; Franz et al., 1996). Bacteriocinogenic enterococci may be used as anti-*Listeria* agents in the dairy industry, particularly in certain types of soft cheese (e.g., Camembert or Taleggio) where pH in the rind increases to a level that allows growth of *L. monocytogenes*. Enterococci often predominate during ripening of cheeses and could produce bacteriocins at sufficient levels to inhibit *Listeria* (Giraffa et al., 1997). Bacteriocinogenic *E. faecium* was tested on a laboratory scale for use in combination with a commercial starter culture for Taleggio cheese making (Giraffa et al., 1995). Bacteriocin was produced during drainage of the whey and activity could be detected in the cheese until the end of the ripening period, while growth and acidifying activity of the thermophilic commercial starter culture was not inhibited (Giraffa et al., 1995). As a further example, an inhibitory starter culture consisting of bacteriocinogenic *E. faecium*, *E. faecalis*, nisin-producing *L. lactis* and *Lactobacillus paracasei* added to milk prior to Camembert cheese making or sprayed onto the surface of the cheese, totally inhibited *Listeria* spp. when surfaces were contaminated with *Listeria* not later than 1.5 days after brining (Sulzer et al., 1992).

Activity of enterococci against *C. tyrobutyricum* has been described. This may be used to preserve packaged cheeses where growth of *C. tyrobutyricum* causes blowing of the packages. Inhibition of these bacteria by enterocins would eliminate this defect. Use of bacteriocinogenic enterococci as starter cultures for cheese manufacture to increase cheese safety or to prolong storage life of the product has not yet been practised on an industrial scale but could be pursued in the future.

6. Enterococci as probiotics

Probiotics may be defined as ‘mono- or mixed cultures of live microorganisms which, when applied

to animal or man, beneficially affect the host by improving the properties of the indigenous flora' (Havenaar et al., 1992). O'Sullivan et al. (1992) reported a marked increase in sales of cultured products containing viable probiotic bacteria originating from the human intestine. Products for human consumption containing probiotic organisms may be grouped into three categories: infant foods, cultured milks and pharmaceutical preparations (O'Sullivan et al., 1992). Claims for beneficial effects include: maintenance or restoration of the normal intestinal microflora and thereby prevention or reduction of gastro-intestinal disorders, alleviation of lactose intolerance, reduction in serum cholesterol levels, anticarcinogenic activity, stimulation of the immune system and improved nutritional value of foods (Fuller, 1989; O'Sullivan et al., 1992; Agerbaeck et al., 1995; Lee and Salminen, 1995; Salminen et al., 1996). Most probiotic bacteria are of intestinal origin and belong to the genera *Bifidobacterium* and *Lactobacillus*. Strains from other genera that have been used as probiotics include: *E. faecium*, *E. faecalis*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, *Propionibacterium freudenreichii*, *Pediococcus acidilactici*, *Sporolactobacillus inulinus*, *Escherichia coli* and *Bacillus cereus* ('toyoi'), and the yeast *Saccharomyces cerevisiae* ('boulardii') (Fuller, 1989; O'Sullivan et al., 1992; Holzapfel et al., 1998). Although both *E. faecium* and *E. faecalis* find application in probiotic preparations for humans, *E. faecalis* is more widely used as an animal feed supplement.

E. faecium strain SF68 has been studied in detail for use as a human probiotic, especially in the treatment of diarrhoea. The strain was originally isolated in Sweden and was patented in Switzerland and other countries (Lewenstein et al., 1979). Its effectiveness for treatment of intestinal disorders can probably be attributed to the fact that it is a commensal of the intestine and that it has a short lag phase and generation time (ca. 20 min generation time under optimal conditions). It is moderately resistant to antibiotics and has an inhibitory effect in vitro to growth of *E. coli*, *Salmonella* spp., *Shigella* spp. and *Enterobacter* spp. In addition, this strain is resistant to low pH, insensitive to bile salts, and individuals show a high tolerance to it with no side effects (Lewenstein et al., 1979; Bellomo et al., 1980; Canganella et al., 1996). Use of *E. faecium* SF68 for treatment of diarrhoea is considered an alternative to antibiotic treatment (Lewenstein et al., 1979; Bellomo et al., 1980). Clinical studies have shown the effectiveness of *E. faecium* in the treatment of enteritis in both children (Bellomo et al., 1980; D'Apuzzo and Salzberg, 1982) and adults (Lewenstein et al., 1979; Bruno and Frigerio, 1981). These controlled, 'double blind' studies on the treatment of enteritis with SF68 showed a statistically significant decrease in the duration of diarrhoea and time for normalisation of stools (Bellomo et al., 1980; Bruno and Frigerio, 1981; D'Apuzzo and Salzberg, 1982). However, in one study with *E. faecium* SF68 no antidiarrhoeal effect was demonstrated in adults suffering from acute diarrhoea due to *Vibrio cholerae* or enterotoxinogenic *E. coli* infections (Mitra and Rabbani, 1990). In another controlled study it was shown that *E. faecium* SF68 significantly decreased the incidence of diarrhoea and prevented mucositis (as a possible consequence of vitamin deficiency) in chronic pulmonary tuberculosis patients receiving long-term antibiotic treatment. Supportive treatment with *E. faecium* probiotic preparation was recommended for these patients (Borgia et al., 1982). A further controlled study showed that use of *E. faecium* SF68 was as effective as lactulose in lowering blood ammonia and for improving mental state and psychometric performance of patients with hepatic encephalopathy (Loguercio et al., 1987). Raised levels of blood ammonia in patients with hepatic encephalopathy is thought to result from putrefactive intestinal microflora, and it was suggested that treatment with *E. faecium* SF68 modified such intestinal microflora, thus lowering the level of ammonia in the blood (Loguercio et al., 1987).

Some enterococcal probiotic preparations are sold in 'health food' stores as nutrition supplements, with vague claims to 'improve the gastrointestinal balance'. Sufficient scientific information on certain probiotic preparations from health food stores is not always supplied. Such preparations containing strains of *E. faecium* are also available on the market. Clearly, at a time when consumer awareness of nutrition and health is increasing, it is important that strains used in probiotic preparations to promote human health should be safe for all potential consumers.

Probiotic preparations are also used in animal husbandry to prevent or treat enteric disease, or as growth promoters. Enteritis in livestock has routinely been cured or prevented by feeding or treatment with antibiotics, but development of antibiotic resistance in pathogenic and opportunistic microorganisms has stimulated interest in probiotics as an alternative treatment (Underdahl, 1983). Gastroenteritis is a major disease of neonatal pigs. Colibacillosis is most severe when pigs are infected in their first week of life, and pigs may develop diarrhoea during the stress of weaning at 3 to 5 weeks of age (Underdahl, 1983). The effectiveness of *E. faecium* cernelle 68 (the same as SF68) preparation to prevent or treat *E. coli* diarrhoea in experimentally infected gnotobiotic pigs was studied by Underdahl (1983). Three strains of *E. coli* were used, one which caused diarrhoea but not death, and two strains which caused severe diarrhoea and death. Pigs fed the probiotic preparation and infected with the less virulent strain of *E. coli* had less diarrhoea, recovered earlier and had higher weight gain than those not receiving the probiotic preparation. Pigs which were infected with the more virulent strains of *E. coli* and which received the probiotic preparation developed low grade diarrhoea, but survived and gained weight, whereas those that did not receive the probiotic developed severe diarrhoea and some died (Underdahl, 1983).

Enterococci and other LAB probiotic preparations may receive more interest in animal management, as a result of gastrointestinal bacterial pathogens developing antibiotic resistance. The use of probiotics in animal husbandry in place of antibiotics is important also to the food microbiologist, as it may prevent the development of dissemination of antibiotic-resistant pathogens from animal foods. In addition, the use of probiotics in animal husbandry may be important also for economic considerations, as costly antibiotic treatments are avoided.

7. Enterococci in disease

Enterococci are considered as emerging pathogens of humans, and their role and importance have been reviewed by Murray (1990), Lewis and Zervos (1990) and Morrison et al. (1997). They have become of major importance in community-acquired and in hospital-acquired (nosocomial) infections and superinfections such as endocarditis, bacteraemia, urinary tract, neonatal, central nervous system (CNS), intraabdominal and pelvic infections. In the USA, their importance has increased from the third leading cause of nosocomial disease, accounting for 10% of such infections in 1984 (Chenoweth and Schaberg, 1990), to the second between 1985 and 1989 (Schaberg et al., 1991). *E. faecalis* clearly predominates among enterococci isolated from human infections, while strains of *E. faecium* are associated with the majority of the remaining infections (Jett et al., 1994; Low et al., 1994; Jones et al., 1997; Simjee and Gill, 1997).

Bacteraemia is the most common form of enterococcal infection (Lewis and Zervos, 1990; Jones et al., 1997; Morrison et al., 1997). Compared with a steady reduction in community-acquired cases of enterococcal bacteraemia, nosocomial cases may have increased three-fold and reach up to 77% of cases (Shlaes et al., 1981; Maki and Agger, 1988; Morrison et al., 1997; Weinstein et al., 1997). Risk factors associated with enterococcal bacteraemia include underlying disease, presence of urethral or intravascular catheters, surgery, major burns, multiple trauma or prior antibiotic therapy (Lewis and Zervos, 1990). Sources of enterococci causing bacteraemia without endocarditis are most commonly from the urinary tract, but the gastrointestinal and hepatobiliary tracts have also been implicated (Chenoweth and Schaberg, 1990; Lewis and Zervos, 1990; Morrison et al., 1997). Mortality from enterococcal bacteraemia is generally high, most probably because of the underlying complicating factors (Murray, 1990; Kaufhold and Ferrieri, 1993).

Enterococci cause an estimated 5 to 15% of cases of bacterial endocarditis with *E. faecalis* more commonly involved than *E. faecium* (Murray, 1990). The enterococci usually originate from the urinary tract (Chenoweth and Schaberg, 1990; Lewis and Zervos, 1990; Aguirre and Collins, 1993). Underlying heart disease is often present, but it is not a prerequisite for development of this infection (Chenoweth and Schaberg, 1990; Murray, 1990). Endocarditis often occurs in patients that had preceding genitourinary instrumentation or urinary tract infections (UTI), abortion, or urinary tract instrumentation (Chenoweth and Schaberg, 1990; Murray, 1990; Moellering, 1992).

Urinary tract infections are commonly caused by enterococci, especially in hospitalised patients. Such infections occur especially in persons who had been medically instrumented, received antibiotics, had structural abnormalities, or had recurrent enterococcal infections (Chenoweth and Schaberg, 1990; Murray, 1990; Moellering, 1992).

Infections of the central nervous system by enterococci are rare and are seen primarily in neonates and persons who have undergone complicated and neurological procedures (Moellering, 1992; Morrison et al., 1997). Enterococci causing neonatal infection are thought to originate from the vagina, because they are detected in the vaginal microflora in 25% of healthy women (Lewis and Zervos, 1990). *E. faecium* and *E. faecalis* have been implicated in outbreaks of neonatal central nervous system infections, although infections of older children and adults have also been reported (Murray, 1990; Tailor et al., 1993).

Enterococci may cause or contribute to abdominal and pelvic abscess formation and sepsis (Murray, 1990). They were reported as a cause of spontaneous peritonitis in cirrhotics and nephrotics, and may be associated with peritonitis in patients on peritoneal dialysis (Murray, 1990). Dialysis catheters and prior use of antibiotics are predisposing factors for intraabdominal infections by enterococci (Chenoweth and Schaberg, 1990; Low et al., 1994).

The use of antibiotics to which enterococci are resistant is an important factor in enterococcal superinfection (Moellering, 1982; Zervos et al., 1988; Murray, 1990; Moellering, 1992; Low et al., 1994; Morrison et al., 1997). Antibiotic resistance of enterococci and gene transfer mechanisms, which often involve antibiotic resistance, are discussed below; however, antibiotic resistance alone cannot explain the virulence of these bacteria.

8. Antibiotic resistance

The enterococci have become a focus of attention in hospitals because of their increasing resistance to antibiotics. Antibiotic resistance and nosocomial infection are mutually reinforcing phenomena because resistance allows enterococci to survive in the hospital environment where antibiotics are used, and the hospital provides the opportunity for dissemination of resistant organisms (Murray, 1990). Examples of intrinsic resistance include the cephalosporins, β-lactams, sulphonamides, and low levels of clindamycin and aminoglycosides (Moellering, 1990; Murray, 1990; Leclercq, 1997; Morrison et al., 1997). Acquired resistance based on acquisition of plasmids and transposons, has relevance for chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, β-lactams (by β-lactamase or penicillinase), fluoroquinolones and glycopeptides (Murray, 1990; Moellering, 1991; Landman and Quale, 1997; Leclercq, 1997; Morrison et al., 1997). For detailed reviews on resistance and mechanisms of resistance in enterococci see Murray (1990), Moellering (1991) and Leclercq (1997).

Intrinsic resistance to many antibiotics suggests that treatment of infection could be difficult. However, combinations of cell-wall-active antibiotics with aminoglycosides (e.g. streptomycin, kanamycin and gentamicin) act synergistically and have been used successfully in treatment of enterococcal infection (Moellering, 1990, 1991; Murray, 1990; Simjee and Gill, 1997). In the early 1970's, a high level of streptomycin resistance was reported, and strains were also found resistant to penicillin–streptomycin combinations. High level gentamicin resistance followed, with enterococci exhibiting resistance to combinations of penicillin and gentamicin (Moellering, 1990). In 1983 a strain of *E. faecalis* producing a β-lactamase identical to that produced by *S. aureus* was reported (Murray and Mederski-Samoraj, 1983), and it is believed that this strain of *Enterococcus* received the gene from *S. aureus* (Murray et al., 1986). The hitherto successful penicillin–aminoglycoside treatment was no longer a viable option, resulting in a major therapeutic problem (Moellering, 1991; Morrison et al., 1997).

Another major concern is the emergence of vancomycin-resistant enterococci (VRE). Different vancomycin resistance phenotypes have been reported for *E. faecalis* and *E. faecium*. VanA and VanB are acquired resistance phenotypes which are transferable by conjugation (Arthur and Courvalin, 1993). The VanA-type confers high level and inducible resistance to both vancomycin and teicoplanin, while the VanB-type displays variable levels of inducible resistance only to vancomycin. The VanC type, comprising constitutive low-level resistance to vancomycin, appears to be an intrinsic property of the

motile species *E. gallinarum*, *E. casseliflavus* and *E. flavescens* (Vincent et al., 1991; Dutka-Malen et al., 1995; Leclercq, 1997). For a review of genetics and mechanisms of glycopeptide resistance in enterococci, see Arthur and Courvalin (1993), Evers et al. (1996), Leclercq and Courvalin (1997) and Murray (1997). A PCR assay was developed by Dutka-Malen et al. (1995) by which glycopeptide resistance genotypes may be detected with simultaneous species level identification of clinically relevant enterococci.

Vancomycin was previously used for treatment of enterococcal infections with strains exhibiting a high level β-lactam resistance, or when β-lactams were poorly tolerated by the patient (Leclercq et al., 1988; Shlaes et al., 1989). Unfortunately, many VRE are also highly resistant to all standard anti-enterococcal drugs, including penicillin–aminoglycoside combinations (Landman and Quale, 1997), and only a few alternatives remain for successful treatment (Fraise, 1996). Therefore, VRE presently constitute a serious risk group among bacterial nosocomial pathogens and their presence in hospitals is met with great concern.

9. Gene transfer mechanisms

Several gene transfer mechanisms in enterococci have been described. They involve both conjugative and nonconjugative plasmids as well as conjugative transposons, and these may carry antibiotic resistance genes (Clewell, 1990; Simjee and Gill, 1997). Conjugative plasmids may have a broad bacterial host range and transfer at a low frequency in broth (e.g., pAMβ1, which has been transferred to lactococci, lactobacilli, *S. aureus* and *Bacillus* spp.), or narrow host range, such as the plasmids of *E. faecalis*, which transfer at a high frequency in broth and respond to sex pheromones (Clewell, 1990; Simjee and Gill, 1997). For a review of gene transfer mechanisms see Clewell (1990).

The sex pheromone response is an interesting system for highly efficient exchange of genetic material in *E. faecalis* (see reviews by Dunny, 1990; Clewell, 1993; Wirth, 1994; Dunny et al., 1995). A plasmidless recipient strain produces chromosomally encoded pheromones which consist of seven to eight amino acid hydrophobic peptides. The pheromones induce genes on the plasmid of the donor strain to produce aggregation substance (AS), which facilitates binding to recipient cells by a complementary receptor on the recipient cell, called enterococcal binding substance (Dunny et al., 1995). A mating channel is formed and plasmid DNA is transferred from donor to recipient cell. The new recipient cell is prevented from responding to its own pheromone by a surface exclusion protein which is plasmid encoded (Dunny, 1990; Clewell, 1993; Wirth, 1994; Dunny et al., 1995). A strain of *E. faecalis* that harbours a specific sex pheromone plasmid will not secrete the corresponding pheromone; however, it may secrete pheromones specific for other sex pheromone plasmids which it does not possess but may subsequently acquire. It is not uncommon for strains of *E. faecalis* to harbour two or three sex pheromone plasmids (Wirth, 1994).

Sex pheromone plasmids may carry one or more antibiotic resistance genes, e.g. tetracycline, penicillin, gentamicin, streptomycin, kanamycin and tobramycin (Clewell, 1990; Wirth, 1994), or they may encode haemolysin/bacteriocin production. This latter protein lyses human, rabbit and horse erythrocytes, but it also has antibacterial activity and inhibits a broad range of gram-positive bacteria (Clewell, 1990). The haemolysin/bacteriocin (or cytolysin) system is determined by two peptides ($CylL_L$ and $CylL_S$) that are activated by proteolytic cleavage involving a serine protease activator component (CylA) (Booth et al., 1996). The two cytolysin peptides are post-translationally modified after transcription, and contain lanthionine and β-methyllanthionine (Booth et al., 1996).

Antibiotic resistance may contribute to an understanding of the establishment of enterococci as nosocomial pathogens. However, pathogenicity of enterococci can obviously not be explained on the basis of antibiotic resistance alone, and virulence factors are to be taken into consideration.

10. Virulence factors

For many years enterococci were considered to be harmless commensals with low pathogenic potential (Moellering, 1990; Jordens et al., 1994; Leclercq, 1997). In general terms this is true because they lack potent virulence factors as compared to other gram-

positive pathogens such as pathogenic streptococci, *S. aureus* and *L. monocytogenes* (Moellering, 1990). However, this view is changing because of the increasing role of enterococci in nosocomial infections, especially under selective pressure of antibiotics (Chenoweth and Schaberg, 1990; Leclercq, 1997). For enterococci to cause infection, they must colonise host tissue, resist host specific and unspecific defense mechanisms, and produce pathological changes; see Jett et al., 1994 and Johnson, 1994 for reviews.

10.1. Colonisation

It is assumed that enterococci are able to colonise the gastrointestinal tract because they are normal inhabitants of the human intestine and enterococcal infections originate from the gastrointestinal and genitourinary tracts (Murray, 1990; Chenoweth and Schaberg, 1990; Johnson, 1994). Evidence exists for patient-to-patient spread, and hospital staff may acquire strains from outbreaks and excrete these in their faeces (Chenoweth and Schaberg, 1990; Johnson, 1994). Colonisation may not constitute a virulence factor as such, but it may amplify potential pathogenicity of a strain in combination with other virulence factors. However, specificity for particular host tissues and the production of aggregation substance (discussed below) may play a major role in pathogenicity.

10.1.1. Adherence

Adherence of pathogens to the extracellular matrix of various host tissues is considered crucial for infection. Both specific adhesin-ligand as well as hydrophobic interactions may be involved (Zareba et al., 1997). In this context, both *E. faecium* and *E. faecalis* were shown to bind specific extracellular matrix proteins, especially thrombospondin, lactoferrin and vitronectin; however, the cell surface components responsible for binding were not isolated (Zareba et al., 1997). The role of enterococci in urinary tract infections and endocarditis suggests that these bacteria are efficient colonisers of these host tissues. The tendency for bacteria to infect specific tissues is often related to their ability to adhere to the respective target cell in vitro (Guzmàn et al., 1989). Strains of *E. faecalis* isolated from UTI's had a greater capacity to adhere to urinary tract epithelial cells and human embryo kidney cells than *E. faecalis* isolated from cases of endocarditis. Conversely, isolates from cases of endocarditis showed better adherence to Girardi heart cells than to urinary tract epithelial cells or human embryo kidney cells. All isolates from cases of endocarditis, but only some from UTI's, exhibited an in vitro potential to be highly invasive, and isolates from cases of endocarditis associated less efficiently with human polymorphonuclear leucocytes than those from UTI (Guzmàn et al., 1989). After growth in serum, strains from UTI and endocarditis adhered more efficiently to both human embryo kidney and Girardi heart cell lines, and associated less efficiently with human polymorphonuclear leucocytes. These findings led the authors to suggest that enterococci causing endocarditis may originate from UTI. More invasive UTI strains could attack the kidneys, causing pyelonephritis, and bacteraemia.

Persistence in blood would change surface antigen expression, making the cell more resistant to phagocytosis and favouring adherence to cardiac cells (Guzmàn et al., 1989). Persistence in a habitat which favours the selection of bacteria with high adherence ability may render some surface modifications induced by blood factors irreversible; this would explain why isolates from cases of endocarditis adhere better to cardiac cells than UTI isolates (Guzmàn et al., 1989). It was subsequently shown that for *E. faecalis* isolates from cases of UTI and endocarditis that were grown in broth, adherence was mediated by surface adhesins containing D-glucose and D-mannose (Guzmàn et al., 1991).

Aggregation substance (AS) produced in response to sex pheromones (see above) mediates adhesion of *E. faecalis* to cultured renal tubular cells (Kreft et al., 1992). An Arg-Gly-Asp-Ser amino acid motif in the AS is thought to be involved in binding to eucaryotic cells. This amino acid motif is also found in fibronectin and it mediates the binding to eucaryotic cells by a class of receptors known as integrins (Kreft et al., 1992). Expression of AS can be induced in serum by an unidentified factor (Kreft et al., 1992), and it was argued that this enables the bacterium to 'sense' the eucaryotic environment and to respond by synthesising AS adhesin (Wirth, 1994). It was also suggested that aggregation substance plays a role in invasion of cultured cells. *E. faecalis* strains expressing AS were internalised by

enterocytes in higher numbers than non-expressing mutant strains (Olmsted et al., 1994). Electron microscopy showed that AS interacted with the surface of the enterocyte microvillus and that intracellular enterococci were localised within membrane-bound vacuoles in the cytoplasm of the enterocyte, clearly demonstrating that AS is an important virulence factor (Olmsted et al., 1994).

10.1.2. Translocation

A large proportion of enterococcal infections are thought to originate from the intestinal tract. According to the translocation model, intestinal epithelial cells or intraepithelial leucocytes phagocytose bacteria adhering to them at the lumen side. The bacteria exit on the apical side of epithelial cells or migrate in phagocytes to mesenteric lymph nodes, proliferate, and spread to distant sites (Jett et al., 1994). A murine model for enterococcal translocation showed that under appropriate conditions of intestinal overgrowth with antibiotic-resistant *E. faecalis*, the bacteria could translocate across an intact epithelium and cause systemic infection (Wells et al., 1990).

10.2. Resistance to host defense mechanisms

Most studies on resistance to host defense mechanisms have concentrated on interaction with polymorphonuclear leucocytes (Johnson, 1994). As mentioned above, Guzmàn et al. (1989) showed that isolates of *E. faecalis* from cases of endocarditis grown in broth adhered to polymorphonuclear leucocytes less readily than isolates from UTI. *E. faecalis* isolates from cases of both endocarditis and UTI that were grown in serum showed decreased adherence to polymorphonuclear leucocytes (Guzmàn et al., 1989). In vitro phagocytosis assays with *E. faecium* and *E. faecalis* showed that efficient killing requires the presence of serum complement, and that antibodies to enterococci enhance polymorphonuclear leucocyte-mediated killing (Harvey et al., 1992; Jett et al., 1994; Johnson, 1994). The enterococci also express a flavin-containing NADH peroxidase which degrades hydrogen peroxide, and they possess an oxygen-inducible superoxide dismutase which catalyses conversion of superoxide to hydrogen peroxide. However, whether these enzymes enhance survival in macrophages after phagocytosis requires further investigation (Jett et al., 1994).

The group D antigen of enterococci is a membrane-associated lipoteichoic acid which may bind reversibly to human erythrocytes (Beachey et al., 1979). This may be relevant to inflammation, because lipoteichoic acid bound to eucaryotic cells retains antigenic specificity and such cells can suffer complement-mediated lysis when exposed to plasma (Hummell and Winkelstein, 1986). Thus, tissue damage may occur at sites of infection from complement activation by membrane-associated bacterial lipoteichoic acid of the host cell (Jett et al., 1994). In addition, lipoteichoic acid from several enterococcal species stimulated monokine production from cultured human monocytes (Bhakdi et al., 1991). Lipoteichoic acid is also thought to be the binding substance of pheromone-producing cells which recognises AS of plasmid-bearing donor cells (see above). Therefore, enterococcal lipoteichoic acid may serve as a virulence factor because it may modulate inflammatory responses and facilitate plasmid transfer (Jett et al., 1994).

10.3. Pathology

Pathological changes associated with enterococci include acute inflammation (Johnson, 1994). Sex pheromones and surface exclusion proteins are thought to be involved in eliciting an inflammatory response. These compounds are chemotactic for human and rat polymorphonuclear leucocytes in vitro, and induce superoxide production and secretion of lysosomal enzymes (Ember and Hugli, 1989; Sannomiya et al., 1990; Johnson, 1994). Another pathological change in bacterial endocarditis involves platelet activation and accumulation, leading to development of endocardial vegetations (Johnson, 1994). *E. faecalis*, *E. faecium* and *E. avium* induce platelet aggregation in vitro, with concomitant release of serotonin (Usui et al., 1991; Johnson, 1994). Many, but not all, pathogenic strains of enterococci produce cytolysin, which has been linked to increased virulence in animal models, such as murine peritonitis and rabbit endophthalmitis (Ike et al., 1984; Jett et al., 1992). Cytolysin induces tissue damage, as shown in the endophthalmitis model (Stevens et al., 1992). In the rabbit model for endocarditis, cytolysin contributed to virulence only when associated with AS (Chow et al., 1993). Batish et al. (1990) observed a close relationship between

haemolysin production of enterococci, lethality in mice and dermonecrosis in rabbit skin. In Japan it was shown that 60% of clinical strains involved in parenteral infection showed a haemolytic phenotype, compared with only 17% of isolates from faeces of healthy individuals (Ike et al., 1987). Cytolysin is involved in, but is not a prerequisite for virulence because nonhaemolytic strains of enterococci may also cause infection (Johnson, 1994). Gelatinase is a protease that hydrolyses gelatin, collagen, haemoglobin and other bioactive peptides (Coque et al., 1995), and it is also considered to be involved in pathogenicity of *Enterococcus* species. Kühnen et al. (1988) showed that 63.7% of strains of *E. faecalis* isolated from surgical intensive care units in Germany produced protease.

Enterococci may produce hyaluronidase. Because production of this enzyme was linked to pathogenesis of other microorganisms, it was suggested that it may also play a role in enterococcal pathogenesis. However, there is no direct evidence for the role of hyaluronidase in disease caused by enterococci (Jett et al., 1994). Coque et al. (1995) investigated the incidence of haemolysin, gelatinase and AS among enterococci isolated from cases of endocarditis, other infections, and from faeces of hospitalised and community-based individuals. Haemolysin production was detected at a higher frequency among isolates from clinical specimens (17 to 60%) compared with isolates from faeces of healthy individuals (17%). This was in accordance with information reported by Ike et al. (1987); however, haemolysin production was not a common trait among isolates from endocarditis (16%), hence haemolysin may not be required for enterococci to cause this type of infection (Coque et al., 1995). Gelatinase and AS both occurred more frequently among clinical isolates than among faecal isolates from healthy volunteers, indicating that these two factors may also play a role in virulence of enterococci. While most clinical isolates of *E. faecalis* exhibited at least one of the three possible virulence factors, more than 45% of isolates from cases of endocarditis lacked haemolysin, gelatinase or AS, indicating that other properties must be operating in the pathogenesis of *E. faecalis* endocarditis (Coque et al., 1995). In addition, none of the non-*E. faecalis* strains (*E. faecium*, *E. gallinarum*, *E. casseliflavus*, *E. raffinosus* and other species) exhibited any of these 'virulence' traits, suggesting that unknown factors may play a role in pathogenesis (Coque et al., 1995).

11. Implications of enterococci in foods

Enterococci, especially *E. faecium* and *E. faecalis*, may be considered as opportunistic pathogens. Infections usually occur nosocomially in persons who are debilitated, have an underlying disease, or have received medical instrumentation. However, the incidence of infections caused by enterococci, their seriousness, and the increasing difficulty of treating such infections because of multiple antibiotic resistance, put these organisms among the most important emerging human pathogens. Sources of enterococci involved in human infection were thought to be from the patient's endogenous microflora; however, person-to-person transmission of enterococci in hospital outbreaks has been reported, as well as stool carriage of strains implicated in outbreaks (Moellering, 1991; Jordens et al., 1994; Gordts et al., 1995). Discussion has focussed on whether pathogenic enterococci can be transmitted by foods and cause disease in a hospital setting, particularly with emphasis on VRE. It was also thought that VRE originated in the hospital environment and that they are disseminated to the community, but several researchers have proposed the opposite (Bates et al., 1993, 1994; Klare et al., 1995a,b; Das et al., 1997). A proposed source of VRE is farm animals in which there has been ergotropic use of avoparcin, a glycopeptide antibiotic (Klare et al., 1995a,b; Das et al., 1997). VRE have been isolated from a wide variety of farm animals, and these constitute an important reservoir of VRE that could be transmitted to the hospital environment via contaminated meat (Klare et al., 1995a,b; Devriese et al., 1996). Chadwick et al. (1996) isolated VRE from chicken, pork and beef samples from retail markets in the UK and suggested that *van*A resistance genes may be introduced into the community via the food chain. VRE were also isolated from raw sewage, farm animals and uncooked chicken by Bates et al. (1994), and, more importantly, they showed that blood and urine isolates from different hospital patients and a porcine isolate shared the same ribotyping pattern. These findings strongly suggest that food transmission occurred and, as a result, two European countries

(Denmark and Germany) banned the use of avoparcin (Morrison et al., 1997), followed by a European Union-wide ban (McDonald et al., 1997). In a recent study, however, Klein et al. (1998) could not establish any direct connection between the occurrence of VRE in minced meat and nosocomial infections. The role of the meat chain in the transfer of vancomycin-resistant enterococci (VRE) has indeed not been fully elaborated yet. In a study of 555 samples of 115 batches of minced beef and pork, Klein et al. (1998) found an incidence of only 0.5% VRE positive samples, at a concentration of 1.0 log CFU/g, when a direct plating method was used. They also found the antibiotic resistance patterns of VRE isolates to differ from those of clinical isolates.

In the USA the situation with respect to nosocomial VRE infections appears to differ considerably from that in Europe, because avoparcin has not been licensed for use (McDonald et al., 1997). A community prevalence survey failed to isolate VRE from healthy volunteers without hospital exposure and from environmental sources or probiotic preparations (Coque et al., 1996). In contrast to Europe, transmission of VRE in the USA does not appear to be from the community to the hospital, and food has not been implicated as a possible vehicle for transmission. This raises the question of the source of VRE isolates in the USA. McDonald et al. (1997) proposed that undetected community transmission of VRE may occur at low levels. Alternatively, it was proposed that enterococci acquired vancomycin resistance genes from an unknown gastrointestinal bacterium (Rice, 1996; Morrison et al., 1997).

The important question is whether enterococci originating from food and from community sources possess an equally pathogenic potential, or whether a difference in pathogenicity exists among enterococci from different sources. Using molecular characterisation of resistance determinants for enterococci isolated from processed meat products and cheeses, Teuber et al. (1996) showed them either to be similar or identical to corresponding determinants known from clinical samples. Valdivia et al. (1996) showed that the incidence of antibiotic resistance, as well as aggregation response to sex pheromones, was much higher in clinical strains than isolates from municipal waste water. Regarding the food chain, it is not clear whether and with which frequency VRE strains are transferred. Therefore, enterococci from food should be investigated for the presence of other potential virulence factors such as AS or particular proteases. In addition, studies should focus on determining possible differences in antibiotic resistance patterns and other potential virulence factors of enterococci isolated from community and clinical sources.

It has been reported that strains of enterococci isolated from dairy products do not produce haemolysin (Arihara et al., 1993; Giraffa, 1995), and it was suggested that absence of haemolytic activity should be a selection criterion for starter strains for dairy use (Giraffa, 1995). It is now known that haemolytic activity is not necessarily associated with all clinical isolates (see above); therefore, absence of haemolytic activity in enterococci isolated from food does not mean that these bacteria are nonvirulent.

Antibiotic-resistant enterococci have been isolated from foods such as raw milk cheeses, raw meats and sausages (Batish and Ranganathan, 1986; Knudtson and Hartman, 1993b; Perreten and Teuber, 1995; Perreten et al., 1997; Wegener et al., 1997). For example, Teuber et al. (1996) showed that enterococci isolated from Salami and Landjäger-types of fermented sausage were frequently resistant to streptomycin and lincomycin, while isolates from Emmental and Appenzeller cheeses showed a high frequency of resistance to erythromycin, gentamicin, tetracycline and/or vancomycin. Enterococci harbouring conjugative antibiotic resistance transposons and (or) plasmids are present in the human food chain and antibiotic resistance was transferred by filter matings to foodborne pathogens, such as *L. monocytogenes* and *S. aureus*, as well as to other bacteria which are commonly associated with foods, such as *L. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* (Perreten et al., 1997). There have not been any comprehensive studies on possible differences in antibiotic resistance patterns and virulence factors of food and clinical isolates. However, molecular characterisation of some resistance determinants showed them to be similar to those from clinical specimens. These observations also included resistance transfer mechanisms such as conjugative plasmids and transposons (Teuber et al., 1996; Perreten et al., 1997).

The principal concern for enterococci in the food supply is their pathogenic potential based on horizontal transfer of genes for factors associated with virulence and antibiotic resistance. In the hospi-

tal setting, enterococci acquire multiple antibiotic resistance from their efficient gene transfer mechanisms, which may also encode factors which are associated with virulence, i.e. aggregation substance, haemolysin or gelatinase (Coque et al., 1995). Whether or not these bacteria should be considered pathogens in food, the concern is that in the hospital setting they can rapidly acquire plasmid-encoded genes for antibiotic resistance and virulence traits, and can become pathogenic. The enterococci used in foods or as probiotics are usually strains of *E. faecium*. Most bacteriocin-producing enterococci described for use as starter cultures are also strains of *E. faecium*. The pathogenic potential of *E. faecalis* is considered to be greater than that of *E. faecium*, because greater than 80% of enterococci associated with human infections are *E. faecalis* (Jett et al., 1994). This may reflect the fact that transfer of plasmids in reponse to sex pheromones appears to be specific for *E. faecalis*. This genetic transfer mechanism is highly efficient and it is thought to be associated with virulence factors. On the other hand, vancomycin-resistance has been linked to strains of *E. faecium* (Gordts et al., 1995; Morrison et al., 1997), and the importance of vancomycin-resistant *E. faecium* in nosocomial disease cannot be disregarded.

Even though *E. faecalis* seems to have a greater pathogenic potential than *E. faecium*, the association of either of these species with food may not be considered desirable. Although present evidence does not suggest enterococci to be regarded as foodborne pathogens, the food chain has clearly been established as an important source of enterococci in the human environment, some strains of which – albeit at a low frequency – may bear resistance traits to glycopeptide and other antibiotics. The incidence of enterococci in human disease, however, does not appear to correlate with the incidence of these organisms in foods, especially when their use as starter cultures or as probiotics is taken into consideration. On the other hand, Teuber et al. (1996) concluded from data obtained over 15 years that fermented foods such as cheese and cured sausages may contribute to the distribution of antibiotic-resistant bacteria (staphylococci and enterococci) to the consumer. With foods as a potential source of enterococci bearing plasmids for antibiotic resistance and factors associated with virulence, it would therefore be prudent for food manufacturers to be cognisant of these organisms and to exercise better control over their presence in and colonisation of foods than is presently the case.

12. Conclusions and recommendations

The implications of food transmission and intestinal colonisation of enterococci for their control in the food supply are not clear. On the positive side, enterococci are associated with desired flavour attributes of some Southern European cheeses, whilst in some probiotics they represent the dominant or sole organism. However, in most cases the enterococci constitute an unnecessary adventitious microflora of foods, especially of heat-treated and processed products. For example, enterococci should not be considered an acceptable microflora of in-package heated meats. While reworking of processed meat in an industrial setting makes economic sense, it may lead to the establishment of enterococci as the predominant microflora of the meat. These bacteria are undesirable because of their potential role in spoilage and quality defects. Moreover, if they carry antibiotic resistance genes and possible virulence factors, they would constitute a definite health risk. Special care should be taken that enterococcal strains used as probiotics or starter cultures should not acquire antibiotic resistance or carry potential virulence factors.

Of further concern is the potential risk of vertical transfer of antibiotic resistance and virulence traits to other LAB in foods. This applies particularly when antibiotic resistance genes or possible virulence traits are encoded on broad-host-range plasmids. Although streptococci and enterococci are the predominating LAB associated with human infections, other LAB have been implicated in human infections, albeit at a low incidence and mainly associated with immunodeficient hosts (Aguirre and Collins, 1993; Gasser, 1994; Adams and Marteau, 1995).

Clearly, the safety of foods that contain enterococci is an issue that the food industry must address. Control of enterococci in foods is important especially for those consumers who are at highest risk, i.e. the elderly, the hospitalised and the immunocompromised, i.e. people belonging to the YOPI-group of vulnerable consumers as defined by Mossel and

Struijck (1993) and Mossel et al. (1998). The food supply should be safe for all, including those who are at risk for opportunistic infections. This point of view was recently advocated by Mossel et al. (1998), who also recommended that in order to obtain protection of the entire citizenry, the minimal infectious range of the pathogenic organisms should be kept low. However, the control of enterococci may constitute a special challenge because of their robust nature, their wide distribution and their stability in the extraenteral environment.

In foods where other LAB grow as a competitive microflora, the use of bacteriocinogenic LAB strains with specific activity against enterococci, offers a strategy for their control. Bacteriocin-producing LAB have already been used successfully in application-type studies to inhibit target pathogens, e.g. *Listeria monocytogenes* (Muriana, 1996; Stiles, 1996). However, the use of bacteriocins or bacteriocin-producing LAB to control enterococci in foods has not been studied yet. Enterocins which are active against a wide range of enterococci other than the producer strain show potential for such a control strategy. The genes necessary for enterocin production may be transferred to other LAB used as starter cultures and which are generally recognised as safe (GRAS). In this way, growth of enterococci could be inhibited by the enterocin produced by a heterologous host, and thereby transmission of potentially pathogenic enterococci in a food could be reduced.

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