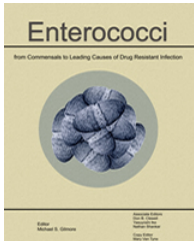




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## Enterococcus Diversity, Origins in Nature, and Gut Colonization

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### Abstract

Enterococci have evolved over eons as highly adapted members of the gastrointestinal consortia of a wide variety of hosts—humans and other mammals, birds, reptiles and insects—but for reasons that are not entirely clear, they emerged in the 1970s as some of the leading causes of multidrug-resistant, hospital-acquired infections. The taxonomy of enterococci has changed considerably over the past ten years, and the genus now includes over forty distinct species with various habitats, tropisms, and metabolic and phenotypic characteristics. These habitats include animal hosts, as well as plants, soil and water, and man-made products, including fermented foods and dairy products. Antibiotic-resistant strains of enterococci have emerged in many of these habitats, and strains with novel resistance mechanisms are isolated with alarming regularity. As a result, the relationship between the non-therapeutic use of antibiotics and the occurrence of enterococci in various non-human habitats is of substantial interest.

### Introduction

The term "enterococcus" has its origin at the end of the 19th century when Thiercelin described a saprophytic coccus, of intestinal origin, capable of causing infection (Thiercelin & Jouhaud, 1899; Thiercelin, 1899). The same year, MacCallum and Hastings characterized a similar organism, now known to be *Enterococcus faecalis*, from a lethal case of endocarditis, thus providing a first detailed description of its pathogenic capabilities (MacCallum & Hastings, 1899). These early descriptions constitute the basis for the paradigm of enterococcal pathogenesis as that of a commensal opportunist. The emergence of multi-drug resistant enterococci over the past 50 years has substantially compounded this problem, and antibiotic resistant enterococci are now leading causes of nosocomial infection worldwide (Prabaker & Weinstein, 2011; Werner, et al., 2008).

Phylogenetically, the genus *Enterococcus* belongs to the low GC branch of Gram-positive bacteria (i.e. 37.5% of GC in the *Enterococcus faecalis* chromosome) (Paulsen, et al., 2003). Enterococci were classified as group D streptococci (Sherman J. M., The streptococci, 1937; Sherman J. M., The enterococci and related streptococci, 1938) until 1984, when *Streptococcus faecalis* and *Streptococcus faecium* were reclassified as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer & Kilpper-Balz, 1984).

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The *Enterococcus* genus is placed in the Enterococcaceae family (Ludwig, Schleifer, & Whitman, 2009), along with genus *Bavariicoccus*, *Catelicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus* and *Vagococcus*, and consists of species that occur in human and animal gastro-intestinal (GI) tracts (Mundt, 1963; Mundt, 1986), as well as in the guts of insects (Martin & Mundt, 1972), traditional fermented food and dairy products (Mundt, 1986), and in various environments including plants ((Mundt, 1963), soil (Mundt, 1961) and water (Ator & Starzyk, 1976). Undoubtedly, an important key to their broad distribution in nature stems from being remarkably resilient organisms that are capable of enduring a broad range in pH and temperatures, as well as hypotonic and hypertonic conditions (Sherman J. M., The enterococci and related streptococci, 1938). Enterococci are ubiquitous in GI tracts even though they constitute a small proportion of the gut consortium, typically comprising less than 1% of the adult microflora (Finegold, Sutter, & Mathisen, 1983; Sghir, Gramet, Suau, Violaine, Pochart, & Dore, 2000). Little is known about the main mechanisms used by enterococci to colonize GI tracts of either healthy individuals or hospitalized patients. Nevertheless, exposure of hospitalized patients to antibiotics results in major modifications of the gut microbiota, which facilitate colonization of the GI tract by drug-resistant enterococci (Donskey, et al., 2000; Ubeda, et al., 2010).

In this chapter, we review the literature on the diversity of species that belong to the *Enterococcus* genus, discuss their origin and distribution in nature, and focus on the gut colonization by the enterococci and their transition from commensal to opportunistic pathogens.

## The *Enterococcus* genus: definition and diversity

### Historical perspective

In his report to the French Society of Biology in 1899, Thiercelin shared his observations of a saprophytic microbe that pullulates in the GI tract of humans along with the *Bacterium coli* (Thiercelin & Jouhaud, 1899). He described this microbe as a Gram-positive diplococcus and proposed the name "Enterocoque" to emphasize its morphology and its intestinal origin (Thiercelin & Jouhaud, 1899). Among the many initial observations in this paper was the observation that this microbe was pathogenic for mice, and susceptible to becoming pathogenic for humans (Thiercelin & Jouhaud, 1899). Microscopically, Thiercelin observed a halo surrounding some cells, similar to that observed for *Pneumococcus*, especially in the context of an intestinal disease, which suggested the presence of a capsule *in situ* (Hancock & Gilmore, 2002; Thiercelin & Jouhaud, 1899). Two months later, he described the morphological polymorphism of enterococcal cells (Thiercelin, 1899). He noted that since his previous report, this microbe had been found to be predominant in the GI tract of a patient with diarrhea, which then translocated from the intestine to the blood, which led to septicemia (Thiercelin, 1899). At nearly the same time, MacCallum and Hastings provided a detailed report of a case of acute endocarditis in a 37-year-old patient, caused by an organism they called *Micrococcus zymogenes* (163), now recognized as a strain of *Enterococcus faecalis*. This bacterium was continuously isolated from the patient's blood until his death due to cardiac failure (MacCallum & Hastings, 1899). The authors described that this bacterium was "very hardy and tenacious of life" (MacCallum & Hastings, 1899), which are now well-recognized traits of the *Enterococcus* genus. MacCallum and Hastings thoroughly investigated the biochemical capabilities of the enterococci and showed that this particular isolate was hemolytic (MacCallum & Hastings, 1899). The accurate detail in the descriptions of the pathogenic traits of these opportunistic commensal bacteria is particularly striking.

In 1906 the name *Streptococcus faecalis* was used by Andrewes and Horder (Andrewes & Horder, 1906) to characterize an organism of fecal origin that clotted milk, similar to the observations of MacCallum and Hastings, and that also fermented mannitol and lactose, but not raffinose. The name *faecalis* was chosen to emphasize that this *Streptococcus* was characteristic of the human intestine, even though the organism was isolated from a patient with endocarditis in this study (Andrewes & Horder, 1906). Orla-Jensen subsequently reported the first description of *Streptococcus faecium*, which differed from the fermentation patterns described for *S. faecalis* (Orla-Jensen, 1919). About twenty years later, a third species, *Streptococcus durans*, was described

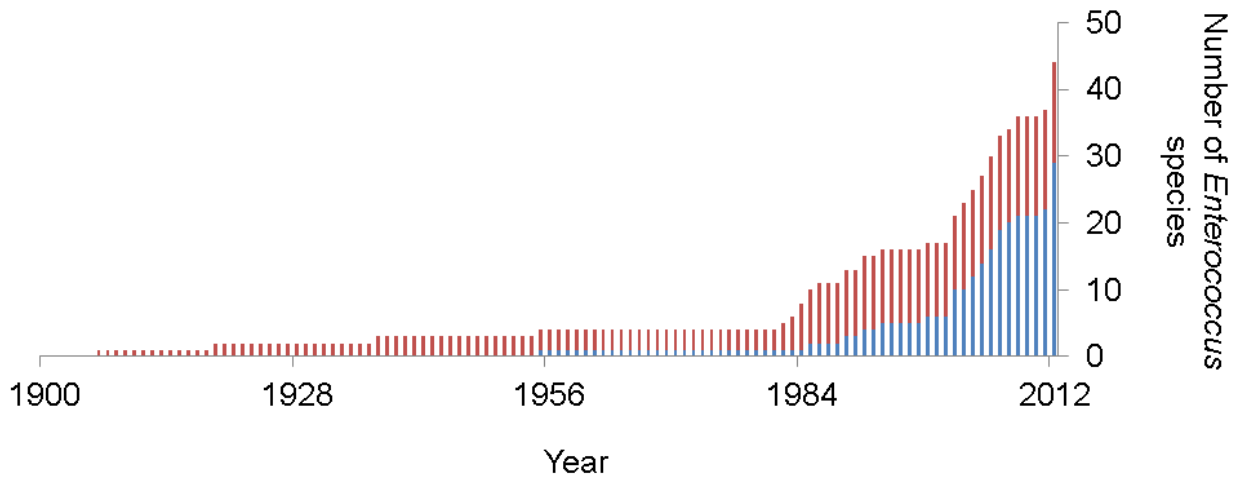
by Sherman and Wing (Sherman & Wing, 1935) as being similar to *S. faecium*, but with more restricted fermentation capabilities. Sherman subsequently proposed a classification scheme that separated streptococci into four groups: pyogenic, viridans, lactic, and enterococcus (Sherman, 1937). The term "enterococcal group" included *S. faecalis* (hemolysis negative and proteolysis negative), *S. faecalis* var. *liquefaciens* (hemolysis negative and proteolysis positive), *S. faecalis* var. *hemolyticus* (hemolysis positive and proteolysis negative), *S. faecalis* var. *zymogenes* (hemolysis positive and proteolysis positive), and *S. durans*; and was used to describe streptococci that grew between 10 and 45° C, grew in broth at pH 9.6, grew in 6.5% NaCl, and survived heating to 60°C for 30 min (Sherman, 1937). Noteworthy, *S. faecium*, described by Orla-Jensen earlier, was considered by Sherman to be the same as *S. faecalis* (Sherman, 1937), and was only officially recognized as a separate species in the mid-1960s (Barnes, 1956; Breed, Murray, & Smith, 1957; Deibel, 1964).

In 1957, motile and yellow-pigmented enterococci were described and the name *S. faecium* var. *casseliflavus* (emphasizing the yellow coloration) was proposed (Graudal, 1957; Mundt, 1986). A decade later, Nowlan and Deibel (Nowlan & Deibel, 1967) added *Streptococcus avium* to the enterococcal group. As early as 1970, Kalina (Kalina, 1970) proposed the creation of the taxon *Enterococcus*, based on cellular arrangement and phenotypic characteristics of species in the enterococcal group. However, this genus was not formally accepted until 1984, when Schleifer and Kilpper-Balz (Schleifer & Kilpper-Balz, 1984) provided genetic evidence that *S. faecalis* and *S. faecium* were sufficiently distant from other members of the *Streptococcus* genus. Collins and collaborators (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984) used a similar genetic approach to show that strains previously named *Streptococcus avium*, *Streptococcus casseliflavus*, *Streptococcus durans* and *Streptococcus gallinarum*, were distinct species but closely related to those of the genus *Enterococcus*. In 2002, an extensive review of the literature (Facklam, Carvalho, & Teixeira, 2002) reported the existence of 23 distinct *Enterococcus* species.

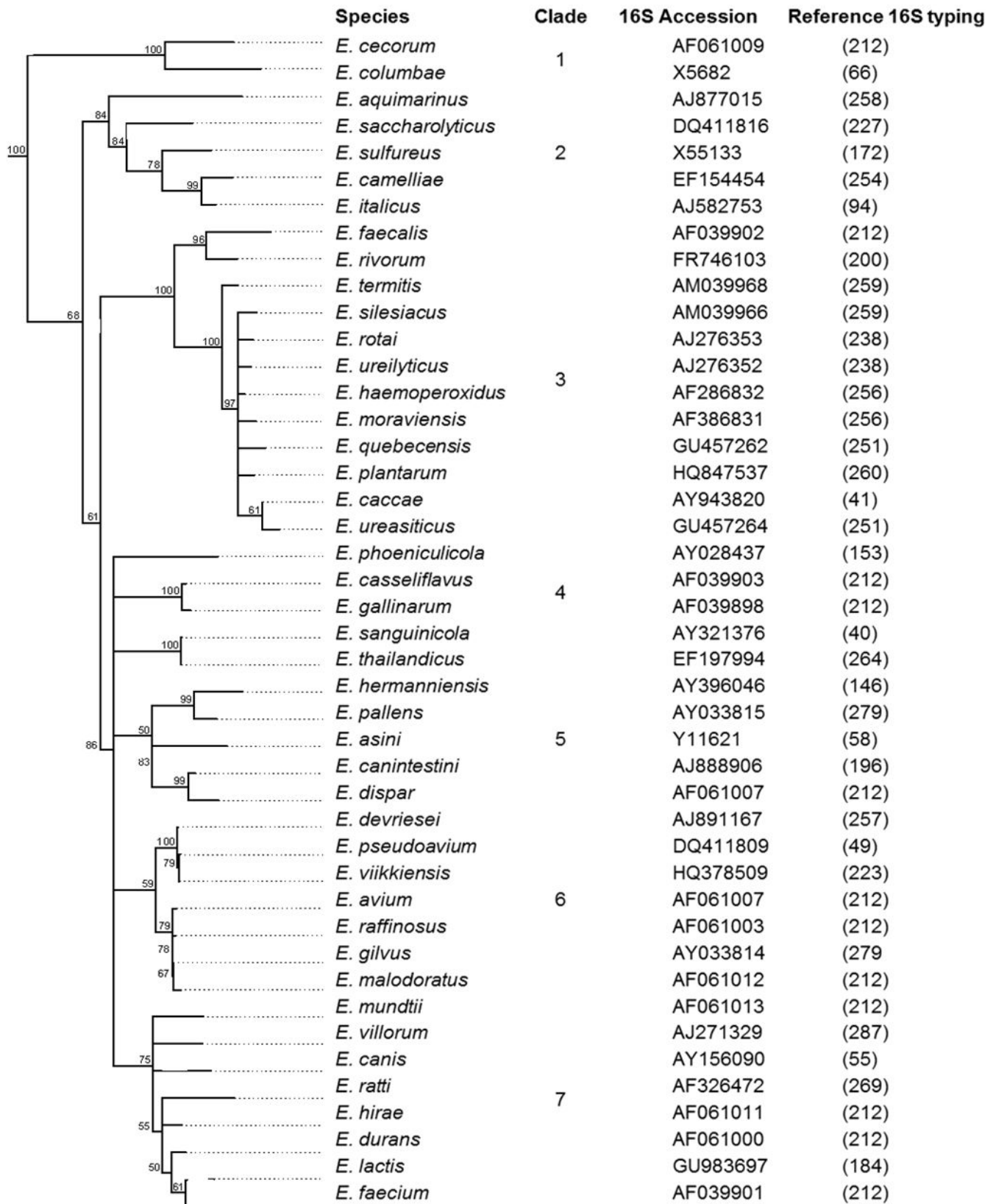
Considering the ubiquitous distribution in nature of the *Enterococcus* genus, it is noteworthy that the three *Enterococcus* species (*E. faecalis*, *E. faecium* and *E. durans*) that were recognized prior to 1950 (Figure 1), are capable of causing human infection. *E. faecalis* and *E. faecium* are considered to be the most abundant enterococci in human feces (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Finegold, Sutter, & Mathisen, 1983; Noble, 1978; Patel, et al., 1998), and account for most enterococcal disease (Malani, Kauffman, & Zervos, 2002). *E. durans* occurs occasionally among the fecal flora of healthy adults (Ruoff, 1990). Until the mid-1990s, *E. faecalis* accounted for 90–95% of clinical isolates, and *E. faecium* infection was rare (Huycke, Sahm, & Gilmore, 1998). Since then, the proportion of *E. faecium* isolates has increased, largely due to the spread of resistance to antibiotics, particularly vancomycin and ampicillin (Arias & Murray, 2012; Gilmore, Lebreton, & van Schaik, 2013; Huycke M. M., et al., 2002). Other species, including *Enterococcus avium*, *gallinarum*, *casseliflavus*, *hirae*, *mundtii*, and *raffinosis*, also have been isolated from human infection (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Hammerum A. M., 2012; Murray, 1990). *Enterococcus gallinarum* and *Enterococcus casseliflavus* infections are of special interest because of their intrinsic resistance to vancomycin, an antibiotic used to treat the aminoglycoside-resistant enterococcal infections that became problematic in the mid-1980s.

Among the 30 species of *Enterococcus* described between 1992 and 2012 (Fig. 1, Table 1), only four (*E. sanguinicola*, *E. gilvus*, *E. pallens* and *E. canintestini*) have been associated with human infection (Carvalho, et al., 2008; Tan, et al., 2010; Tyrell, et al., 2002). Additionally, *E. caccae* has been isolated from human feces (Figure 2) (Carvalho, et al., 2006). The remaining species described since 1992 were isolated from non-human sources, including animals (De Graef, et al., 2003; de Vaux, Laguerre, Diviès, & Prévost, 1998; Devriese, Pot, & Collins, 1993; Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004; Law-Brown & Meyers, 2003; Švec, et al., 2005), plants (Rahkila, Johansson, Säde, & Björkroth, 2011; Sedláček, et al., 2013; Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007; Švec, et al., 2011), water (Niemi, et al., 2012; Sedláček, et al., 2013; Sistik, et al., 2012; Svec, et al., 2001; Svec, et al., 2005; Svec, et al., 2006) and food (Fortina, Ricci, Mora, & Manachini, 2004; Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004; Morandi, Cremonesi, Povolo, & Brasca, 2012;

Tanasupawat, Sukontasing, & Lee, 2008) (Figure 2). Although much of the interest in enterococci historically has been driven by their association with infection and human carriage, it is clear that this is only the tip of the *Enterococcus* genus diversity iceberg, and comparison of these species to enterococci from other environments will reveal much about the basic features and origins of the genus.



**Figure 1.** Cumulative *Enterococcus* species identified. Red bars show the number of species associated with human disease.



**Figure 2.** Dendrogram of the genus *Enterococcus*. A dendrogram of the available 16S rRNA gene sequences for members of the *Enterococcus* genus was compiled using the Geneious software (Biomatters Ltd) using the neighbour-joining algorithm and the 16S sequence of *Tetragenococcus solitarius* as an outgroup. Bootstrap values were generated over 1000 iterations. (Adapted from Gilmore et al., 2013)

**Table 1.** Species part of the *Enterococcus* genus

a.	m.
<i>Enterococcus aquimarinus</i> (Svec, et al., 2005)	<i>Enterococcus malodoratus</i> (Pette, 1955)
<i>Enterococcus asini</i> (de Vaux, Laguerre, Diviès, & Prévost, 1998)	<i>Enterococcus moraviensis</i> (Svec, et al., 2001)
<i>Enterococcus avium</i> (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984)	<i>Enterococcus mundtii</i> (Collins, Farrow, & Jones, 1986)
c.	p.
<i>Enterococcus caccae</i> (Carvalho, et al., 2006)	<i>Enterococcus pallens</i> (Tyrell, et al., 2002)
<i>Enterococcus camelliae</i> (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007)	<i>Enterococcus phoeniculicola</i> (Law-Brown & Meyers, 2003)
<i>Enterococcus canintestini</i> (Naser, et al., 2005)	<i>Enterococcus plantarum</i> (Švec, et al., 2011)
<i>Enterococcus canis</i> (De Graef, et al., 2003)	<i>Enterococcus pseudoavium</i> (Collins, Facklam, Farrow, & Williamson, 1989)
<i>Enterococcus casseliflavus</i> (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984)	q.
<i>Enterococcus cecorum</i> (Devriese, Dutta, Farrow, Van De Kerckhove, & Phillips, 1983)	<i>Enterococcus quebecensis</i> (Sistek, et al., 2012)
<i>Enterococcus columbae</i> (Devriese, Ceysens, Rodrigues, & Collins, 1990)	r.
d.	<i>Enterococcus raffinosus</i> (Collins, Facklam, Farrow, & Williamson, 1989)
<i>Enterococcus devriesei</i> (Švec, et al., 2005)	<i>Enterococcus ratti</i> (Teixeira, et al., 2001)
<i>Enterococcus dispar</i> (Collins, Rodrigues, Pigott, & Facklam, 1991)	<i>Enterococcus rivorum</i> (Niemi, et al., 2012)
<i>Enterococcus durans</i> (Sherman & Wing, 1935)	<i>Enterococcus rotai</i> (Sedláček, et al., 2013)
f.	s.
<i>Enterococcus faecalis</i> (Andrewes & Horder, 1906)	<i>Enterococcus saccharolyticus</i> (Farrow, Kruze, Phillips, Bramley, & Collins, 1984)
<i>Enterococcus faecium</i> (Orla-Jensen, 1919)	<i>Enterococcus sanguinicola</i> (Carvalho, et al., 2008)
g.	<i>Enterococcus silesiacus</i> (Svec, et al., 2006)
<i>Enterococcus gallinarum</i> (Bridge & Sneath, 1982)	<i>Enterococcus sulfureus</i> (Martinez-Murcia & Collins, 1991)
<i>Enterococcus gilvus</i> (Tyrell, et al., 2002)	t.
h.	<i>Enterococcus termitis</i> (Svec, et al., 2006)
<i>Enterococcus haemoperoxidus</i> (Svec, et al., 2001)	<i>Enterococcus thailandicus</i> (Tanasupawat, Sukontasing, & Lee, 2008)
<i>Enterococcus hermanniensis</i> (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004)	u.
<i>Enterococcus hirae</i> (Farrow & Collins, 1985)	<i>Enterococcus ureasiticus</i> (Sistek, et al., 2012)
i.	<i>Enterococcus ureilyticus</i> (Sedláček, et al., 2013)
<i>Enterococcus italicus</i> (Fortina, Ricci, Mora, & Manachini, 2004)	v.
l.	<i>Enterococcus viikkiensis</i> (Rahkila, Johansson, Säde, & Björkroth, 2011)

Table 1. continued from previous page.

Enterococcus lactis (Morandi, Cremonesi, Povolò, & Brasca, 2012)	Enterococcus villorum (Vancanneyt, et al., 2001)
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## Definition of the genus and criteria for identification

The core morphological and physiological features of all enterococci include being Gram-positive spherical or ovoid cells arranged in pairs or chains (Thiercelin, 1899). They are non-spore-forming facultative anaerobes and obligatory fermentative chemoorganotrophs. They typically have an optimum growth temperature of 35°C and a growth range from 10 to 45°C (Sherman, 1937). They typically grow in broth containing 6.5% NaCl, and hydrolyze esculin in the presence of 40% bile salts (Facklam, 1973). They are catalase reaction negative, and do not express complete cytochromes, although some species produce a catalase and appear catalase positive with weak effervescence. They are usually homofermentative, producing lactic acid as the end product of glucose fermentation, without production of gas (Klein, 2003; Murray, 1990). Some species are motile, such as *E. gallinarum* and *E. casseliflavus* (Graudal, 1957; Mundt, 1986). Pigmentation is a variable trait, with yellow-pigmented species including *E. sulfureus*, *E. casseliflavus*, and *E. mundtii* (Martinez-Murcia & Collins, 1991; Graudal, 1957; Mundt, 1986), and pigmented species are commonly found among plants (Aarestrup, Butaye, & Witte, 2002). Most enterococci, apart from *E. cecorum*, *E. columbae*, *E. pallens*, and *E. saccharolyticus*, *E. devriesei* (variable among strains), *E. canintestini*, *E. termitis* and *E. viikiensis* are capable of hydrolyzing pyrrolidonyl  $\beta$  naphthylamide and producing leucine aminopeptidase (Facklam, Carvalho, & Teixeira, 2002; Naser, et al., 2005; Rahkila, Johansson, Säde, & Björkroth, 2011; Švec, et al., 2005; Svec, et al., 2006).

Phylogenetic analysis of catalase-negative Gram-positive cocci, based on the comparison of ~1400 bases of the 16S rRNA gene, shows that the *Enterococcus* are more closely related to the *Vagococcus*, *Tetragenococcus*, and *Carnobacterium* than they are to the *Streptococcus* and *Lactococcus* (Facklam, Carvalho, & Teixeira, 2002). The G + C content of DNA ranges from 37 to 45 mol% (237). *Enterococcus faecalis* has long been known to require a number of amino acids (including Val, Leu, Ile, Ser, Met, Glu, Arg, His and Trp) and vitamins (including biotin, nicotinic acid, pantothenate, pyridoxine, riboflavin, and sometimes folic acid) for maximal growth, with other species being similar in their fastidiousness (Facklam, Carvalho, & Teixeira, 2002). As a reflection of their highly evolved role as members of a consortium in an extremely competitive environment, enterococci have reduced genomes that range from 2.7 Mb to 3.6 Mb across species sequenced so far (Gaechter, Wunderlin, Schmidheini, & Solioz, 2012; Qin, et al., 2012; Palmer, et al., 2012; van Schaik & Willems, 2010).

Species of the *Enterococcus* genus identified by DNA-DNA reassociation, 16S rRNA gene sequencing, and/or whole-cell protein analysis to rigorously establish the novel species, and the dates of their initial description are shown in Table 1. DNA-DNA reassociation remains the gold standard for establishing bacterial species (Johnson & Ordal, 1968; Tindall, Rosselló-Móra, Busse, Ludwig, & Kämpfer, 2010), and this analysis has been carried out for most enterococci (Facklam, Carvalho, & Teixeira, 2002). Sequencing of 16S rDNA has become a practical and reliable additional tool (Vandamme, et al., 1996). Additionally, whole-cell protein analysis using standardized techniques correlate well with DNA hybridization, and give unique signatures for each *Enterococcus* species tested (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Merquoir, Netz, Camello, & Texeira, 1997). For practical reasons, 16S rDNA sequencing and whole-cell protein analysis are beginning to replace DNA-DNA reassociation, and are increasingly being used to describe new species. These methodological improvements are important developments, since many enterococcal species vary by only one phenotypic trait (Knudtson & Hartman, 1992; Moore, Zhouwandai, Ferguson, McGee, Mott, & Stewart, 2006; Scheidegger, Fracalanza, Texeira, & Caradarelli-Leite, 2009; Bodnar, Noskin, Suriano, Cooper, Reisberg, & Peterson, 1996) using conventional biochemical tests or commercial test systems (Devriese, Pot, & Collins, 1993; Facklam, Carvalho, & Teixeira, 2002). By itself, 16S rRNA sequence analysis does not appear to provide the resolution needed to distinguish *Enterococcus* species such as *E. casseliflavus* and *E. gallinarum*, which are 99.9%



identical by this criterion (Patel, et al., 1998) (as opposed to 97% identity being the threshold usually used for species identification). As a consequence, additional complementary rapid molecular methods have been developed for routine identification of enterococcal isolates (Facklam, Carvalho, & Teixeira, 2002), including randomly amplified polymorphic DNA (RAPD) (Descheemaeker, Lammens, Pot, Vandamme, & Goossens, 1997; Monstein, Tiveljung, Kraft, Borch, & Jonasson, 2000; Quednau, Ahrné, Petersson, & Molin, 1998), sequencing of the domain V of the 23S rRNA gene (Poyart, Quesnes, & Trieu-Cuot, 2000), amplification of rRNA or tRNA intergenic spacers (Naimi, Beck, Monique, Lefèbvre, & Branlanti, 1999; Tyrell, Bethune, Willey, & Low, 1997), sequencing of the D-ala:D-ala ligase genes (*ddl*) (Ozawa, Courvalin, & Gaiimand, 2000) or the manganese-dependent superoxide dismutase (*sodA*) genes (Poyart, Quesnes, & Trieu-Cuot, 2000), sequencing and hybridization of the chaperonin 60 (*cpn60*) gene (Goh, et al., 2000), and amplification and probing of the *Enterococcus* protein A (*efaA*) gene (Singh, Coque, Weinstock, & Murray, 1998). Several additional molecular tests have been recently introduced to differentiate the *Enterococcus* species, including sequencing genes encoding the RNA polymerase  $\alpha$ -subunit (*rpoA*), the phenylalanyl-tRNA synthase (*pheS*) or the elongation factor Tu (*tufA*) (Naser, et al., 2005; Naser, et al., 2005; Picard, et al., 2004), sequencing repetitive extragenic palindromic PCR (REP-PCR) or BOX-PCR (Nayak, Badgley, & Harwood, 2011), pulsed-field gel electrophoresis (PFGE) (Gordillo, Singh, & Murray, 1993; Tenover, Arbeit, & Goering, 1997), and multilocus sequence typing (MLST) (Homan, et al., 2002; Ruiz-Garbajosa, et al., 2006). MLST has proven to be useful in epidemiological studies of *E. faecalis* and *E. faecium* (Leavis, Bonten, & Willems, 2006), and shows similar accuracy to PFGE for the identification of organisms at the subspecies level (Nallapareddy, Duh, Singh, & Murray, 2002).

## New species and reclassifications

The taxonomy of the enterococci has changed considerably, as the genus consisted of only 20 species at the end of the 20th century. Numerous new species have been described as the result of improvements in methods for differentiation, combined with growing interest in the enterococci (Figure 1) (Euzéby, 2013). Currently, the genus *Enterococcus* consists of 35 recognized species (Euzéby, 2013), with 8 more species (*Enterococcus lactis*, *plantarum*, *quebecensis*, *rivorum*, *rotai*, *ureasiticus*, *ureilyticus*, and *viikiensis*) likely to be added (Morandi, Cremonesi, Povolo, & Brasca, 2012; Niemi, et al., 2012; Rahkila, Johansson, Säde, & Björkroth, 2011; Sedláček, et al., 2013; Sistek, et al., 2012; Švec, et al., 2011). *E. sanguinicola* (Carvalho, et al., 2008) was recently described as likely to be a junior synonym of *Enterococcus thailandicus* (Shewmaker, et al., 2011), and probably will be removed. The list of *Enterococcus* species (Table 1) will doubtlessly continue to increase and be subject to reevaluation in the rapidly expanding genomic era, which offers new insights into distinctions between bacterial species (Konstantinidis, Ramette, & Tiedje, 2006). Since the diversity of the *Enterococcus* genus was extensively reviewed in 2002 (Facklam, Carvalho, & Teixeira, 2002), this review focuses on new enterococcal species that have been reported since then.

*Enterococcus aquimarinus* sp. nov. was isolated from sea water and was described by Švec et al. (Svec, et al., 2005) based on whole-cell protein analysis and 16S rDNA sequence. The 16S rDNA sequence shares 96.1–96.9% identity with its closest phylogenetic neighbors, *E. saccharolyticus*, *E. sulfureus*, and *E. italicus* (Svec, et al., 2005). Additional distinguishing features include *pheS* gene sequence polymorphism, (GTG)<sub>5</sub>-PCR fingerprinting (which identifies short sequence repeats (Lupski & Weinstock, 1992)), and biochemical characteristics. For example, *E. aquimarinus* is not able to produce acid from ribose, which distinguishes it from *E. sulfurous*, *E. saccharolyticus*, and the majority of other enterococcal species; but produces acid from L-Arabinose and other substrates which distinguishes it from *E. italicus* (Svec, et al., 2005). *E. aquimarinus* LMG 16607T was identified as the type strain (Svec, et al., 2005).

*Enterococcus caccae* sp. nov. (Carvalho, et al., 2006) was recovered from human stool, and was distinguished from other species by DNA–DNA hybridization, whole-cell protein analysis, and sequencing of the 16S rDNA (Carvalho, et al., 2006). Comparative 16S rDNA sequence analysis showed that *E. caccae* shared 99.6% identity with *E. moraviensis* (Carvalho, et al., 2006). However, the level of DNA–DNA relatedness between this novel

taxon and any of the currently recognized species of *Enterococcus* was surprisingly low (32% for *E. moraviensis*), and the differences in the protein pattern supported the novelty of this species (Carvalho, et al., 2006). *E. caccae* can be differentiated from *E. moraviensis* by its ability to grow at 45 °C and a positive Voges-Proskauer test, as well as its inability to produce acid from arabinose, lactose and mannitol (Carvalho, et al., 2006). The type strain of the *E. caccae* species is 2215-02T (Carvalho, et al., 2006).

*Enterococcus camelliae* sp. nov., isolated from fermented tea leaves in Thailand, was proposed as a new species based on sequence analysis of its 16S rDNA and *rpoA* genes, and global DNA–DNA hybridization analysis (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). On the basis of the 16S rRNA and the *rpoA* sequence comparison, *E. camelliae* is closely related to *Enterococcus italicus* (99.2% and 93.8% sequence identity, respectively) (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). However, it shared only 33.8% genome identity based on DNA-DNA hybridization, and possessed phenotypic characteristics, including its inability to produce acid from D-galactose and lactose, which distinguishes it from *E. italicus* and other enterococci (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). Its type strain is *E. camelliae* FP15-1T (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007).

The taxonomic position of isolates obtained from healthy dog faeces and originally classified as *Enterococcus dispar* was reinvestigated by Naser and collaborators (Naser, et al., 2005), who determined them to be a different species that they named *Enterococcus canintestini* sp. nov. The 16S rDNA gene sequence showed the highest similarities (between 98–99%) with *E. dispar*, *E. canis* and *E. asini*; however, *E. canintestini* shared only 33.8% genome identity, based on DNA-DNA hybridization with *E. dispar*, and showed a substantially different whole-cell protein profile (Naser, et al., 2005). Multilocus sequence analysis (MLSA) of the genes that encode the alpha subunit of ATP synthase (*atpA*), *rpoA*, and *pheS* was also performed (Naser, et al., 2005). Additionally, biochemical characteristics, including the inability to produce acid from raffinose and D-melibiose, distinguished *E. canintestini* from *E. dispar* and other enterococci (Naser, et al., 2005). The type strain is *E. canintestini* LMG 13590T (Naser, et al., 2005).

*Enterococcus canis* sp. nov. was proposed by De Graef and collaborators (De Graef, et al., 2003) for strains isolated from rectal swabs and chronic otitis in dogs. While the 16S rDNA sequence shared 98.4% to 99% similarity with members of the *E. faecium* species group, the level of DNA-DNA reassociation with these species was very low (7–13%) (De Graef, et al., 2003). *E. canis* shares a number of phenotypic characteristics with the *E. faecium* species group, but can be distinguished by its inability to hydrolyse arginine or to grow on selective media that contain 0.04% sodium azide (55). The type strain is *E. canis* LMG 12316T (De Graef, et al., 2003).

The species *Enterococcus devriesei* sp. nov. was proposed by Švec *et al.* (Švec, et al., 2005), after having reinvestigated the taxonomic position of two isolates of bovine origin that were originally assigned to the species *Enterococcus raffinosus* based on biochemical reactions. These strains, as well as two additional strains obtained from a charcoal-broiled river lamprey and from the air of a poultry slaughter by-product processing factory, showed key differences from *E. raffinosus* and all other enterococcal species (Švec, et al., 2005). The 16S rDNA sequence indicated a phylogenetic position in the *Enterococcus avium* species group, and showed *Enterococcus pseudoavium* as the closest phylogenetic relative (99.8–99.9% identity) (Švec, et al., 2005). However, whole cell protein electrophoresis, (GTG)<sub>5</sub>-PCR fingerprinting, ribotyping, and DNA-DNA hybridization (with reassociation values between 25% and 34% with the closest phylogenetic neighbors) confirmed that these strains were a novel species (Švec, et al., 2005). A combination of phenotypic traits, including the ability to produce acid from lactose (but not from melibiose or 2-ketogluconate) is helpful for distinguishing *E. devriesei* from species of the *E. avium* group (Švec, et al., 2005). However, there is variability within the phenotypic traits of this species (Švec, et al., 2005). The type strain is *E. devriesei* LMG 14595T (Švec, et al., 2005).

*Enterococcus hermanniensis* sp. nov. was proposed by Koort (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004) for strains isolated from modified-atmosphere-packaged broiler meat, and was also detected on canine tonsils. *E. hermanniensis* isolates were placed in the *Enterococcus avium* group, with *E. pallens* as their closest

phylogenetic neighbor, based on 16S rDNA sequence (99% sequence similarity) (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004). Whole-cell protein analysis as well as a low level of DNA-DNA hybridization (12-30%), however, distinguishes this species from *E. pallens* and other known enterococci (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004). Interestingly, *E. hermanniensis* strains fails to produce acid from most common substrates, further distinguishing this species from other enterococci (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004). The type strain is *E. hermanniensis* LMG 12317T (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004).

Fortina *et al.* (Fortina, Ricci, Mora, & Manachini, 2004) investigated the taxonomic position of atypical *Enterococcus* strains isolated from artisanal Italian cheeses, and proposed the name *Enterococcus italicus* sp. nov. for strains that were found to be unique. The closest relatives are *Enterococcus sulfureus* and *Enterococcus saccharolyticus*, which possess 96% and 97% similarity based on 16S rDNA sequence, respectively (94). *E. italicus* strains can be easily differentiated from the other *Enterococcus* species by DNA-DNA hybridization and phenotypic characteristics (*e.g.*, lack of growth in 6.5% NaCl, and lack of acid production from L-arabinose, melezitose, melibiose, raffinose or ribose). The type strain is *E. italicus* DSM 15952T (Fortina, Ricci, Mora, & Manachini, 2004).

*Enterococcus lactis* sp. nov. was proposed by Morandi *et al.* (Morandi, Cremonesi, Povolo, & Brasca, 2012) for other atypical *Enterococcus* strains isolated from raw milk Italian cheeses. On the basis of 16S rDNA sequence analysis, the strains were found to be closely related to *E. hirae*, *E. durans*, and *E. faecium*, with a sequence similarity greater than 98.8% (Morandi, Cremonesi, Povolo, & Brasca, 2012). Since no DNA-DNA hybridization or whole-cell protein analysis have been conducted, the authors based the description of this new species on the analysis of the cellular fatty acid composition between *E. lactis* and its phylogenetic neighbors *E. faecium* and *E. durans* (Morandi, Cremonesi, Povolo, & Brasca, 2012). The authors indicated that *E. lactis* strains can be differentiated from other *Enterococcus* species based on the *pheS* gene sequence, RAPD-PCR, 16S-23S rRNA gene intergenic region analysis (Morandi, Cremonesi, Povolo, & Brasca, 2012). The proposed type strain is *E. lactis* DSM 23655T (Morandi, Cremonesi, Povolo, & Brasca, 2012).

*Enterococcus phoeniculicola* sp. nov. was proposed by Law-Brown and Meyers (Law-Brown & Meyers, 2003) to identify a single strain isolated from the uropygial gland of wild red-billed woodhoopoes (*Phoeniculus purpureus*). Examination of the 16S rDNA sequence showed that its closest relatives are *E. faecium* and *E. avium* (97.3% and 97.5% respectively). However, neither DNA-DNA hybridization nor whole-cell protein analyses were conducted. *E. phoeniculicola* may be differentiated from *E. faecium* and *E. avium* by its inability to form acid from lactose, D-mannitol, D(+)-melezitose or D-sorbitol, as well as its inability to grow in the presence of 40% bile or 6.5% NaCl (153). Its type strain is *E. phoeniculicola* DSM 14726T (Law-Brown & Meyers, 2003).

*Enterococcus plantarum* sp. nov. was proposed after investigation of the taxonomic position of eight strains isolated during characterization of enterococcal populations on plants (Švec, *et al.*, 2011). Rep-PCR fingerprinting indicated that the isolates constituted a single cluster that was distinct from known enterococcal species (Švec, *et al.*, 2011). In addition, the 16S rDNA sequence showed that these isolates clustered with the *E. faecalis* species group (Švec, *et al.*, 2011). *E. plantarum* was confirmed to be a novel species by whole-cell protein fingerprinting, and can be differentiated from its closest phylogenetic neighbors by *pheS* and *rpoA* gene sequence analysis (Švec, *et al.*, 2011). The authors described the colonies as "yellowish" on agar, but did not describe this species as pigmented (Švec, *et al.*, 2011). The type strain is *E. plantarum* LMG 26214T (Švec, *et al.*, 2011).

*Enterococcus quebecensis* sp. nov. was recently proposed by Sistek and collaborators (Sistek, *et al.*, 2012) to identify a single isolate that originated from water sampled in the Province of Québec, Canada. Sequence analysis of genes 16S rRNA, *rpoA*, *pheS*, *tufA*, and *atpD*, as well as the results of AFLP DNA fingerprinting and DNA-DNA hybridization, confirmed the status of this strain as distinct from other enterococcal species (Sistek, *et al.*, 2012). Based on the 16S rDNA phylogeny, *E. quebecensis* is closely related to *E. moraviensis*, and clusters in the *E. faecalis* group. *E. quebecensis* strains are negative for the Voges-Proskauer test, meaning that they do not

produce acetoin as a result of fermentation (Sistek, et al., 2012). The type strain is *E. quebecensis* DSM 23327T LMG 26306T (Sistek, et al., 2012).

*Enterococcus rivorum* sp. nov. was proposed by Niemi and collaborators (Niemi, et al., 2012) after taxonomic investigation of strains that originated from pristine waters in Finland. The authors showed by genotypic analysis that *E. rivorum* strains are closely related to the *E. faecalis* group (Niemi, et al., 2012). However, DNA-DNA hybridization confirms that they are a new species (Niemi, et al., 2012). Growth at 45 °C, or in broth containing 6.5% NaCl, is weak or absent; and production of the D antigen is variable (200). Slow growth occurs at 10 °C (Niemi, et al., 2012). The type strain is *E. rivorum* LMG 25899T = CCM 7986T (Niemi, et al., 2012).

Very recently, Sedláček et al. (Sedláček, et al., 2013), proposed the name *Enterococcus rotai* sp. nov. for urease-producing, yellow-pigmented enterococci isolated from environmental sources, including drinking water, plants, and mosquitoes, and placed them in the *Enterococcus faecalis* species group. These strains show resistance to clindamycin, chloramphenicol, and oxacillin, with most also being resistant to ofloxacin—but they are sensitive to imipenem, and most strains are sensitive to gentamicin as well (Sedláček, et al., 2013). The type strain is *E. rotai* LMG 26678T (Sedláček, et al., 2013).

Two isolates from drinking water in the region of Silesia in Czech Republic were shown to be distinct from other enterococcal species by DNA-DNA hybridization, and were named *Enterococcus silesiacus* sp. nov. (Svec, et al., 2006). The 16S rRNA gene sequence analysis classified these strains in the *E. faecalis* species group. The type strain is *E. silesiacus* LMG 23085T (Svec, et al., 2006). During the same study, Švec and collaborators (Svec, et al., 2006) identified an isolate from the gut of a termite, and proposed the name *Enterococcus termitis* sp. nov. for this species. As for *E. silesiacus*, *E. termitis* is part of the *E. faecalis* species group based on 16S rDNA analysis (Svec, et al., 2006). *E. termitis* strains are pyruvate and Voges-Proskauer test negative, which is unusual among the enterococci (Svec, et al., 2006). The type strain is *E. termitis* LMG 8895T (Svec, et al., 2006).

In 2008, a single strain was isolated from fermented sausage in Thailand, named *Enterococcus thailandicus* sp. nov., was examined and proposed as a new species (Tanasupawat, Sukontasing, & Lee, 2008). By DNA-DNA hybridization, this strain shared less than 70% identity with known species, but was found to be related to *E. hirae*, *E. durans*, and *E. faecium*, based on 16S rDNA and *rpoA* gene sequence analysis (Tanasupawat, Sukontasing, & Lee, 2008). The type strain is *E. thailandicus* FP48-3T (Tanasupawat, Sukontasing, & Lee, 2008).

*Enterococcus ureasiticus* sp. nov. (which refers to the presence of urease activity) was proposed based on the study of two enterococcal strains isolated from water samples that differed from other *Enterococcus* species by more than 30%, based on DNA-DNA hybridization (Sistek, et al., 2012). 16S rDNA sequence analysis classified them in the *E. faecalis* species group, but this new species harbored divergent *rpoA*, *pheS*, *tufA*, and *atpD* sequences, as well as AFLP-DNA fingerprinting patterns (Sistek, et al., 2012). The type strain is *E. ureasiticus* FP48-3T (Sistek, et al., 2012).

*Enterococcus ureilyticus* sp. nov. was proposed as a name for a novel *Enterococcus* species identified in a study of strains that inhabit both water and plants (Sedláček, et al., 2013). These urease-producing, yellow-pigmented strains were placed in the *E. faecalis* species group, based on 16S rDNA sequence (Sedláček, et al., 2013). Analysis of *pheS* and *rpoA* genes sequences, as well as as whole-cell protein electrophoresis and DNA-DNA hybridization, indicated significant divergence from other enterococci (Sedláček, et al., 2013). Strains of this species showed resistance to chloramphenicol, oxacillin, and tetracycline, as well as sensitivity to imipenem (Sedláček, et al., 2013). The type strain is *E. ureilyticus* LMG 26676T (Sedláček, et al., 2013).

Finally, Rahkila *et al.* (Rahkila, Johansson, Säde, & Björkroth, 2011) proposed the species *Enterococcus viikkiensis*, with strain LMG 26075T being the type strain. This strain and four others were isolated from air from a broiler processing plant. Based on 16S rDNA sequence analysis, they belong to the *E. avium* group, with *E. devriesei* being their closest neighbor (Rahkila, Johansson, Säde, & Björkroth, 2011). Notably, these strains are also negative for pyruvate utilization (Rahkila, Johansson, Säde, & Björkroth, 2011).

The identification of enterococcal species is dynamic and is continually being revised. The species *Enterococcus seriolicida*, originally proposed by Kusuda (Kusuda, Kawai, Salati, Banner, & Fryer, 1991), has been reclassified as *Lactococcus garvieae*, based on DNA relatedness (Elliott, Collins, Pigott, & Facklam, 1991; Teixeira, et al., 1996)). Additionally, based on biochemical and genetic evidence, *Enterococcus solitarius* (Collins, Facklam, Farrow, & Williamson, 1989), was moved to the *Tetragenococcus* genus, and was named *Tetragenococcus solitarius* (Ennahar & Cai, 2005). Within the *Enterococcus* genus, *E. saccharominimus* (Vancanneyt, et al., 2004) was shown to be a synonym of *Enterococcus italicus* (Fortina, Ricci, Mora, & Manachini, 2004; Naser, Vancanneyt, Hoste, Snauwaert, Vandemuelebroecke, & Swings, 2006), and *E. casseliflavus* was shown to be the senior subjective synonym of *Enterococcus flavescens* (Naser, Vancanneyt, Hoste, Snauwaert, Vandemuelebroecke, & Swings, 2006; Teixeira, et al., 1997). In 2001, *Enterococcus porcinus*, which was isolated and associated with enteric disorders in animals (Teixeira, et al., 2001), was shown to be the same species as *Enterococcus villorum* (Vancanneyt, et al., 2001), as no phenotypic or genotypic differences could be found between these species (De Graef, et al., 2003). Finally, *Enterococcus sanguinicola* sp. nov. was proposed by Carvalho *et al.* (Carvalho, et al., 2008) in 2008, after taxonomic analysis of two isolates recovered from the blood of patients in the United States and in Sweden (Carvalho, et al., 2008), and the proposed type strain was *E. sanguinicola* SS-1729T (Carvalho, et al., 2008). One of the isolates showed a vancomycin-resistant phenotype, due to the presence of the *vanA* operon. However, the authors noted that this species has not been fully validated (Carvalho, et al., 2008), and in fact, a recent study reported that *E. sanguinicola* should be reclassified as *E. thailandicus*, based on DNA-DNA reassociation and sequence of the 16S rDNA and *rpoB* genes (Shewmaker, et al., 2011).

## Phenotypic characteristics of the *Enterococcus* genus

The phenotypic tests routinely used to identify members of the *Enterococcus* genus and species have been previously reviewed (Facklam, Carvalho, & Teixeira, 2002). Briefly, Facklam and collaborators (Facklam, Carvalho, & Teixeira, 2002) divided enterococcal species into 5 groups based on acid formation in mannitol and sorbose broth, as well as hydrolysis of arginine. This clustering was applied to the 22 recently identified *Enterococcus* species, based on phenotypic tests performed along species descriptions (Table 2). It is important to note that this phenotypic classification should be distinguished from the 16S rRNA sequence relationship, so although these tests are potentially useful for diagnostics, it does not appear to reflect the evolutionary relationships between *Enterococcus* species.

Group I consists of enterococcal species that form acid in both carbohydrate broths, but do not hydrolyze arginine (Facklam, Carvalho, & Teixeira, 2002). The new species *E. phoeniculicoa*, *E. devriesei*, and *E. canis* (De Graef, et al., 2003; Law-Brown & Meyers, 2003; Švec, et al., 2005) belong to Group I, along with *E. avium*, *E. malodoratus*, *E. raffinosus*, *E. pseudoavium*, *E. saccharolyticus*, *E. pallens*, and *E. gilvus* (Table 2). Group II includes *E. faecalis* and *E. faecium*, and consists of species that form acid in mannitol broth and hydrolyze arginine but fail to form acid in sorbose broth (Facklam, Carvalho, & Teixeira, 2002). New species *E. canintestini*, *E. lactis*, *E. thailandicus*, and *E. sanguinicola* belong to this group (40, 184, 196, 264) (Carvalho, et al., 2008; Morandi, Cremonesi, Povo, & Brasca, 2012; Naser, et al., 2005; Tanasupawat, Sukontasing, & Lee, 2008). Group III includes species that are not able to form acid in either mannitol or sorbitol broth, but that hydrolyze arginine (Facklam, Carvalho, & Teixeira, 2002). This group now includes *E. villorum*, *E. durans*, *E. dispar*, *E. hiraе*, *E. silesiacus*, and *E. rotai* (Sedláček, et al., 2013; Svec, et al., 2006), as well as variants of *E. faecalis* and *E. faecium* (Table 2) (Facklam, Carvalho, & Teixeira, 2002). *E. asini*, *E. sulfureus*, and *E. cecorum* previously formed Group IV, to which we may now add *E. aquamarinus*, *E. plantarum*, *E. caccae*, and *E. termitis* (Carvalho, et al., 2006; Facklam, Carvalho, & Teixeira, 2002; Švec, et al., 2005; Svec, et al., 2006; Švec, et al., 2011). These species are negative for acid formation in mannitol and sorbose broth and do not hydrolyze arginine (Table 2). Finally, Group V consists of the species that form acid on mannitol, but not sorbose broth, and fail to hydrolyse arginine (Facklam, Carvalho, & Teixeira, 2002). This group includes *E. columbae*, *E. rivorum*, *E. hermaniensis*, *E. camelliae*, and *E. viikiensis*, as well as variants of *E. casseliflavus*, *E. gallinarum*, and *E. faecalis* that fail to hydrolyse arginine (Table 2) (Facklam, Carvalho, & Teixeira, 2002; Koort, Coenye, Vandamme, Sukura, &

Björkroth, 2004; Niemi, et al., 2012; Rahkila, Johansson, Säde, & Björkroth, 2011; Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). The recently described *E. ureilyticus* (Sedláček, et al., 2013) does not appear to fit into any existing group, and we place it as the sole member of Group VI, based on its ability to formate acid from sorbose but not in mannitol broth, as well as its inability to hydrolyze arginine. Because of lack of available information on the fermentation capabilities of *E. quebecensis*, *E. italicus*, and *E. ureasiticus*, they are unable to be classified by this scheme (Table 2) (Fortina, Ricci, Mora, & Manachini, 2004; Sisteck, et al., 2012). Zhang and collaborators have identified a gene cluster in the *E. faecium* strain E980 that is responsible for the metabolism of the  $\alpha$ -galactoside sugar raffinose (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011). While (Table 2) *E. faecium* is placed in Group II in the presented table, which is the group unable to use raffinose, these authors showed that raffinose utilization is actually a trait carried by megaplasmids, which indicates that these mobile elements can have important roles in shaping the competitive fitness of the enterococci in the environment; for example, by expanding their metabolic repertoire (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011).

**Table 2.** Phenotypic characteristics of recently identified *Enterococcus* species

Species	Phenotypic characteristic <sup>a</sup>										Group
	MAN	SOR	ARG	ARA	SBL	RAF	MOT	PIG	SUC	MGP	
<i>E. phoeniculicola</i>	+	+	-	+	+	-	-	-	+	nd	I
<i>E. devriesei</i>	+	+	-	v	v	v	-	-	+	-	I
<i>E. canis</i>	+	+	-	+	+	-	-	-	v	+	I
<i>E. canintestini</i>	+	-	+	-	-	-	-	-	+	+	II
<i>E. lactis</i>	+	-	+	+	-	-	-	-	-	-	II
<i>E. thailandicus</i>	+	-	+	-	-	-	-	-	+	-	II
<i>E. sanguinicola</i>	+	-	+	-	-	-	-	-	+	-	II
<i>E. silesiacus</i>	-	-	+	-	-	-	-	-	-	-	III
<i>E. rotai</i>	-	-	+	-	-	-	-	+	+	-	III
<i>E. ratti</i>	-	-	+	-	-	-	-	-	-	-	III
<i>E. aquimarinus</i>	-	-	-	+	-	+	-	-	+	-	IV
<i>E. caccae</i>	-	-	-	-	-	-	-	-	+	-	IV
<i>E. plantarum</i>	-	-	-	-	-	-	-	w	+	-	IV
<i>E. termitis</i>	-	-	-	-	-	-	-	-	-	+	IV
<i>E. rivorum</i>	+	-	-	-	+	-	-	-	+	-	V
<i>E. hermaniensis</i>	+	-	-	-	-	-	-	-	-	nd	V
<i>E. camelliae</i>	+	-	-	-	-	-	-	-	+	-	V
<i>E. viikkiensis</i>	+	-	-	-	-	-	-	-	nd	-	V
<i>E. ureilyticus</i>	-	+	-	-	-	-	-	+	+	-	VI
<i>E. quebecensis</i>	+	nd	-	-	-	-	-	-	+	nd	NA
<i>E. italicus</i>	v	-	-	-	v	-	-	-	+	+	NA
<i>E. ureasiticus</i>	v	nd	-	-	-	-	-	-	+	nd	NA

<sup>a</sup> Abbreviations and symbols: MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; MOT, motility; PIG, pigment; SUC, sucrose; MGP, methyl-  $\alpha$ -D-glucopyranoside; +, >80% positive; -, <20% positive; v, variable.

## Phylogeny of the *Enterococcus* genus

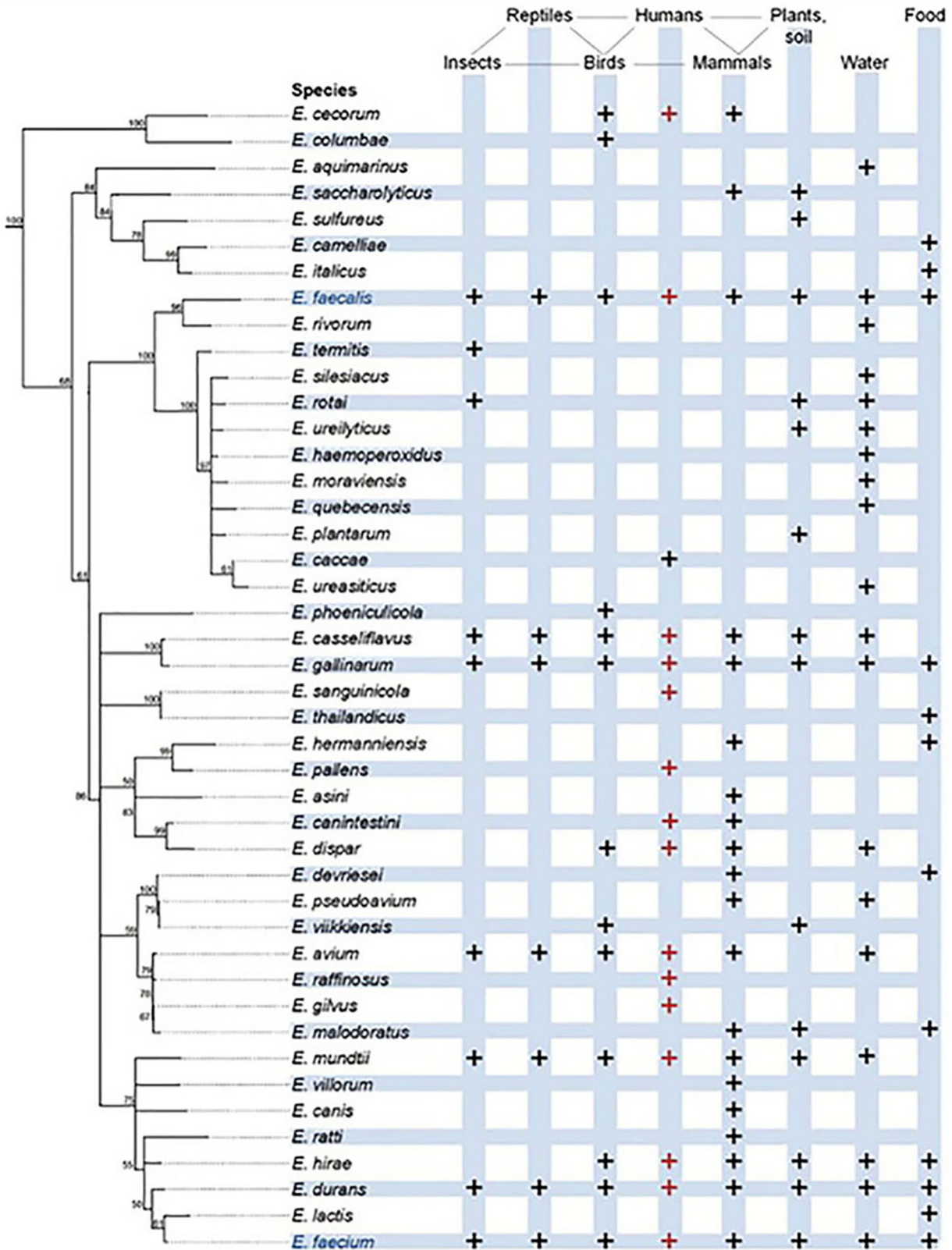
As a reflection of specialization for life in the gut of a host, over millions of years of co-evolution, genome analysis have highlighted the selective advantage for enterococci of their acquisition of many nutrients required for growth and survival from their habitats (usually either from the diet of the host, or through cross-feeding relationships with other microbes in the gut consortium), as opposed to carrying the additional genetic material necessary for their biosynthesis from simpler precursors (Gilmore, Lebreton, & van Schaik, 2013). Figure 2 shows the phylogenetic relationship among the different species of *Enterococcus* based on single-nucleotide polymorphism (SNP) analysis of the 16S rDNA sequences (Fig. 2). This tree can be divided into at least 7 groups, based on deep divides within the genus (Fig. 2). Some patterns emerge with respect to the habitats in which these species were identified and/or reported (Figure 3). Group I members are species that are farthest from other enterococci, which likely means that their speciation from the rest of the genus occurred comparatively early (Fig. 3). This is translated phenotypically by their unusual negative result in the pyruvate utilization test. *E. columbae* was exclusively isolated from pigeon faecal flora (Devriese, Ceysens, Rodrigues, & Collins, 1990), and *E. cecorum* also occurs mainly in the gut of birds and less frequently in the GI tract of mammals (some mammals like cattle or swine are fed with poultry) (Aarestrup, Butaye, & Witte, 2002). These strains have not yet been isolated from insects that are at the base of the food chain for many birds (Fig. 3), leading to speculation that speciation may have occurred after the evolutionary split among mammals, reptiles, birds, and insects. For strains of Group II, there appears to be a tropism for mammals, as none of these five strains have been isolated from insects, lizards, or birds (Fig. 3). The presence of the strains in the environment may be the consequence of their occurrence in the GI tract of humans and animals (Fig. 3), whereas their presence in food products reflects intentional use and not a natural habitat.

*E. faecalis* in Group III is found ubiquitously along the food chain, in addition to having emerged as an important opportunistic hospital pathogen (Fig. 3). This would suggest that since at least the early Devonian period, ~412 MYA (the time of the last common ancestor of mammals, reptiles, birds, and insects), this species has been a member of gut microbiomes (Gilmore, Lebreton, & van Schaik, 2013). *E. faecalis* is closely related to the recently described *E. rivorum*, which has been isolated from natural water (Niemi, et al., 2012). It would be very interesting to know the extent to which the genome of these two species is conserved. Other species found in Group III have a strong tropism (and almost specificity) to environments like water or plants. In several cases, an attempt was made to exclude contamination from human or animal feces, but it still remains unknown whether these species live freely in the environment (and if so, how their metabolic requirements are satisfied), or whether they are associated with simple eukaryotes like amoeba, as suggested by recent reports (Huws, Morley, Jones, Brown, & Smith, 2008).

Species found in Groups IV, V, and VI show some tropism for humans and animals (especially mammals) (Fig. 3). Enterococcal species that belong to Group V appear to be specific to humans and mammals (Fig. 3). In contrast, species of Groups IV and VI are more ubiquitous, and can be found along the food chain from insects to mammals. Their occurrence in environments such as water or on plants may stem from faecal contamination (Casanovas-Massana & Blanch, 2013; Staley, Reckhow, Lukasik, & Harwood, 2012). It will be of substantial interest to determine precisely how the nutritional requirements of the various species relate to their host or environmental specializations.

## Enterococcal species: Natural and man-made habitats

In the 1960's and 1970's Mundt and collaborators explored a variety of environments and found enterococci in the GI tracts and feces of mammals (71.3%), reptiles (85.7%), and birds (31.8%) (Mundt, 1963), as well as insects (53%) (Martin & Mundt, 1972). Culture-positive rates underestimate the presence of microbes (Ward, Weller, & Bateson, 1990), which suggests that the actual rate of colonization may be closer to 100%. This widespread pattern of colonization suggests that since at least the early Devonian period, ~412 MYA (the time of the last



**Figure 3.** Distribution in nature of species of the genus *Enterococcus*. The dendrogram shows phylogenetic relationships. The sources of isolation are indicated for each species. A simplified food chain is shown. Red and black symbols indicate species that has been described in human infections or colonization, respectively.



common ancestor of mammals, reptiles, birds and insects (Selden, 2007), enterococci have been members of gut microbiomes, which likely place them among the earliest members of GI tract consortia (Gilmore, Lebreton, & van Schaik, 2013).

## Enterococci in humans: gut colonization and infection

The human body (especially the GI tract, but also the skin, the upper respiratory tract, the oral cavity, and the vagina) is colonized by microbial communities that, together, constitute the “normal microbiota.” Each colonized body site represents an ecosystem that is defined by unique physicochemical and histological characteristics that constitute a competitive environment and select for adapted microbes (Tannock, 1988). The human colon contains about  $10^{12}$  diverse bacteria per gram of contents, with the highest counts in the colon (Sartor, 2008; Vollaard & Clasener, 1994), which contribute to tissue development and immune system homeostasis (Berg, 1996) (Figure 4).

*Infections in humans.* Enterococcal infection and its treatment are comprehensively covered elsewhere in this volume. Here, we briefly highlight select features of infection that inform the dynamic between colonization and infection by diverse species of enterococci. Enterococci began to emerge as leading causes of multidrug-resistant hospital-acquired infections in the 1970s and 1980s (Huycke, Sahm, & Gilmore, 1998; Jett, Huycke, & Gilmore, 1994), and they now rank among leading causes of hospital-acquired infections of the bloodstream, urinary tract, surgical wounds, and other sites (Hidron, et al., 2008). They are also associated with obligate anaerobes in mixed infections that result in intra-abdominal abscesses (Onderdonk, Bartlett, Louie, & Sullivan-Seigler, 1976). The most recent data available on enterococcal infection from all infection sites and all classes of hospitals in the US, which covers the period 01/01/10–06/30/12, identified 9,309 bloodstream isolates, 54,709 urinary tract isolates, and 20,032 wound isolates (a total of 84,050 isolates) (TSN® Database, Eurofins, Inc., personal communication Daniel F. Sahm, Ph.D.). Of the total, 17,360 are vancomycin-resistant (20.6%) and 64,015 (76%) are *E. faecalis*. Although 24% of isolates are *E. faecium*, they represent 14,998 of the 20,038 (75%) of the vancomycin-resistant isolates. Similar trends have been reported for the European Union (56, 294). *E. durans*, *E. avium*, *E. gallinarum*, *E. casseliflavus*, *E. hirae*, *E. muntzii*, *E. dispar*, and *E. raffinosus* only occasionally cause infection in humans (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Tannock & Cook, 2002). In 2002, Tyrrell and collaborators reported the isolation of *E. gilvus* and *E. pallens* from the bile of a patient with cholecystitis and the peritoneal dialysate of another patient with peritonitis, respectively (Tyrrell, et al., 2002). This has been the only description of these species causing human disease thus far. Recently, Ahmed and coworkers (Ahmed, Baig, Gascoyne-Binzi, & Sandoe, 2011) reported a case of human aortic valve infective endocarditis caused by *E. cecorum*, while a study conducted between 2000 and 2008 identified this species as responsible for 0.2% (2 out of 1887) of the bacteremia cases diagnosed at a medical center in Taiwan (Tan, Liu, Li, Huang, Sun, & Qu, 2013). These authors also reported a unique case of bacteremia caused by *E. canintestini* (Tan, Liu, Li, Huang, Sun, & Qu, 2013). While we lack studies that characterize the prevalence of the recently described species in the genus, we can generally assume, based on their recent discovery, that these are not often found as etiological agents of infections in humans.

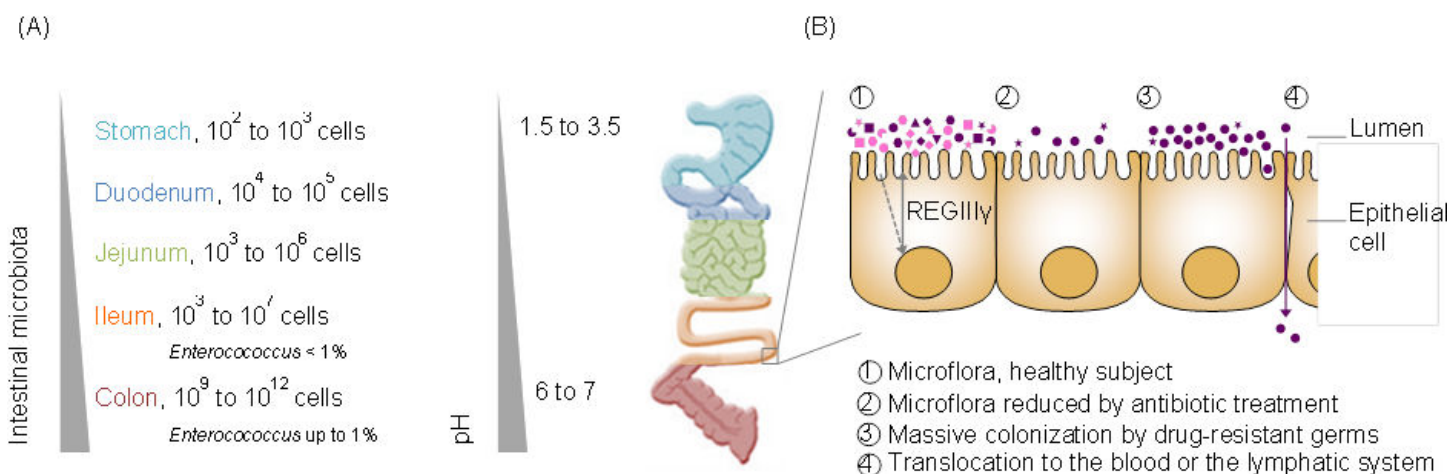
*Gut colonization.* Much of what is known about enterococcal colonization of the gut stems from studies of the human GI tract and feces. Enterococci are primarily localized to the human small and large intestine, where enterococci are prominent members of jejunal, ileal, cecal, and recto-sigmoidal consortia (Hayashi, Takahashi, Nishi, Sakamoto, & Benno, 2005). They are also found in human feces, although they constitute a minority population (up to 1%) within the gut microflora (Eckburg, et al., 2005; Sghir, Gramet, Suau, Violaine, Pochart, & Dore, 2000). Enterococci are common in the oral cavity (Smyth, Matthews, Halpenny, Brandis, & Colman, 1987), but occur more rarely in the stomach (Bik, et al., 2005; Monstein, Tiveljung, Kraft, Borch, & Jonasson, 2000). *E. faecalis* and *E. faecium* are most common in human feces, and *E. durans* and *E. avium* are occasionally detected (Finegold, Sutter, & Mathisen, 1983; Tannock & Cook, 2002). *E. caccae* was isolated from human feces, but its prevalence is low (Carvalho, et al., 2006). One study quantified *E. avium* (11%), *E. durans* (33%), *E.*

*faecalis* (78%), *E. faecium* (100%), *E. gallinarum* (33%), and *E. hirae* (11%) in human fecal specimens (Layton, Walters, Lam, & Boehm, 2010), while *E. casseliflavus* and *E. saccharolyticus* were absent from the nine samples tested (Layton, Walters, Lam, & Boehm, 2010). Other members of the genus *Enterococcus* identified in clinical samples are rarely part of the normal human intestinal microflora, but are present in the gut of other animals or on plants (Fig. 3).

Some members of the microbiota, including indigenous commensal enterococci, can act as opportunistic pathogens and translocate across the mucosal barrier to cause systemic infection in immune-compromised hosts (Berg, 1996; Donskey, 2004). More commonly, however, infection results from the colonization, overgrowth, and translocation of hospital-adapted antibiotic-resistant strains with enhanced pathogenicity. Studies using animal models have shed light on the mechanisms of microflora-mediated colonization resistance to enterococci (Brandl, et al., 2008; Kinnebrew, Ubeda, Zenewicz, Smith, Flavell, & Pamer, 2010). Components of bacterial cells, which include lipoteichoic acid, lipopolysaccharide, and flagellin, are recognized by Toll-like and Nod receptors expressed by intestinal epithelial and Paneth cells. By using a mouse model, Brandl and co-workers (Brandl, et al., 2008) showed that antibiotic reduction of the population of Gram-negative bacteria in the intestine resulted in decreased production of RegIII $\gamma$  by Paneth cells, which resulted in intestinal overgrowth of vancomycin-resistant enterococci (VRE) (Brandl, et al., 2008). RegIII $\gamma$  is a secreted C-type lectin that selectively kills Gram-positive bacteria, including VRE. RegIII $\gamma$  expression was restored in antibiotic treated animals by oral administration of the TLR4 agonist LPS, or systemic administration of the TLR5 agonist flagellin, which reduced VRE counts in the gut of mice (Figure 4B) (Brandl, et al., 2008; Kinnebrew, Ubeda, Zenewicz, Smith, Flavell, & Pamer, 2010). The same mechanisms may also take place in humans, as antibiotic intake by hospitalized patients is associated with an *Enterococcus* outgrowth (Ruiz-Garbajosa, et al., 2012; Ubeda, et al., 2010) that often precedes VRE bloodstream invasion (Ubeda, et al., 2010). Despite increasing knowledge about the interplay between Gram-negative commensals, the host's immune system, and *Enterococcus*, the understanding of synergies and antagonisms between enterococci and other microbes is far from complete. A recent study by Ubeda and coworkers (Ubeda, et al., 2013) observed that the reintroduction of a diverse intestinal microbiota to the GI tracts of mice densely colonized with VRE resulted in a substantial reduction in VRE numbers. They further showed that obligate anaerobic commensal bacteria belonging to the *Barnesiella* genus specifically promoted the clearance of intestinal VRE (Ubeda, et al., 2013). These authors further showed that, in patients undergoing allogeneic hematopoietic stem cell transplantation, intestinal colonization with *Barnesiella* confers resistance to intestinal domination and bloodstream infection with VRE (Ubeda, et al., 2013), which may provide a novel approach for the prevention of infection and the spread of highly antibiotic-resistant bacteria.

## Enterococci in animals: gut colonization and infection

Animal GI tracts likely represent the greatest reservoir for enterococci (Gilmore, Lebreton, & van Schaik, 2013). As in humans, enterococci also can infect animals (Aarestrup, Butaye, & Witte, 2002). Ostrolenk and Hunter (Ostrolenk & Hunter, 1946) recovered low levels of enterococci from 49 of 51 fecal samples from various animals, including human, cat, mouse, guinea pig, rabbit, dog, rat, chicken, fly, and monkey. Similarly, Haenel and Mueller-Buethow (Haenel & Muller-Buethow, 1957) obtained enterococci from human, rat, chicken, and dog, but not in the samples isolated from rabbit, guinea pig, or horse. In 1963, Mundt and colleagues (Mundt, 1963) conducted a thorough analysis of the occurrence of enterococci in animals and natural environments. Enterococci were obtained from the feces of 71% of 216 mammals, 86% of 70 reptiles, and 32% of 22 birds sampled in Great Smoky Mountains National Park (Mundt, 1963). Enterococci occurred only sporadically among the primarily herbivorous mammals, whereas enterococci appear to naturally colonize rodents and larger animals that have varied diets (Mundt, 1963). Enterococci were obtained from most specimens of bats and from the carnivorous mammals, such as fox, bear, raccoon, skunk, and boar (Mundt, 1963). Most probably, microbiota composition in relation to some animals' particular diets determine whether enterococci are able to survive and thrive in their GI tracts (Mieth, 1960).



**Figure 4.** (A) Barriers for gut colonization by the enterococci. The intestinal microbiota, as well as the acidic pH in the stomach, the duodenum, and the jejunum, limit the presence of *Enterococcus* species. Enterococci are found in the ileum and the colon but represent only a small proportion of the microbiota. (B) The role of antibiotics in the gut colonization by multi-drug resistant enterococci. In response to the Gram-negative bacteria (pink), abundant in the microflora, the intestinal epithelial cells secrete the lectin REGIII $\gamma$  that inhibits Gram-positive bacteria (purple) (panel 1). In the course of an antibiotic treatment, the amount of Gram-negative bacteria is reduced and, as a consequence, the production of REGIII $\gamma$  decreases (panel 2). Drug-resistant enterococci will benefit of the absence of this natural barrier and massively colonize the gut if their host (panel 3). The novel promiscuity between the bacteria and the epithelial cells may lead to the translocation of enterococcal cells to the blood or the lymphatic system (panel 4).

As new species are described, their associations with hosts are discovered. *E. columbae* is specific to pigeons so far (Devriese, Ceysens, Rodrigues, & Collins, 1990), and *E. asini* has only been found in donkeys (de Vaux, Laguerre, Diviès, & Prévost, 1998). *E. asini* has not been reported in additional studies since its description in 1998, and as a result, is likely to be rare. *E. columbae* is difficult to isolate, as this species does not grow on selective media that are commonly used for isolation of enterococci and also requires CO<sub>2</sub> for growth, so the prevalence of this species may therefore be underestimated (Devriese, Ceysens, Rodrigues, & Collins, 1990).

The most commonly encountered enterococcal species in the gut of mammals are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* (Devriese & De Pelsmaecker, 1987). Other species are found only occasionally (Devriese & De Pelsmaecker, 1987), or in particular age groups (such as *E. cecorum* in older poultry) (Aarestrup, Butaye, & Witte, 2002). In fact, an age-dependent succession of enterococcal species colonization appears to occur in chickens (Devriese, Hommez, Wijnfels, & Haesebrouck, 1991; Kaukas, Hinton, & Linton, 1987). Chickens are initially colonized by *E. faecalis*, but this population is then displaced, mainly by *E. faecium*, and it was proposed that the use of tylosin (to which *E. faecium* is commonly resistant) as a growth promoter is the reason of this replacement (Kaukas, Hinton, & Linton, 1987). These species then appear to be replaced by *E. cecorum* in the mature chicken. It is of interest that *E. avium* and *E. gallinarum*, originally described from chickens, were rarely found, suggesting that these species may not belong to the normal intestinal flora of poultry (Devriese, Hommez, Wijnfels, & Haesebrouck, 1991). *E. avium* was originally described from human feces (Guthof, 1955), but is common in chicken feces (Nowlan & Deibel, 1967). The extent to which the flora of chickens now reflects that native to poultry, or reflects the consequences of intensive production and modern poultry husbandry practices (namely, the frequent use of antibiotics including aminoglycosides), is controversial, but has undoubtedly impacted the representation of various species of enterococci (Aarestrup, Butaye, & Witte, 2002; Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994). Kobayashi and collaborators reported that aminoglycoside modifying enzymes are less prevalent in *E. avium* strains, as compared to *E. faecium* and *E. faecalis* strains isolated from poultry (Kobayashi, Alam, Nishimoto, Urasawa, Uehara, & Watanabe, 2001).

Age-dependent enterococcal colonization also has been described in cattle (Devriese, Laurier, De Herdt, & Haesebrouck, 1992). In preruminant calves, the enterococcal flora mainly consists of *E. faecalis*, *E. faecium*, and

*E. avium*. This flora is gradually replaced by *E. cecorum* (Devriese, Laurier, De Herdt, & Haesebrouck, 1992). Enterococci occur on the tonsils of preruminating calves; mainly *E. faecalis*, as well as *E. raffinosus*, to a lesser extent (Devriese, Laurier, De Herdt, & Haesebrouck, 1992).

The enterococcal species most frequently isolated from the intestines of swine are *E. faecalis* and *E. faecium*; however, the latter species occurs in low numbers (Devriese & De Pelsmaecker, 1987; Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994). Other enterococcal species found among swine flora include *E. hirae* and *E. cecorum* (Devriese & De Pelsmaecker, 1987; Devriese & Haesebrouck, 1991; Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994).

A study on the flora of cats and dogs found that *E. faecalis* was the most frequently encountered enterococcal species in the gut, as well as on the tonsils of these animals, with *E. faecium* and *E. hirae* occurring infrequently (Devriese, Cruz Colque, De Herdt, & Haesebrouck, 1992). Other species, including *E. avium*, *E. raffinosus*, *E. durans*, *E. cecorum*, and *E. gallinarum* were also occasionally isolated (Devriese, Cruz Colque, De Herdt, & Haesebrouck, 1992). As noted above, *E. canis* and *E. canintestini* have more recently been isolated from healthy dogs (De Graef, et al., 2003; Naser, et al., 2005). *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, *E. casseliflavus*, and *E. mundtii* have also been found in horses (Devriese & De Pelsmaecker, 1987; Thal, et al., 1995).

Recent studies in Portugal identified enterococci in a broad range of environments, including wild animals. A total of 144 enterococci (120 *E. faecium*, 14 *E. hirae*, 8 *E. faecalis*, 2 *E. gallinarum*) were recovered in echinoderms collected from Azorean waters (Marinho, et al., 2013). Resistance to erythromycin, ampicillin, tetracycline, and ciprofloxacin in these enterococci was found to be common among them (Marinho, et al., 2013). VRE (*E. faecium*, *E. gallinarum* and *E. casseliflavus*) were recovered in 8 of the 365 analysed fecal samples from Iberian wolf and lynx (Gonçalves, et al., 2013). Similarly, vancomycin-resistant *E. faecalis*, *E. faecium*, and *E. durans* were isolated in 7 of 118 fecal samples (5.9%) of natural gilthead seabream recovered off the coast of Portugal (Barros, et al., 2012).

In other studies, Radhouani and collaborators (Radhouani, Poeta, Gonçalves, Pacheco, Sargo, & Igrejas, 2012) examined 31 enterococcal isolates from 42 common buzzard fecal samples, and found *E. faecium* as the most commonly encountered species (48.4%) recovered. *E. faecium*, *E. durans*, and *E. gallinarum* isolates were detected in fecal samples of other wild birds (Silva, et al., 2011). Enterococci were also isolated from wild boars, partridges and fish (Almeida, et al., 2011), red foxes (Radhouani, et al., 2011), and wild rabbits (Silva, et al., 2010). In addition, enterococci have been found in wild geese (Han, et al., 2011) and from cattle reared with traditional practices in Ethiopia (Bekele & Ashenafi, 2010). In this last study, a total of 298 enterococcal isolates were obtained, which consisted of *E. faecium* (49.6%), *E. durans* (26.9%), *E. hirea* (11.9%), and *E. faecalis* (11.5%).

A large variety of insects, including beetles, flies, bees, termites, and worms have also been found to harbor enterococci. *E. faecalis* and *E. faecium* are predominant but other species occur at lower prevalence. Martin and Mundt (Martin & Mundt, 1972) observed the association of *E. faecalis*, *E. faecium*, and *E. casseliflavus* with a broad range of insect orders. *E. faecalis* (32%), *E. faecium* (22.4%), and *E. casseliflavus* (43.5%) were associated with wild insects from 37 different taxa (Martin & Mundt, 1972). In a survey of laboratory-reared and wild *Drosophila*, Cox and Gilmore (Cox & Gilmore, 2007) found several species of enterococci, including *E. faecalis*, *E. faecium*, *E. gallinaraum*, and *E. durans*, and localized them to the digestive tract. Differences between human and *Drosophila* GI tract anatomy and physiology undoubtedly play a role in determining the composition and physical location of their respective microbial populations.

Anatomically, the GI tract of *Drosophila* (Bodenstein, Cooper, Ferris, Miller, Poulson, & Sonnenblick, 1950) has an overall organization that has parallels to that of humans. With the exception of the *Drosophila* crop, the two systems possess a single alimentary canal that begins at the esophagus, connects to a ventriculus (stomach), extends to the intestine, proceeds to the rectum, and terminates at the anus (Figure 5). In terms of digestive

function, however, they are quite different. *Drosophila* possesses an acidic crop, but maintains an alkaline ventriculus and a neutral to acidic hindgut (Clark, 1999; Dow & Harvey, 1988). Another major difference is the absence of obligate anaerobic phylotypes in *Drosophila* (Cox & Gilmore, 2007), while the majority of human intestinal microbial phylotypes are obligate or microaerophilic anaerobes (Eckburg, et al., 2005).

Enterococci have been detected on insects in hospitals, and concerns have been raised about this possible reservoir for human contamination (Fotedar, Banerjee, Singh, Shriniwas, & Verma, 1992; Pai, Chen, & Peng, 2004). The importance of insects to the transmission of resistant or infectious enterococci has not been established. Ahmed and collaborators (Ahmed, Baig, Gascoyne-Binzi, & Sandoe, 2011) showed that house flies and cockroaches in the confined swine production environment may act as vectors and/or reservoirs of antibiotic resistant and potentially virulent enterococci. Macovei *et al.* (Macovei, Miles, & Zurek, 2008) showed that houseflies captured in fast-food restaurants commonly carry antibiotic-resistant germs. Among newly characterized *Enterococcus* species, *E. termitis* was isolated from the gut of a termite, while some *E. rotai* strains were isolated from mosquitoes (Sedláček, et al., 2013; Svec, et al., 2006).

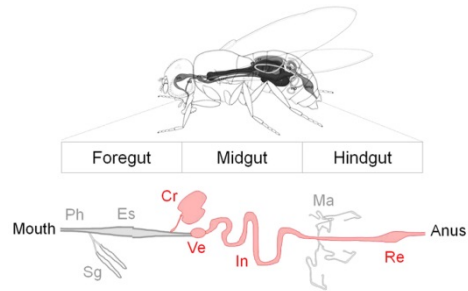
## Comparison of enterococci isolated from human and animals

In 1993, Bates and collaborators (Bates, Jordens, & Selkon, 1993) described the first vancomycin-resistant enterococci isolated from a non-human reservoir. These authors detected a vancomycin-resistant strain of *E. faecium* (VREF) in farm animals in the UK (Bates, Jordens, & Selkon, 1993). To understand the relationship between enterococci, and more particularly, between antibiotic-resistant strains of agricultural and human origin, *E. faecium* isolates were compared using PFGE, and similar PFGE profiles were found for VRE isolated from stool samples from both humans and animals (Hammerum, et al., 2004; van den Bogaard & Stobberingh, 2000). Besides PFGE, amplified fragment length polymorphism (AFLP) analysis and MLST were used to compare these *E. faecium* isolates (Willems R. J., et al., 2000). VREF strains were largely found to be host-specific, and strains isolated from hospitalized patients were genetically different from the prevailing VREF strains present in the fecal flora of non-hospitalized humans (Willems R. J., et al., 2000). Further, MLST showed that outbreak isolates from hospitalized patients clustered in a subgroup, which was originally termed a clonal complex 17 (CC17), while *E. faecium* isolated from mainly domesticated animals belonged to other types (Willems R. J., et al., 2005; Willems R. J., et al., 2012). Although there is some evidence of host adaptation to *E. faecium* strains, typical hospital-associated clones have been identified in farm animals (Hammerum, 2012), and dogs are frequently colonized by strains similar to those identified in clinical isolates (Damborg, Sørensen, & Guardabassi, 2008; de Regt, et al., 2012). This indicates that sharing a habitat is an important risk factor for the spread of these organisms.

Similarly to *E. faecium*, *E. faecalis* isolates from hospitalized patients tend to derive from select STs and CCs (McBride, Fischetti, LeBlanc, Moellering Jr., & Gilmore, 2007; Ruiz-Garbajosa, et al., 2006), but a diversity of STs can be detected in *E. faecalis* recovered from animals, meat, fecal samples from humans in the community, and patients with bloodstream infections (Kawalec, et al., 2007; Kuch, et al., 2012; McBride, Fischetti, LeBlanc, Moellering Jr., & Gilmore, 2007; Nallapareddy, Wenxiang, Weinstock, & Murray, 2005; Ruiz-Garbajosa, et al., 2006). *E. faecalis* human isolates have been found to be highly related to pig isolates, based on their antimicrobial resistance pattern, virulence gene profile, and MLST/ PFGE types (Larsen, et al., 2011). Similarly, the presence of the pathogenicity island (PAI) of *E. faecalis* can be found in isolates from pigs (Shankar, Baghdayan, Willems, Hammerum, & Jensen, 2006).

## Enterococci in the environment (plants, water and soil)

Enterococci are widely used as indicators of fecal contamination from animal or human origin in the environment (see Enterococci as indicators of environmental fecal contamination). However, the extent to which enterococci in the environment (including in water sources) reflects human or animal waste contamination continues to be debated (Harwood, Whitlock, & Withington, 2000). Comparing the genomes of enterococci



**Figure 5.** Schematic diagram of the enterococcal colonization of *Drosophila* GI tract. Red indicates portions of the GI tract naturally colonized by *Enterococcus*. Ph, pharynx; Sg, salivary gland; Es esophagus; Ve, proventriculus; In, intestine; Mal, Malpighian tubule; Re, rectum. Adapted from Hartenstein, 1993.

obtained from environmental sources to those derived from humans and animals will shed considerable light on this question. Among the extra-enteric niches where enterococci are routinely isolated include soil and sediments, aquatic and terrestrial plants, and ambient waters (Fig. 3). These are heterothermic habitats, in which temperatures are variable—in contrast to the gastrointestinal tract of warm-blooded animals, where the temperature is relatively constant. In addition, a variety of environmental stressors such as UV sunlight, salinity, starvation, and predation also occur (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). The transition from the animal gastrointestinal tract, an environment rich in many nutrients with key nutrients restricted, to oligotrophic environments that are generally nutrient-poor, exposes enterococci to nutrient starvation. One of the first reports on the survival of enterococci under nutrient-starvation conditions indicated that *E. faecalis* survived for extended periods in sterilized sewage (presumably due to the availability of organic nutrients), but declined rapidly in sterile lake water and in water with a phosphate buffer, indicating that oligotrophic conditions (exemplified by the sterile lake water and phosphate buffer) were deleterious to the survival of enterococci (Sinclair & Alexander, 1984). Indeed, enterococci are auxotrophic bacteria. It is important to note that although the authors observed survival of *E. faecalis* in sterilized sewage, they did not observe growth (Sinclair & Alexander, 1984).

*In and on aquatic and terrestrial vegetation.* While various enterococcal species can be found on plants (Fig. 3), it is unclear whether or not these strains are contaminants from fecal consortia that are simply surviving, or whether these environments actually constitute a habitat. Ostrolenk and Hunter (Ostrolenk & Hunter, 1946) considered their presence in these locations more an indication of survival, whereas Sherman (Sherman, 1937) considered as evidence against accidental occurrence and survival, given the fact that, of the many cultures isolated from plants, none of the hemolytic types of enterococci had been obtained. Mundt (Mundt, 1961; Mundt, 1963) recovered enterococci from the surface of a variety of plant species, and proposed that enterococci were transient populations, most likely introduced by insects and wind, since their occurrence was seasonal (Mundt, 1961; Mundt, 1963). In another study, however, he demonstrated what appeared to be the ability of *E. faecalis* to grow on plants (Mundt, Coggin, Jr., & Johnson, 1962). Occurrence in seed or soil, movement from the seed to the emergent parts, and ability to reproduce on the growing plant are criteria for bacterial plant epiphytes (Burri, 1903; Dueggeli, 1904; Thaysen & Galloway, 1930; Voznyakovskaya & Khudyakov, 1960). In his work (Mundt, Coggin, Jr., & Johnson, 1962), Mundt suggested that *S. faecalis* is a potential epiphyte on plants, since it was found consistently on most plant parts, it established a cycle on plants in which transmission occurs via the seed, and it reproduced on the growing plant. Although no details are provided, the author concluded that these findings indicate that these bacteria are capable of adaptation to an environment substantially different from that of the intestinal tract in its quantity of nutrients, temperature, and moisture (Mundt, Coggin, Jr., & Johnson, 1962). However, it is important to note that the possibility that the enterococcal cells were commensals of macro or micro-invertebrates, or that they may have benefitted from a more complex bacterial population, were not addressed. Huws and collaborators (Huws, Morley, Jones, Brown, & Smith, 2008) investigated the interactions of

*E. faecalis*, among other bacteria, with the amoeba *Acanthamoeba polyphaga*. They found evidence of protozoal predation of *E. faecalis*. Interestingly, the extracellular numbers of *E. faecalis* JH2-2 were significantly higher when cultured with amoebae, as compared to growth in the absence of amoebae (Huws, Morley, Jones, Brown, & Smith, 2008). The authors stated that, since not all bacteria are digested by protozoa, outgrowth of select populations occurs, as was observed for the tested enterococcal strain (Huws, Morley, Jones, Brown, & Smith, 2008).

The occurrence of enterococci has been investigated in association with various vegetation, including algae (Whitman, Shively, Pawlik, Nevers, & Byappanahalli, 2003), beach wrack (Anderson, Turner, & Lewis, 1997; Grant, et al., 2001; Inamura, Thompson, Boehm, & Jay, 2011), submerged vegetation (Badgley, Nayak, & Harwood, 2010; Badgley, Thomas, & Harwood, 2010), flowering plants (Mundt, 1963), and forage crops (Müller, Ulrich, Ott, & Müller, 2001; Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). Whitman and collaborators (Whitman, Shively, Pawlik, Nevers, & Byappanahalli, 2003) showed that the *Cladophora* mats, a green alga found in both fresh and marine waters, were a significant source of *E. coli* and enterococci. In addition to these fecal bacteria, enteric pathogens such as *Shigella*, *Campylobacter*, and *Salmonella* also were isolated (Whitman, Shively, Pawlik, Nevers, & Byappanahalli, 2003). The high densities of enterococci in fresh *Cladophora* have been attributed to *in situ* growth (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003). Indeed, these authors stated that algal leachate readily supported the *in-vitro* multiplication of enterococci (100-fold in undiluted algal leachate at 35 °C over 24 h), and that such growth was directly related to the concentration of algal leachate. This suggests that leachates contain the necessary growth-promoting substances to sustain these bacteria (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003). However, other adherent bacteria present on these algae may contribute nutrients that enterococci require. Enterococci can persist and survive on submerged vegetations (Badgley, Nayak, & Harwood, 2010; Badgley, Thomas, & Harwood, 2010) or dried algae (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003), and enterococci have also been isolated from plankton and macro-invertebrates (Maugeri, Carbone, Fera, Irrera, & Gugliandolo, 2004; Signoretto, et al., 2004).

Several recently characterized *Enterococcus* species were identified on plants. *E. plantarum* was isolated from plant samples obtained from a meadow in the Czech Republic, not influenced by farm animals and exploited only for hay harvesting; and *E. rotai* and *E. ureilyticus* were isolated from plants, as well as water samples (Švec, et al., 2011).

*In water.* Large quantities of human and animal wastes are distributed into the environment through sewage or non-sewage systems. Human wastes empty into waterways, usually through a sewage treatment plant, while animal wastes are often used composted (but otherwise untreated) as fertilizers on fields. Enterococci are found in both fresh (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012; Fujioka, Tenno, & Kansako, 198) and marine water (Boehm, et al., 2002; Korajkic, Brownell, & Harwood, 2011). They can survive and persist in fresh (Anderson, Whitlock, & Harwood, 2005) as well as marine water (Litopoulo-Tzanetaki & Tzanetakis, 1992), but it is not clear from the literature whether enterococci can also multiply in these environments. Growth in the absence of essential nutrients obviously will not occur, and studies that report growth in water in the presence of algae (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003) or sediments (Yamahara, Walters, & Boehm, 2009) rarely precisely define their position in more complex food webs.

Shifts in *Enterococcus* species composition generally occur during domestic wastewater treatment. While *E. faecalis* was predominant, different species including *E. pseudoavium*, *E. casseliflavus*, *E. faecium*, *E. mundtii*, *E. gallinarum*, *E. dispar*, *E. hirae*, *E. durans*, *E. flavescens*, *Enterococcus haemoperoxidus*, and *Enterococcus moraviensis* also can be recovered (Kühn, et al., 2003). This may have implications for the identification of fecal pollution based on the presence of specific bacterial types associated with domestic wastewater (Graves & Weaver, 2010). In water, the species considered as fecal contaminants are mainly *E. faecium* and *E. faecalis*, and the origins of other species are less clear (Aarestrup, Butaye, & Witte, 2002). Data reported by Signoretto *et al.*

(Signoretto, et al., 2004) suggested that the adhesion to plankton of viable but non-culturable cells contribute to the prolonged survival of enterococci in marine waters. Numerous recently-characterized *Enterococcus* species have been isolated from water samples. *E. rotai* and *E. ureilyticus* were isolated from drinking water, *E. aquimarinus* was isolated from sea water, and *E. rivorum* was isolated from a pristine brook (Niemi, et al., 2012; Svec, et al., 2005). Similarly, *E. moraviensis*, *E. silesiacus*, *E. ureasiticus*, and *E. quebecensis* have been isolated from surface water (Sistek, et al., 2012; Svec, et al., 2001; Svec, et al., 2006). Whether they represent flora of microscopic eukaryotes or other larger organisms remains to be definitively determined.

*In soil and sediments.* Enterococci occur in temperate and tropical soils (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012; Mundt, 1961), fresh or marine water sediments (Ferguson, Moore, Getrich, & Zhouandai, 2005; Obiri-Danso & Jones, 2000), and can be recovered from sand (Halliday & Gast, 2011; Yamahara, Layton, Santoro, & Boehm, 2007). The prolonged survival of enterococci has been observed in freshwater and estuarine sediments (Anderson, Whitlock, & Harwood, 2005). van Donsel *et al.* (Van Donsel, Geldreich, & Clarke, 1967) found that the rates of survival of *S. faecalis* were higher than those of fecal coliforms during spring and winter, but that fecal coliforms survived longer than *S. faecalis* during the summer months. Interestingly, many of the early investigations of the survival and persistence of enterococci in soil environments focused on watersheds impacted by anthropogenic activities, particularly in cattle grazing and field lot operations (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). All samples taken from urban sewage, and from farmland using pig manure, as well as crops grown on this land, readily yielded *Enterococcus* species (Kühn, et al., 2003). In crops to which animal fertilizer had not been applied, the incidence of *Enterococcus* species was reduced to 33% (Kühn, et al., 2003). *Enterococcus* species can be recovered from sand from freshwater and marine beaches (*E. faecium*, *E. casseliflavus*, *E. durans*), and from marine sediments (*E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundtii*) (Ferguson, Moore, Getrich, & Zhouandai, 2005). As for other environments, whether enterococci grow as free living organisms in sediments, sands, or soils remains highly speculative. While the growth requirements of many bacteria, including *E. coli*, are relatively simple (Andrews, 1991), enterococci require complex nutrients.

## Enterococci in food

It is likely that because of their natural occurrence as contaminants of raw meat and dairy products (Franz, Holzapfel, & Stiles, 1999), thermotolerance, low toxicity, and their ability to acidify an environment, enterococci have become an important ingredient in fermented foods. Enterococci are often used for the preparation of traditional cheeses manufactured in Mediterranean countries, including Greece, Italy, Spain and Portugal, from raw or pasteurized goat, sheep, or bovine milk (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Levels of enterococci in different cheeses range from  $10^4$  to  $10^6$  CFU/g, with *E. faecium* and *E. faecalis* being the most common (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Enterococci contribute to the ripening and development of aroma in these products because of their proteolytic and esterolytic properties, as well as the production of diacetyl and other important volatile compounds (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Enterococci play an important role in improving flavor development and overall quality of other traditional fermented foods as well, including vegetables and sausages (Franz, Holzapfel, & Stiles, 1999; Giraffa, 2002), including salami and chorizo (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). In addition to desired fermentation properties, both *E. faecalis* and *E. faecium* have been implicated in the spoilage of cured meat products, including canned hams and chub-packed luncheon meats (Magnus, Ingledew, & McCurdy, 1986; Magnus, McCurdy, & Ingledew, 1988). Finally, enterococci commonly occur in large numbers in vegetables (Ben Omar, et al., 2004; Fernandez-Diaz, 1983; Franz, Holzapfel, & Stiles, 1999; Giraffa, 2002; Mundt, 1963); and occur in Spanish-style green olive fermentations (de Castro, Montañó, Casado, Sánchez, & Rejano, 2002; Fernandez-Diaz, 1983; Floriano, Ruiz-Barba, & Jiménez-Díaz, 1998), in which *E. faecalis* and *E. faecium* are frequent contaminants.

While enterococci are used as an indicator of fecal contamination in the environment, their value as hygiene indicators in the industrial processing of foods has been questioned (Birolo, Reinheimer, & Vinderola, 2001).



The resistance of enterococci to pasteurization and their adaptability to different substrates and growth conditions (extreme pH and salinity) explains their occurrence in food products manufactured from raw materials or in nominally-heat-treated food products. The contribution of enterococci to the organoleptic properties of fermented food products (Coppola, Villani, Coppola, Coppola, Parente, & Parente, 1990; Giraffa & Carminati, 1997; Pisano, Fadda, Deplano, Corda, Casula, & Cosentino, 1994; Litopoulou-Tzanetaki & Tzanetakis, 1992; Manolopoulou, Sarantinopoulos, Zoidou, Aktypis, Moschopoulou, & Kandarakis, 2003), and their ability to produce bacteriocins (enterocins) (Cleveland, Montville, Nes, & Chikindas, 2002; De Vuyst & Vandamme, 1994; Ennahar & Cai, 2005; Klaenhammer, 1993; Moll, Konings, & Driessen, 1999; Nes, Diep, Håvarstein, Brurberg, Eijnsk, & Holo, 1996), are important characteristics in food technology. Despite their use in traditionally fermented products, the continuing use of enterococci in food processes is somewhat controversial, because of their association with human infection (De Vuyst, Foulquié Moreno, & Revets, 2003; Vancanneyt, et al., 2002).

*E. faecium* and *E. faecalis* are generally the most frequently encountered species of *Enterococcus* in food products (Lopez-Diaz, Santos, Gonzalez, Moreno, & Garcia, 1995; Suzzi, et al., 2000). Martín and collaborators (Martín, Corominas, Garriga, & Aymerich, 2009) investigated the species distribution of enterococci in traditional fermented sausages by species-specific multiplex PCR, and by *atpA* and 16S rDNA gene sequencing. They identified *E. faecalis* (31.4%) and *E. faecium* (30.7%), and more rarely *E. sanguinicola* (14.9%), *E. devriesei* (9.7%), *E. malodoratus* (7.2%), *E. casseliflavus* (3.4%), *E. gallinarum* (1.3%), *E. gilvus* (1.0%), *E. hermannienseis* (0.2%), and *E. durans* (0.2%) (Martín, Corominas, Garriga, & Aymerich, 2009). The species *E. camelliae*, *E. italicus*, *E. thailandicus*, and *E. lactis* were originally isolated from tea leaves, an Italian cheese, fermented sausages, and raw milk cheese, respectively (Fortina, Ricci, Mora, & Manachini, 2004; Morandi, Cremonesi, Povolò, & Brasca, 2012; Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007; Tanasupawat, Sukontasing, & Lee, 2008). The natural habitat of these *Enterococcus* species is very likely not fermented food, but elsewhere in nature in ecologies yet to be defined.

Enterococci are permitted in some countries as probiotics (Franz, Holzapfel, & Stiles, 1999; Franz, Stiles, Schleifer, & Holzapfel, 2003). One *E. faecium* strain (SF68) used as a probiotic, has been suggested to be clinically effective in the treatment of diarrhea in children (Bellomo, Mangiagle, Nicastro, & Frigerio, 1980) and to prevent diarrhea caused by antibiotic treatments (Wunderlich, et al., 1989). In a comprehensive review on the various enterococcal strains used as probiotics, Franz and collaborators (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011) concluded that data in support of the use of enterococci as probiotics is controversial. While it is possible to argue for the probiotic benefit of some strains, the emergence of enterococci in human disease and as multidrug-resistant germs raises concern.

## Drug resistant enterococci in non-human reservoirs

Besides the treatment of human infection, antimicrobial agents are used in food preservation, to treat and promote the growth of animals and pets, in plant production, and for other non-health-related purposes (Dibner & Richards, 2005; Turnidge, 2004). The contribution of each of these applications to resistance among human pathogens is still debated.

*In animals.* Enterococcal infections of animals are rarely treated with antimicrobial agents; however, enterococci are exposed to antimicrobial selection as members of the GI tract during the course of treatment of other infections, or when antibiotics are used as growth promoters or prophylaxis (Turnidge, 2004). The virtues of antibiotics as growth promoters have been debated since the 1960s (Swann, 1969). Studies conducted in the 1950s confirmed that the use of tetracycline for treatment or growth promotion selected for tetracycline resistance among group D streptococci (enterococci) in chickens (Barnes, 1958; Elliott & Barnes, 1959). More recently, epidemiological studies and feeding experiments have shown that the use of different antimicrobial growth promoters, including glycopeptides (avoparcin), macrolides (tylosin) and oligosaccharides (avilamycin), select for resistance among enterococci in poultry and pigs (Aarestrup, Bager, Jensen, Madsen, Meyling, &

Wegener, 1998; Aarestrup, Bager, Jensen, Madsen, Meyling, & Wegener, 1998; Aarestrup F. M., 2000; Bager, Madsen, Christensen, & Aarestrup, 1997; Christie, Davidson, Novick, & Dunny, 1983; Hinton, Kaukas, Lim, & Linton, 1986). Most interest has focused on the use of avoparcin and the selection of VRE in animal husbandry (Aarestrup F. M., 1995; Bager, Madsen, Christensen, & Aarestrup, 1997). A multitude of global studies describe the occurrence of VRE in many different non-human reservoirs, including pet cats and dogs, horses, birds, wood frogs, ostriches, pigs, pork, broilers, poultry meat, environmental samples, and sewage, as well as from stool samples from farmers and non-hospitalized humans in the community, mainly in Europe (Hammerum A. M., 2012). The linkage of VRE in non-human reservoirs to the use of avoparcin as a growth promoter is debated. Avoparcin was used in animal husbandry in the EU, Australia, and several other locations, but was not allowed for growth promotion in the USA and Canada. As a likely result, there is a low prevalence of VRE in the US and Canada, but nevertheless, there is a high level of occurrence of VRE in US hospitals (Hammerum A. M., 2012). Feeding animals low doses of antimicrobials, in certain conditions, increases productivity by improving feed conversion and decreasing morbidity and mortality caused by infection (Butaye, Baele, Devriese, & Haesebrouck, 2002). The glycopeptide avoparcin was first introduced for growth promotion in 1975 (Hammerum, Lester, & Heuer, 2010), and was mainly used for broilers and pigs, but also for turkeys, veal calves, and other animals (Bates J., 1997; Hammerum, Lester, & Heuer, 2010; Hammerum A. M., 2012). At that time, it was used extensively in most parts of Europe and the rest of the world, with the notable exceptions of Canada and the USA (McDonald, Kuehnert, Tenover, & Jarvis, 1997). As avoparcin confers cross-resistance to vancomycin, its use selected for outgrowth of VRE, and as a consequence, VRE was common in the intestinal flora of farm animals in Europe during the 1990s (Bager, Madsen, Christensen, & Aarestrup, 1997). In contrast, until 2008, VRE had never been isolated from farm animals in the US. Because of this connection, the use of avoparcin as a growth promoter was banned and the prevalence of VRE in farm animals in Europe rapidly declined, but VRE did not disappear (Aarestrup F. M., 2000). To explain why VRE persist among farm animals, it has been suggested that the use of the macrolide tylosin in pigs co-selects for vancomycin resistance among enterococci, since both the resistance determinants are often on the same plasmid (Aarestrup F. M., 2000). Besides tylosin, Johnsen and collaborators (Johnsen, et al., 200) hypothesized that plasmid addiction systems may contribute to retention of the resistance.

High-level aminoglycoside resistance has been observed among enterococci isolated from a variety of animals. Highly gentamicin-resistant strains were isolated from bovine mastitis (Jayaro & Oliver, 1992) and pigs (Aarestrup, Bager, Jensen, Madsen, Meyling, & Wegener, 1998; Aarestrup F. M., 2000). These gentamicin-resistant isolates include *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* (Aarestrup, Butaye, & Witte, 2002). Resistance to kanamycin has been observed in 30% of *E. faecalis* and *E. faecium* isolates from bovine mastitis (Jayaro & Oliver, 1992), 62% of 62 isolates from chickens, and 36% of 72 isolates from pigs (Rollins, Lee, & LeBlanc, 1985). Streptomycin resistance has been observed among enterococcal isolates from bovine mastitis (80%) (Jayaro & Oliver, 1992), chickens (64%), beef cattle (6%), and pigs (57%) (Rollins, Lee, & LeBlanc, 1985). Thal and collaborators (Thal, et al., 1995) also isolated streptomycin-resistant enterococci from horses and birds. Chloramphenicol use has been limited in food animals for the last several decades. There are few reports on the occurrence of resistance to chloramphenicol among enterococci isolated from animals. Saikia *et al.* (Saikia, Dutta, Devriese, & Kalita, 1995) found that one-third of *E. faecalis* from ducks in India were resistant to chloramphenicol. A low frequency (1–7%) of chloramphenicol resistance occurs in *E. faecium* and *E. faecalis* isolates from broilers and pigs in Denmark (Aarestrup F. M., 2000).

The first finding of quinupristin-dalfopristin-resistant *E. faecium* in non-hospitalized humans was reported in the United Kingdom (UK) by Woodford and collaborators (Woodford, Adebisi, Palepou, & Cookson, 1998) in the late 1990s. Interestingly, quinupristin-dalfopristin and other streptogramins used for human therapy were not available at that time in the UK for use in humans. Similarly to vancomycin and avoparcin, the finding of quinupristin-dalfopristin-resistant *E. faecium* isolates outside hospitals was assumed to be associated with the use of virginiamycin in animals (McDonald, et al., 2001). The genetic determinant that encodes streptogramin A resistance has been detected in *E. faecium* isolates from poultry, pigs, pork, and sewage worldwide. Enterococci

from animals are also frequently resistant to penicillin or ampicillin. Frequencies of resistance comparable to those observed among isolates from humans have also been reported (Aarestrup F. M., 2000; Yoshimura, Ishimaru, Endoh, & Kojima, 2000).

*In food.* Antibiotic-resistant enterococci occur in meat products, dairy products, and even within strains used as probiotics (Giraffa, 2002). The contamination of meat with antibiotic resistant enterococci during slaughter of animals to which antibiotics were administered has been reported (Bager, Madsen, Christensen, & Aarestrup, 1997; Del Grosso, et al., 2000; Klare, Badstübner, Konstabel, Böhme, Claus, & Witte, 1999; Kruse, Johansen, Rørvik, & Schaller, 1999). In 2002, Giraffa and collaborators (Giraffa, 2002) highlighted the role played by food reservoirs of VRE in the dissemination of antibiotic resistance traits in the environment. VRE and tetracycline-resistant enterococci have been found among food animals in several countries in Europe (Aarestrup, Butaye, & Witte, 2002). Recently, Ronconi and collaborators (Ronconi, Merino, & Fernández, 2002) assessed the presence of highly aminoglycosides and glycopeptide-resistant enterococci in uncooked foods, and more particularly, in lettuce. The most frequently detected species was *E. faecium* (32.61%), followed by *E. faecalis* (21.74%), *E. gallinarum* (13.04%), *E. casseliflavus* and *E. mundtii* (7.60%), *E. hirae*, (6.52%), *E. durans* (4.35%), *E. raffinosus* and *E. saccharolyticus* (2.17%), and *E. avium* and *E. malodoratus* (1.10%) (Ronconi, Merino, & Fernández, 2002). Susceptibility profiles were found to be similar to those of strains occurring in hospitals (Ronconi, Merino, & Fernández, 2002).

High-level aminoglycoside resistance has been observed among enterococci isolated from food of animal origin (Aarestrup, Butaye, & Witte, 2002). In a US study, gentamicin resistance was found in 7 of 18 enterococci isolates from chicken meat (Thal, et al., 1995). Streptomycin resistance occurred at a similar rate (Thal, et al., 1995). Streptomycin resistance was found at a lower rate (1% of enterococcal isolates) from food in Germany; and has been detected among *E. faecalis* (10%) and *E. faecium* isolates from broilers (2-3%) in Denmark (Aarestrup F. M., 2000). The use of chloramphenicol has been banned in food animal production for more than 30 years, and is largely restricted in human medicine to limited use for systemic treatment or for the treatment of eye infections. As a consequence, a low frequency (1-7%) of chloramphenicol resistance, encoded by the gene *cat<sub>IP501</sub>*, has been observed among *E. faecium* and *E. faecalis* isolates from broilers (Aarestrup F. M., 2000). However, Pavia *et al.* (Pavia, Nobile, Salpietro, & Angelillo, 2000) found more than 40% of enterococci from raw meat in Italy to be resistant to chloramphenicol, whereas Klein *et al.* (Klein, Pack, & Reuter, 1998) found 1% of vancomycin susceptible and 29% of vancomycin-resistant enterococci from raw meat in Germany to also be chloramphenicol-resistant.

*In the environment.* Sewage is an important reservoir of resistant enterococci. As early as the 1970s, tetracycline-resistant enterococci were isolated from sewage (van Embden, Engel, & van Klingeren, 1977). Glycopeptide resistance in *E. faecium* was first detected in 1986 in clinical isolates from hospitals in France (Leclercq, Derlot, Duval, & Courvalin, 1988) and the United Kingdom (Uttley, et al., 1989). The first indication of a reservoir outside hospitals was the detection of VRE in wastewater treatment plants of small towns in Germany that had no hospital (Klare, Heier, Claus, & Witte, 1993); and in sewage in the United Kingdom (Bates, Jordens, & Selkon, 1993). High-level aminoglycoside resistance has been observed worldwide among enterococci isolated in the environment (Aarestrup, Butaye, & Witte, 2002). More specifically, high-level resistance to kanamycin has been observed among 34% of 248 enterococcal isolates from sewage and water in the US (Rice, Messer, Johnson, & Reasoner, 1995). Streptomycin resistance was found in 5% of sewage isolates in the US. Highly gentamicin-resistant enterococci have been isolated from sewage and water in the US (Rice, Messer, Johnson, & Reasoner, 1995) and Germany (Klare, Heier, Claus, & Witte, 1993) as well.

## Conclusions

It is clear that the few *Enterococcus* strains and species regularly associated with human infection represent just the tip of the iceberg, as microbes of the genus *Enterococcus* are mainly ancient and highly evolved members of

GI tract consortia of various hosts. More information is sorely needed on the natural habitats of many of the species, including new species that have recently come to our attention, due to of their rare occurrence in clinical specimens. The extent to which enterococcal species naturally inhabit and proliferate in and on plants, soil, and water (as opposed to contaminating them through animal excretion) remains controversial. Genes mirror the requirements of life. As our understanding of enterococcal genomics grows, bacterial genomics will become an important tool for providing new insights into the nature, biology, and habitats of the enterococci.

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