Functional and safety aspects of enterococci in dairy foods

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Abstract The genus *Enterococcus* like other LAB has also been featured in dairy industry for decades due to its specific biochemical traits such as lipolysis, proteolysis, and citrate breakdown, hence contributing typical taste and flavor to the dairy foods. Furthermore, the production of bacteriocins by enterococci (enterocins) is well documented. These technological applications have led to propose enterococci as adjunct starters or protective cultures in fermented foods. Moreover, enterococci are nowadays promoted as probiotics, which are claimed for the maintenance of normal intestinal microflora, stimulation of the immune system and improvement of nutritional value of foods. At the same time, enterococci present an emerging pool of opportunistic pathogens for humans as they cause disease, possess agents for antibiotic resistance, and are frequently armed with potential virulence factors. Because of this 'dualistic' nature, the use of enterococci remains a debatable issue. However, based on a long history of safe association of particular enterococci with some traditional food fermentations, the use of such strains appears to bear no particular risk for human health. Abundance of knowledge as well as progress in molecular techniques has, however, enabled exact characterization and safety assessment of strains. Therefore, a balanced evaluation of both, beneficial

and undesirable nature of enterococci is required. A clear understanding of their status may, therefore, allow their safe use as a starter, or a probiotic strain. The present review describes the broader insight of the benefits and risks of enterococci in dairy foods and their safety assessment.

Keywords *Enterococci · Food safety · Virulence and Functional properties*

Introduction

The Enterococci are lactic acid bacteria (LAB) which form an important part of environmental, food and clinical microbiology. Depending on the strain, they are considered as starter or adjuncts starter, probiotics, spoilage, and pathogenic organisms. They play a beneficial role in the development of the organoleptic characteristics of cheeses (especially those originating in the Mediterranean countries) and have been successfully used as starter and adjuncts cultures. Many enterococci also produce a diverse and heterogeneous group of ribosomally synthesized antimicrobial peptides or bacteriocins, known as enterocins [1, 2]. Enterocins usually belong to the Class II bacteriocins. Enterocins are peptide in nature and are often cationic, amphiphilic, membrane-permeabilizing molecules [3, 4]. Bacteriocins may inhibit pathogenic bacteria with a beneficial impact as protective cultures [3]. In the same manner, enterococci are also acknowledged as contributors to human's digestibility and, therefore, are additionally known for their role as probiotics. Undesirable aspects among others may include the spoilage of foods and the production of biogenic amines [2, 5].

However, some of the enterococcal strains are typical opportunistic pathogens that cause disease especially in the nosocomial settings. Moreover, due to the higher incidence

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of infections by enterococci in young, older and immuno-compromised patients, and to their extended resistance to antibiotics, they are being considered as emerging pathogens [6, 7]. The majority of infections are caused by either *E. faecalis* or *E. faecium* strains, [8, 9] However, strains of *E. gallinarum* [10], *E. hirae* [11], and *E. mundtii* [12] have been also associated with endophthalmitis and native valve endocarditis in humans.

In addition, many enterococcal isolates of different origin carry potential virulence factors [13–15]. Which can be transferred through mobile genetic elements [16]. These also participate in molecular communication between bacteria of the animal and human microflora [17], giving the enterococci a new dimension regarding their potential pathogenicity to immunocompromised persons. This dualistic nature of enterococci gives rise to concern in the food industry.

The Enterococci

Phylogeny and taxonomy of enterococci

Enterococci were described for the first time by Thiercelin in 1899 and genus *Enterococcus* was proposed by Thiercelin and Jouhaud in 1903 for the Gram positive diplococci of intestinal origin. Andrewes and Horder (1906), however, renamed Thiercelin's enterococci as *Streptococcus faecalis* based on their ability to form short or long chains. The species epithet 'faecalis' was suggested because of their close resemblance to strains isolated from the human intestine. This explains why the history of enterococci cannot be separated from that of the genus *Streptococcus* [18, 2].

In 1933, a serological typing system, for enterococci was developed by Lancefield in which those of 'faecal origin' possessed the group D antigen [19]. This correlated with the grouping system of Sherman (1937), which proposed a new classification scheme for the genus *Streptococcus* that separated it into four divisions designated as *pyogenic*, *viridans*, *lactis* and 'enterococcus' [20]. The 'enterococcus' group included *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus bovis* and *Streptococcus equinus* as the 'enterococcal' or group D strains.

The classical taxonomy of the enterococci is rather vague because there are no phenotypic characteristics that unequivocally distinguish them from other Gram-positive, catalase-negative, coccus-shaped bacteria [21]. The majority of *Enterococcus* species, however, can be distinguished from other Gram-positive, catalase-negative cocci by their ability to grow from 10 °C to 45 °C, survive heating at 62.8 °C for 30 min, tolerate 6.5 % NaCl and 40 % bile and grow between pH 4.0 and 9.6 [22].

DNA–DNA hybridisation and 16S rRNA sequencing studies carried out by Schleifer and Kilpper-Ba"lz (1984) [23] indicated that the species *Streptococcus faecium* and *Streptococcus faecalis* were sufficiently distinct from other *streptococci*, and they proposed their transfer to the genus *Enterococcus*. To date, 28 species of enterococci have been added to the genus on the basis of phylogenetic evidence strengthened by 16S rRNA-DNA sequencing and/or DNA–DNA hybridization studies [2].

Enterococci in dairy foods

Presence of enterococci in dairy foods can have conflicting effects, of either as a risk or as a foreign or intrusive flora indicating poor hygiene during milk handling and processing (if in excessive numbers), or as a benefit in contributing to produce unique traditional and emerging by-products, in protecting against diverse spoilers, and as probiotics.

Enterococci as contaminants or natural starter in milk

Traditionally, the prevalence of enterococci in milk is considered as a result of faecal contamination. However, the ability of enterococci to grow in milking equipment, processing plants, and other environments, put in doubt the reliability of enterococcal counts as a reflection of faecal contamination [24]. Mundt (1986) [25] also demonstrated that the common presence of *E. faecalis* in many food products is not always related to direct faecal contamination. Due to their psychotropic nature, heat resistance and adaptability to different substrate and growth conditions, they can grow during refrigeration and survive after pasteurization. Therefore, enterococci are a part of both raw and pasteurized milk microflora [26]. A study of the levels of enterococci in raw cow's milk from 10 New Zealand farms, revealed an enterococcal range from 10¹ cfu/ml to 1.2 x 10⁴ cfu/ml [27]. Different species of enterococci are found in dairy products, but *E. faecalis* and *E. faecium* remain the species of greatest importance. *E. faecalis* is often the predominating over *E. faecium* [28, 29]. Other sources report numbers in European raw milk varying from 10³ cells/ml to 10⁵ cells/ml, or more, without any of the species being markedly represented. [30] In 1992, the European Union established a maximum level for the presence of coliforms and *E. coli*, both considered as indicators of hygiene, while no acceptable levels of enterococci can be stated for foods [31]. Furthermore, it has been shown that enterococci had little value as hygiene indicators in the industrial processing of foods [32]. Therefore, it is now accepted that enterococci naturally occur in raw milk and whey and act as a starter culture during manufacture of a variety of milk products.

Enterococci in cheese

Enterococci are associated with traditional European cheeses manufactured in Mediterranean countries, such as Greece, Italy, Spain and Portugal, from raw or pasteurized milk [33, 34]. The source of enterococci in milk and cheese is thought to be, the faeces of animals, contaminated water sources, milking equipment, and bulk storage tanks [26, 35]. Nevertheless, many reports describe the abundance of enterococci in traditional cheeses thus fortifying their position as normal part of cheese microflora [36, 37]. More interestingly, in cheeses such as Mozzarella [38], Cheddar [29], Picante de Beira Baixa [39], Cebreiro [40], Tetitta [41], and Pecorino Sardo [42] enterococci are the predominant microorganisms in the fully ripened product. Levels of enterococci in different cheese curds range from 10^4 to 10^6 cfu/g and in the fully ripened cheeses from 10^5 to 10^7 cfu/g⁵, [37], with *E. faecium* and *E. faecalis* being the dominant species. Recently Serio *et al.*, (2007) [43] reported the presence of enterococci at the level of 10^4 cfu/g to 10^6 cfu/g at beginning which increased up to 10^9 cfu/g in ripened Pecorino Abruzzese cheese. Likewise, twenty three out of twenty six samples were positive for enterococci and their counts varied from 10^2 cfu/g to 10^6 cfu/g in Schabziger and Appenzeller cheese [44]. Varying levels of enterococci in different cheeses result from cheese type, production season, extent of milk contamination and survival in the dairy environment (dependent on seasonal temperature), along with survival and growth under the particular conditions of cheese manufacture and ripening [45–47]. High levels of contaminating enterococci could lead to deterioration of sensory properties in some cheeses but they also play the beneficial role in cheese ripening and aroma development [48].

Functional properties of enterococci

From the positive side enterococci are closely involved in the production of traditional fermented foods and probiotic preparations. Enterococci show higher proteolytic activities than other LAB, which is important for cheese ripening. Their beneficial effect in cheese making has also been attributed to hydrolysis of milk fat by esterases and production of flavor components such as acetaldehyde, acetoin and diacetyl due to citrate breakdown. This beneficial role of enterococci in the development of cheese aroma has led to the inclusion of enterococcal strains as starter cultures or starter adjuncts [28]. The effect of enterococci as starter cultures or co-cultures was studied in different type of cheeses, such as Cheddar, [49, 50] Feta, [33, 51] water-buffalo Mozzarella, [38, 52] and Cebreiro, [40, 53] In most of these studies, *E. faecalis* strains have been used, and only

in the case of Feta cheese, two strains belonging to the species of *E. faecium* (*E. faecium* FAIR-E 198 and FAIR-E 243) were used. [33] Similarly, Menendez *et al.* 2004 [54] added selected enterococci in Tetilla cheese production, with an ideal starter with the aim of achieving a high lipolysis and proteolysis. The British Advisory Committee on Novel Foods and Processes (ACNFP) accepted the use of *E. faecium* strain K77D as a starter culture in fermented dairy products [55].

Contribution of enterococci to food is not only limited to final taste development through their primary and secondary metabolisms; but they also produce bacteriocins known as enterocins that are linked to food bio-preservation [26]. Enterocins are small, ribosomally synthesised, extracellularly released, antibacterial peptides mainly produced by *E. faecalis* and *E. faecium* strains [56]. Enterocins usually belong to class II bacteriocins, i.e. they are small and heat-stable and membrane active non-lantibiotics, being stable in milk. They are insensitive to rennet, have stability over a wide range of pH values, and a general compatibility with other starter LAB species [57]. The enterocins have been found to display inhibitory spectrum towards foodborne pathogens such as *Listeria spp.* and *Clostridium spp.* [58–61] Activity towards Gram-negative bacteria such as *E. coli* [62, 63] and *Vibrio cholerae* [64] has been shown as well. Two main applications concerning bacteriocins are possible: the use of either antimicrobial peptide as additives or bacteriocinogenic strains directly in the food system. Many reports consider possible use of enterocins/bacteriocinogenic enterococci as protective starters in different food models, where it can efficiently serve as a barrier in a hurdle technology. [65] Well-characterised enterocins are A, P, CRL35, 1071A, and B; bacteriocin 31, 32, RC714, and T8; enterolysin A, AS-48, EJ97, RJ11, Q, L50A and B [66].

Enterocins with broad inhibitory spectra against food spoilage and pathogenic organisms undoubtedly indicate their use as biopreservative agents in dairy systems. As proposed by Giraffa (1995), careful identification followed by thorough testing based upon certain criteria must be accomplished before putting any of the enterocins (or enterocin producing strains) to practical use in the dairy industry [58].

Therapeutic properties of enterococci are manifested through probiotic activities including maintenance of the normal intestinal microflora and thereby reduction of gastrointestinal disorders, alleviation of lactose intolerance, reduction in serum cholesterol levels, anticarcinogenic activity, stimulation of the immune system and improved nutritional value of foods [2]. Enterococci are important members of the microflora inhabiting GI tract of humans with *E. faecalis*, *E. faecium* and *E. durans* as prevailing species [67]. Therefore, *Enterococcus spp.* besides

Lactobacillus spp. and *Bifidobacterium* spp. are used as probiotics [5].

A well-studied *Enterococcus* strain used as a probiotic is *E. faecium* SF 68, which is produced in Switzerland. It has been proposed to be clinically effective in the prevention of antibiotic-associated diarrhea [68] and in the treatment of diarrhea in children [69]. *E. faecium* SF 68 has been tested in adults with acute diarrhea in two hospitals in Belgium. [70] Besides this Benyacoub et al. (2005) [71] also reported that *E. faecium* SF 68 stimulates immune system against *Giardia intestinalis* in mice. *E. faecium* SF 68 has also been studied as a probiotic supplement in feed and has been used in a dry dog food where it significantly enhanced cell-mediated and humoral immune responses [72]. Another probiotic *Enterococcus* is the Causido® culture that consists of two strains of *S. thermophilus* and one strain of *E. faecium*. This probiotic has been claimed to be hypocholesterolaemic in the short-term reduction of LDL-cholesterol, [73] but long-term reduction was not demonstrated. [74, 75] Hlivak et al., (2005) [76] conducted one year study on humans and reported a decrease in 12% serum cholesterol level by probiotic *E. faecium* M-74 strain. Rossi et al., (1999) [77] used the strain *E. faecium* CRL 183 in combination with *Lactobacillus jugurti* for the development of a novel fermented soymilk product. This formulated product showed prominent hypocholesterolemic effect under *in vitro* trials. *E. faecium* PR88, another probiotic strain was studied in a human clinical trial which alleviated the symptoms of irritable bowel syndrome [78]. Likewise, Gardiner et al., (1999) [79] used *E. faecium* PR88 as adjunct starter for the production of a probiotic Cheddar cheese.

E. faecalis and *E. faecium* strains have also been widely used as veterinary feed supplements. Since February 2004, 10 preparations (9 different strains of *E. faecium*) are authorized as additives in feeding stuffs in the European Union (European Commission, 2004) [80].

However, despite the well-established probiotic benefits of several enterococcal strains, the controversy that they may pose some risks for the human health has made the application of enterococci as probiotic within the food industry a debatable issue [65].

Undesirable activities of enterococci

Besides their positive traits, several risk factors are also associated with enterococci. They can, therefore, become a problem in traditionally fermented food products especially if present initially in high numbers [81]. Production of biogenic amines seems to be unfavorable activity of enterococci in fermented dairy products, because biogenic amines have been associated with a number of food

poisoning episodes [82]. It has been observed that the prolific growth of enterococci in milk and milk products leads to the formation of significant levels of biogenic amines [4, 43, 83]. In fact, tyramine is the only relevant biogenic amine produced by enterococci isolated from dairy products [84].

It is known that casein degradation performed by enterococci play an important role in the development of flavor and texture in cheese. This statement could be true to the certain level as some peptides contribute to the flavor; while others undesirable bitter-tasting peptides can lead to off-flavors of cheese [65]. The lipolytic activity of enterococci in cheese contributes towards cheese flavor due to the formation of fatty acids. Oxidation of these fatty acids, leads to the formation of methyl ketones and lactones which thereby form unsaturated and favored aldehydes causing a flavour defect referred to as oxidative rancidity [2].

More seriously, a number of nosocomial infections such as endocarditis, bacteraemia, urinary tract and neonatal infections have been also associated with an increasing incidence of enterococci, predominantly by the strains of *E. faecalis* and, to a lesser extent, *E. faecium*. Over the last two decades, enterococci, formerly viewed as organisms of minimal clinical impact, have emerged as opportunistic pathogens of humans [8]. Several virulence factors of enterococci have been described and the number of antibiotic resistant enterococci (ARE), especially vancomycin-resistant enterococci (VRE), is increasing [2].

Virulence of enterococci

For enterococci to cause infection, they must first colonize the host tissue, resist host specific and unspecific defense mechanisms, and produce pathological changes. [28] Known virulence traits in enterococci include aggregation substance (*agg*), extracellular surface protein (*esp*) and adhesin-like *E. faecalis* and *E. faecium* antigens (*efaAfs*, *efaAfm*) which play a major role in adherence to host tissues. Besides these, secretion of cytolysin (*cyl*), other toxic products as gelatinase (*gelE*), hyaluronidase and production of plasmid-encoded sex pheromones (*cpd*, *cob*, *ccf*, *cad*) are also considered as virulence traits. Cytolysins are responsible for lysing a broad range of eukaryotic and prokaryotic cells, while gelatinase (*gelE*) acts on collagenous material in tissues and facilitates in invasion. Sex pheromone facilitates the conjugation mediated uptake of antibiotic resistance and virulence traits [5, 13].

Some enterococci possess a plasmid collection mechanism which is based on production of chromosomally encoded 'sex pheromones'. Sex pheromones are small, linear peptides of 7 or 8 amino acids that are excreted by *E. faecalis* strains which promote the acquisition of

plasmid DNA. When pheromones bind to receptors on the cell surface of strains that contain plasmid DNA, this signal is transduced and leads to induction of the aggregation substance (AS) gene. When this gene expresses, AS mediates the formation of cell clumps by binding to a complementary receptor termed binding substance, that allows the highly efficient transfer of the pheromone plasmid on which the AS gene is encoded [85, 86]. The pheromones, however, not only have a role in transfer of plasmid DNA, but also serve as chemo-attractive substances for human neutrophils and induce inflammation and superoxide production [87, 88].

Gelatinase is an extracellular metallo-endopeptidase involved in hydrolysis of gelatin, collagen, and other bioactive peptides [89]. The production of gelatinase was shown to increase pathogenicity in an animal model, [90] which confirms its role in virulence. Enterococcal lipoteichoic acid from the cell wall was shown to induce production of interleukin-1 β , interleukin-6 and TNF- α *in vitro* and may, therefore, contribute to local tissue damage. Hyaluronidase is a cell surface-associated enzyme which cleaves the mucopolysaccharide moiety of connecting tissues or cartilage. This enzyme has been implicated to act as 'spreading factor' for dissemination of some microorganisms [88].

Virulence factors are mainly detected among clinical enterococcal isolates, although studies done on the prevalence of virulence traits among enterococcal strains isolated from food suggest that some strains harbour virulence traits as well [5]. In this regard Eaton and Gasson (2001) [13] showed that enterococcal virulence factors were present in clinical, food and starter culture isolates and the prevalence was higher among clinical strains, followed by food isolates; the lowest prevalence was observed for starter isolates. Among enterococcal species, *E. faecalis* generally harbour more and multiple virulence determinants and in *E. faecium* the frequencies was very low [6, 13, 91].

Antibiotic resistance

Virulence of enterococci is strongly enhanced by their frequent resistance to commonly used antibiotics, which makes them effective opportunists in nosocomial infections [81]. Antibiotic resistance encompasses both natural (intrinsic) resistance and acquired (transferable) resistance. Enterococci possess a broad spectrum of antibiotic resistances within these two types [92]. Examples of intrinsic antibiotic resistance include resistance to cephalosporins, β -lactams, sulphonamides and low levels of clindamycin and aminoglycosides, while examples of acquired resistance include resistance to chloramphenicol, erythromycin, high levels of β -lactams, fluoroquinolones and glycopeptides, such as vancomycin [5].

Vancomycin resistance is of special concern because emergence of vancomycin resistant enterococci (VRE) in hospitals has led to serious infections that cannot be treated with conventional antibiotic therapy. Six different gene clusters mediating glycopeptide resistance have been described in enterococci: *vanA*, *vanB*, *vanD*, *vanE* and *vanG* which are known to be acquired traits, while *vanC* is an intrinsic property of motile enterococci. The *vanA* type of enterococcal glycopeptide resistance is the most important one and its main reservoir is *E. faecium* [92].

After considering the positive and negative attributes of this genus, the question whether enterococci are safe for use as starter cultures remains difficult to answer. The principal concern for enterococci in the food is their pathogenic potential based on horizontal transfer of genes for factors associated with virulence and antibiotic resistance. With *in vitro* studies, Eaton and Gasson (2001) [13] were able to show that virulence genes on a pheromone-responsive plasmid could be transferred to strains of *E. faecalis* used as starter cultures in food, but they were not able to transfer virulence genes into strains of *E. faecium* starter cultures. Though the food chain has clearly been established as an important source of enterococci in human environment, where some strains may bear antibiotic resistance and virulence traits. The present evidence, however, does not suggest enterococci as food borne pathogens. The control and safety of foods that contain enterococci is a special challenge to food industry because of their robust nature, their wide distribution and their stability in the environment.

Safety assessment of enterococci

The increasing number of enterococcal infections in recent decades has raised questions regarding their use in foods and probiotics. Studies on the incidence of virulence traits showed that food isolates can also harbour such traits [6, 13]. Thus, before using enterococci for food production or in probiotic preparations, safety of these strains should be evaluated. Recently, many authors have assessed the safety of enterococci isolated from different origins [93–97]. Safe strains of enterococci that can be used in food and as probiotics ideally should possess neither any of virulence factors, nor should be able to acquire antibiotic resistance gene [8]. Furthermore, biogenic amines producing strains are also not preferable in food [82]. Opsonophagocytic killing is considered to be additional and an important test to assess the safety of enterococcal strains. It is used as an *in vitro* test to detect a protective immune response of host against enterococci [98, 99]. On the basis of all these tests the proper selection of enterococci for use in food industry is possible.

Conclusion

Enterococci are widely present in nature and dairy products. They can grow in a cheese environment and develop typical taste and flavor in traditional Mediterranean cheeses. Some strains of enterococci produce bacteriocins inhibitory against spoilage or pathogenic bacteria. Therefore, they can be used as starter or adjuncts starter as well as protective cultures in dairy industry. Enterococci also have been used as probiotics because of their possible health-promoting capabilities. However, very few enterococci isolated from dairy products, carry potential virulence factors and can display pathogenic traits. Furthermore, the number of VRE has been increasing during the last decade. Fortunately, these important features are strain-dependent. For these reasons, the selection of *Enterococcus* strains of interest in the food industry should be based on the absence of any possible pathogenic properties, or transferable antibiotic resistance genes. Modern analysis techniques as well as up-to-date knowledge of these strains and their properties will help the food processor and the consumer to accept enterococci like other LAB as important players in certain food and dairy products.

References

- Cintas LM, Casaus P, Herranz C, Nes IF and Hernández PE (2001) Review: bacteriocins of lactic acid bacteria. *Int Food Sci Technol* 7:281–305
- Foulque Moreno MR, Sarantinopoulos P, Tsakalidou E and De Vuyst L (2006) The role and application of enterococci in food and health. *Int J Food Microbiol* 106:1–24
- Cotter PD, Hill C and Ross RP (2005) Bacteriocins: developing innate immunity for food. *Natu Rev* 3:777–788
- Fimland G, Johnsen L, Dalhus B and Nissen-Meyer J (2005) Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. *J Peptide Sci* 11:688–696
- Franz CMAP, Stiles ME, Schleifer KH and Holzapfel WH (2003) Enterococci in foods—a conundrum for food safety. *Int J Food Microbiol* 88:105–122
- Franz CMAP, Muscholl-Silberhorn AB, Yousif NMK, Vancanneyt M, Swings J and Holzapfel WH (2001) Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl Environ Microbiol* 67:4385–4389
- Pillar CM and Gilmore MS (2004) Enterococcal virulence – pathogenicity island of *Enterococcus faecalis*. *Front Biosci* 9:2335–2346
- Kayser FH (2003) Safety aspects of enterococci from the medical point of view. *Int J Food Microbiol* 88:255–262
- Nallapareddy N, Wenxiang H, Weinstock GM and Murray B (2005) Molecular characterization of a widespread, pathogenic, and antibiotic resistance receptive *Enterococcus faecalis* lineage and dissemination of its putative pathogenicity island. *J Bacteriol* 187:5709–5718
- Dargere S, Vergnaud M, Verdon R, Saloux E, Le Page O, Leclercq R and Bazin C (2002) *Enterococcus gallinarum* endocarditis occurring on native heart valves. *J Clin Microbiol* 40:2308–2310
- Poyart C, Lambert T, Morand P, Abassade P, Quesne G, Baudouy Y and Trieu-Cuot P (2002) Native valve endocarditis due to *Enterococcus hirae*. *J Clin Microbiol* 40:2689–2690
- Higashide T, Takahashi M, Kobayashi A, Ohkubo S, Sakurai M, Shirao Y, Tamura T and Sugiyama K (2005) Endophthalmitis caused by *Enterococcus mundtii*. *J Clin Microbiol* 43:1475–1476
- Eaton TJ and Gasson MJ (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol* 67:1628–1635
- Semedo T, Almeida Santos M, Silva Lopes MF, Figueiredo Marqués JJ, Barreto Crespo MT and Tenreiro R (2003a) Virulence factors in food, clinical and reference enterococci: a common trait in the genus? *Syst Appl Microbiol* 26:13–22
- Martín M, Gutiérrez J, Criado R, Herranz C, Cintas LM and Hernández PE (2006) Genes encoding bacteriocins and their expression and potential virulence factors of enterococci isolated from wood pigeons (*Columba palumbus*). *J Food Prot* 69:520–531
- Cocconcilli PS, Cattivelli D, and Gazzola S (2003) Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. *Int J Food Microbiol* 88(2–3):315–323
- Saavedra L, Taranto MP, Sesma F and de Valdez GF (2003) Homemade traditional cheeses for the isolation of probiotic *Enterococcus faecium* strains. *Int J Food Microbiol* 88:241–245
- Devriese LA and Pot B (1995) In: The Lactic Acid Bacteria, the genera of lactic acid bacteria, the genus *Enterococcus*, vol 2 (Wood BJB and Holzapfel WH eds). Blackie Academic and Professional, London, United Kingdom, pp 327–367
- Lancefield RC (1933) A serological differentiation of human and other groups of hemolytic streptococci. *J Exp Med* 57:571–595
- Sherman JM (1937) The streptococci. *Bacteriol Rev* 1:3–97
- Devriese LA, Pot B and Collins MD (1993) Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* 75:399–408
- Stiles M (2002) Safety aspects of enterococci from food point of view. In: Symposium on Enterococci in Foods—Functional and Safety aspects, Berlin, Abstract Book.
- Schleifer KH and Kilpper-Bälz R (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J Syst Bacteriol* 34:31–34
- Hartman PA, Deibel RH and Sieverding LM (2001) Enterococci. In Compendium of methods for the microbiological examination of foods, (Downes FP and Ito K eds). Amer Public Health Assoc, Washington, DC, USA, pp 83–87
- Mundt OJ (1986) Enterococci. In: Bergey's Manual of Systematic Bacteriology, vol 2 (Sneath PHA, Mair NS, Sharpe ME and Holt JG eds). Williams, Wilkins and Baltimore pp. 1063–1065

26. Giraffa G (2003) Functionality of enterococci in dairy products. *Int J Food Microbiol* 88:215–222
27. Hill BM and Smythe BW (1997) A study of the microbiological quality of raw milk from individual farms. *New Zealand Dairy Res Inst* 1–35
28. Franz CMAP, Holzapfel WH and Stiles ME (1999) Enterococci at the crossroads of the food safety? *Int J Food Microbiol* 47:1–24
29. Gelsomino R, Vancanneyt M, Condon S, Swings J and Cogan TM (2001) Enterococcal diversity in the environment of an Irish Cheddar-type cheese making factory. *Int J Food Microbiol* 71:177–188
30. Perez BS, Lorenzo PL, Garcia ML, Hernandez PE and Ordóñez JA (1982) Heat-Resistance of Enterococci. *Milchwissenschaft-Milk Sci Int* 37(12):724–726
31. Anonymous (1992) EEC council directive of 16 June 1992 on milk hygiene (92/46EEC). *Off J Euro Commu* L268/1.
32. Birollo GA, Reinheimer JA and Vinderola CG (2001) Enterococci vs. nonlactic acid microflora as hygiene indicators for sweetened yoghurt. *Food Microbiol* 18:597–604
33. Sarantinopoulos P, Kalantzopoulos G and Tsakalidou E (2002a) Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. *Int J Food Microbiol* 76:93–105
34. Manolopoulou E, Sarantinopoulos P, Zoidou E, Aktypis A, Moschopoulou E, Kandarakis IG and Anifantakis EM (2003) Evolution of microbial populations during traditional Feta cheese manufacture and ripening. *Int J Food Microbiol* 82(2):153–161
35. Gelsomino R, Vancanneyt M, Cogan TM, Condon S and Swings J (2002) Source of enterococci in a farmhouse raw-milk cheese. *Appl Environ Microbiol* 68:3560–3565
36. Andrighetto C, Knijff E, Lombardi A, Torriani S, Vancanneyt M, Kersters K, Swings J and Dellaglio F (2001) Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. *J Dairy Res* 68:303–316
37. Čanžek Majhenič A, Rogelj I and Perko B (2005) Enterococci from Tolminc cheese: population structure, antibiotic susceptibility and incidence of virulence determinants. *Int J Food Microbiol* 102:239–244
38. Coppola S, Parente E, Dumontet S and Lapeccerella A (1998) The Microflora of Natural Whey Cultures Utilized as Starters in the Manufacture of Mozzarella Cheese from Water-Buffered Milk. *Lait* 68(3):295–309
39. Freitas AC, Pais C, Malcata FX and Hogg TA (1995) Microbiological characterization of Picante de Beira Baixa cheese. *J Food Prot* 59:155–160
40. Centeno JA, Mene'ndez S and Rodriguez-Otero JL (1996) Main microbial flora present as natural starters in Cebreiro raw cow's-milk cheese (Northwest Spain). *Int J Food Microbiol* 33:307–313
41. Mene'ndez S, Godý'nez R, Centeno JA and Rodry'guez-Otero JL (2001) Microbiological, chemical and biochemical characteristics of "Tetilla" raw cows-milk cheese. *Food Microbiol* 18:151–158
42. Mannu L and Paba A (2002) Genetic diversity of lactococci and enterococci isolated from home-made Pecorino Sardo ewes' milk cheese. *J Appl Microbiol* 92:55–62
43. Serio A, Paparalla A, Chaves-Lopez C, Corsetti A and Suzzi G (2007) *Enterococcus* populations in Pecorino Abruzzese cheese: biodiversity and safety aspects. *J Food Prot* 70(7):1561–1568
44. Templer SP and Baumgartner A (2007) Enterococci from appenzeller and schabziger raw milk cheese: antibiotic resistance, virulence factors, and persistence of particular strains in the products. *J Food Prot* 70(2):450–455
45. Litopolou-Tzanetaki E (1990) Changes in numbers and kinds of lactic acid bacteria during ripening of Kefalotyri cheeses. *J Food Sci* 55:111–113
46. Macedo CA, Malcata FX and Hogg TA (1995) Microbiological profile in Serra ewe's cheese during ripening. *J Appl Bacteriol* 79:1–11
47. Sarantinopoulos P, Andrighetto C, Georgalaki MD, Rea MC, Lombardi A, Cogan TM, Kalantzopoulos G and Tsakalidou E (2001a) Biochemical properties of enterococci relevant to their technological performance. *Int Dairy J* 11:621–647
48. Lo'pez-Diaz TM, Santos JA, Gonzalez CJ, Moreno B and Garcia ML (1995) Bacteriological quality of traditional Spanish blue cheese. *Milchwissenschaft* 50:503–505
49. Jensen JP, Reinbold GW, Washam CJ and Vedamuthu ER (1975a) Role of enterococci in Cheddar cheese: free fatty acid appearance and citric acid utilization. *J Milk Food Technol* 38:78–83
50. Jensen JP, Reinbold GW, Washam CJ and Vedamuthu ER (1975b) Role of enterococci in Cheddar cheese: proteolytic activity and lactic acid development. *J Milk Food Technol* 38:3–7
51. Litopolou-Tzanetaki, E, Tzanetakis N and Vafopouloumastrojiannaki A (1993) Effect of the Type of Lactic Starter on Microbiological Chemical and Sensory Characteristics of Feta Cheese. *Food Microbiol* 10(1):31–41.
52. Villani F and Coppola S (1994) Selection of enterococcal strains for water-buffalo Mozzarella cheese manufacture. *Annali di Microbiologia ed Enzimologia* 44:97–105
53. Centeno JA, Menendez S, Hermida MA and Rodry'guez-Otero JL (1999) Effects of the addition of *Enterococcus faecalis* in Cebreiro cheese manufacture. *Int J Food Microbiol* 48:97–111
54. Menendez S, Godinez R, Hermida M, Centeno JA and Rodriguez-Otero JL (2004) Characteristics of "Tetilla" pasteurised milk cheese manufactured with the addition of autochthonous cultures. *Food Microbiol* 21: 97–104
55. ACNFP (1996) Report on *Enterococcus faecium*, strain K77D. In: MAFF Advisory Committee on Novel Foods and Processes, Report, Ergon House c/o Nobel House, 17 Smith Square, London SW1 3JR, United Kingdom
56. Leroy F, Foulquie Moreno MR, and De Vuyst L (2003) *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. *Int J Food Microbiol* 88(2-3):235–240
57. Van Belkum MJ and Stiles ME (2000) Nonantibiotic antibacterial peptides from lactic acid bacteria. *Nat Pro Rep* 17:323–335
58. Giraffa G (1995) Enterococcal bacteriocins: their potential as anti-*Listeria* factors in dairy technology. *Food Microbiol* 12:291–299
59. Maisnier-Patin S, Forni E and Richard J (1996) Purification, partial characterization and mode of action of enterococcal EFS2, an antilisterial bacteriocins produced by a strain of

- Enterococcus faecalis* isolated from a cheese. Int J Food Microbiol 30:255–270
60. Cintas LM, Casaus P, Fena'ndez M and Herna'ndez PE (1998a) Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A and lactocin S against spoilage and foodborne pathogenic bacteria. Food Microbiol 115: 289–298
 61. Mohar P, Čanžek Majhenič A and Rogelj I (2005) Phenotypic characterisation, antibiotic susceptibility and antimicrobial activity of enterococcal population from Karst ewe's cheese. In: 2nd Int ASM-FEMS Conference on Enterococci, Helsingor, Denmark (poster)
 62. Ga'ívez A, Gime'nez-Gallego G, Maqueda M and Valdivia E (1989) Purification and amino acid composition of peptide antibiotic AS-48 produced by *Streptococcus (Enterococcus) faecalis* subsp. *liquefaciens* S-48. Antimicro Agents Chemo 33:437–441
 63. Tomita H, Fujimoto S, Tanimoto K and Ike Y (1997) Cloning and genetic organization of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. J Bacteriol 179:7843–7855
 64. Simonetta AC, Moragues de Velasco LG and Friso'n LN (1997) Antibacterial activity of enterococci strains against *Vibrio cholerae*. Lett Appl Microbiol 24:139–143
 65. Čanžek Majhenič A (2006) Enterococci: yin-yang microbes. Mljekarstvo 56(1):5–20
 66. Franz CMAP, van Belkum MJ, Holzapfel WH, Abrioul H and Galvez A (2007) Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 31:293–310
 67. Tannock GW and Cook G (2002) Enterococci as members of the intestinal microflora of humans, In: The enterococci: pathogenesis, molecular biology, and antibiotic resistance, (Gillmore MS eds). ASM Press, Washington, pp 101
 68. Wunderlich PF, Braun L, Fumagalli I, D'Apuzzo V, Heim F, Karly M, Lodi R, Politta G, Vonbank F and Ja Zeltner L (1989) Double-blind report on the efficacy of lactic acid-producing *Enterococcus* SF68 in the prevention of antibiotic-associated diarrhoea and in the treatment of acute diarrhoea. J Int Medi Res 17:333–338
 69. Bellomo G, Mangiagale A, Nicastro L and Frigerio G (1980) A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. Curr Therap Res 28:927–936
 70. Buydens P and Debeuckelaere S (1996) Efficacy of SF 68 in the treatment of acute diarrhea. A placebo-controlled trial. Sc J Gastroenterol 31:887–891
 71. Benyacoub J, Perez PF, Rochat F, Saudan KY, Reuteler G, Antille N, Humen M, De Antoni GL, Cavadini C, Blum S and Schiffrin EJ (2005) *Enterococcus faecium* SF68 enhances the immune response to *Giardia intestinalis* in mice. J Nutr 135:1171–1176
 72. Benyacoub J, Czarnecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schiffrin EJ and von der Weid T (2003) Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. J Nut 133: 1158–1162
 73. Agerholm-Larsen L, Bell ML, Grunwald GK and Astrup A (2000) The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. Euro J Clin Nutr 54(11):856–860
 74. Richelsen B, Kristensen K and Pedersen SB (1996) Long-term (6 months) effect of a new fermented milk product on the level of plasma lipoproteins - A placebo-controlled and double blind study. Euro J Clin Nut 50(12):811–815
 75. Sessions VA, Lovegrove JA and Taylor GRJ (1997) The effect of a new fermented milk product on total plasma cholesterol, LDL-cholesterol and apolipoprotein B concentrations in middle-aged men and women. In: Functional Foods: The Consumer, The Product, and The Evidence (Sadler MJ and Saltmarsh M eds). The Royal Society of Chemistry, London, United Kingdom, pp15–19
 76. Hilvak P, Odraska J, Ferencik M, Ebringer L, Jahnova E and Mikes Z (2005) One-year application of probiotic strain *Enterococcus faecium* M-74 decreases serum cholesterol levels. Bratisl Lek Listy 106(2):67–72
 77. Rossi E.A, Vendramini RC, Carlos IZ, Pei YC and de Valdez GF (1999) Development of a novel fermented soymilk product with potential probiotic properties. Euro Food Res Technol 209:305–307
 78. Allen WD, Linggood MA and Porter P (1996) *Enterococcus* organisms and their use as probiotics in alleviating irritable bowel syndrome symptoms. Euro Patent 0508701 (B1)
 79. Gardiner GE, Ross RP, Wallace JM, Scanlan FP, Jagers P, Fitzgerald GF, Collins JK and Stanton C (1999) Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of cheddar cheese. J Agri Food Chem 47(12): 4907–4916
 80. European Commission (2004) List of the authorized additives in feeding stuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feeding stuffs. Official J Euro Union C50
 81. Giraffa G (2002) Enterococci from foods. FEMS Microbiol Rev 26:163–171
 82. Karovacova J and Kohajdova Z (2005) Biogenic Amines in food. Chem Pap 59(1):70–79
 83. Martuscelli M, Gardini F, Torriani S, Mastrocola D, Serio A, Chaves-Lopez C, Schirone M and Suzzi G (2005) Production of biogenic amines during the ripening of Pecorino Abruzzese cheese. Int Dairy J 15:571–578
 84. Bover-cid S, Hugas M, Izquierdo-Pulido M and Vidal-Carou MC (2001) Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. Int J Food Microbiol 66:185–189
 85. Clewell DB (1993) Bacterial sex pheromone-induced plasmid transfer. Cell 73:9–12
 86. Dunny GM, Leonard BAB and Hedberg PJ (1995) Pheromone-Inducible Conjugation in *Enterococcus faecalis*: Interbacterial and Host-Parasite Chemical Communication. J Bacteriol 177(4):871–876
 87. Johnson AP (1994) The Pathogenicity of Enterococci. J Antimicro Chemo 33(6):1083–1089
 88. Witte W, Wirth R and Klare I (1999) Enterococci. Chemo 45:135–145
 89. Su YA, Sulavik MC, He P, Ma'kinen KK, Ma'kinen P, Fiedler S, Wirth R and Clewell DB (1991) Nucleotide sequence of the gelatinase gene (gelE) from *Enterococcus faecalis* subsp. *liquefaciens*. Infect Immun 59:415–420

90. Qin X, Singh KV, Weinstock GM and Murray BE (2000) Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. *Infect Immun* 68:2579–2586
91. Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Dupre I and Sechi LA (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int J Food Microbiol* 88:291–304
92. Klare I, Konstabel C, Badstubner D, Werner G and Witte W (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. *Int J Food Microbiol* 88: 269–290
93. Yousif NMK, Dawyndt P, Abriouel H, Wijaya A, Schillinger U, Vancanneyt M and Swings J (2005) Molecular characterization, technological properties and safety aspects of enterococci from “Hussuwa”, an African fermented sorghum products. *J Appl Microbiol* 98:216–228
94. Perez-Pulido R, Abriouel H, Ben Omer N, Lucas R, Martinez-Canamero M and Galvez A (2006) Safety and potential risks of enterococci isolated from traditional fermented capers. *Food Chem Toxicol* 44:2070–2077
95. Omar BN, Castro A, Lucas R, Abriouel H, Yousif NMK, Franz CAMP, Holzapfel WH, Perez-Pulido R, Martinez-Canamero M and Galvez A (2004) Functional and safety aspects of enterococci isolated from different Spanish foods. *Syst Appl Microbiol* 27:118–130
96. Lopes MFS, Simões AP, Tenreiro R, Marques JJF and Crespo MTB (2006) Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *Int J Food Microbiol* 112:208–214
97. Sánchez J, Basanta A, Gómez-Sala B, Herranz C, Cintas LM and Hernández PE (2007) Antimicrobial and safety aspects and biotechnological potential of bacteriocinogenic enterococci isolated from mallard ducks (*Anas platyrhynchos*). *Int J Food Microbiol* 117:295–305
98. Hufnagel M, Koch S, Kropec A and Huebner J (2003) Opsonophagocytic assay as a potentially useful tool for assessing safety of enterococcal preparations. *Int J Food Microbiol* 88: 263–267
99. Kropec A, Hufnagel M, Zimmermann K and Huebner J (2005) *In vitro* assessment of the host response against *Enterococcus faecalis* used in probiotic preparations. *Infect* 33(5/6):377–379