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The Genus *Enterococcus* As Probiotic: Safety Concerns

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ABSTRACT

Species from the genus Enterococcus *have been used as probiotic for humans or animals, although this genus is not considered "generally recognized as safe" (GRAS). While enterococci are considered "positive" in food technology, isolates of this genus have emerged as opportunistic pathogens for the humans. The aim of this review is to summarize the characteristics that can determine the use of this genus as probiotics. According to the guidelines used to define the genus* Enterococcus *strains as probiotic a case-by-case evaluation of each potential technological strain is presented and research perspectives for using enterococci as probiotic is also discussed.*

Key words: Disease, *Enterococcus,* Probiotic, Safety aspects

INTRODUCTION

The genus *Enterococcus* belongs to the family *Enterococcaceae* along with the genera *Atopobacter, Catellicoccus, Melissococcus, Pilibacter, Tetragenococcus* and *Vagococcus.* (Devriese et al. 2006; Euzéby 2010). In general, the enterococci may occur in the form of isolated cocci, in pairs or in short chains. They do not produce spores, are oxidase negative and facultative anaerobe. They are classified as lactic acid bacteria (LAB) as they carry most of the phenotypes of the other components of the group such as Gram positive, catalase negative and the ability to convert glucose into lactic acid as main product (homofermentative) of primary metabolism. Moreover, individuals of this genus grow at an optimum temperature of 35 °C, although some species of the genus grow in temperatures ranging from 10 to 45 ° C. Most of them grow at high NaCl concentrations (up 6.5%), pH 9.6, survive at 60 ° C for 30 min, hence are considered thermoduric. Many of these organisms can hydrolyze esculin in the presence of 40% bile

salts, which is one of the traits for phenotypic identification (Holt et al. 1994; Devriese et al. 2006; Leblanc 2006). The main species of this genus, frequently found both in food and in clinical samples, are *Enterococcus faecalis* and *E. faecium* (Facklam et al. 1995; Hardie and Whiley 1997). Although nowadays molecular tools are available for the identification of the strains and species of the genus, phenotype characterizations are still important in some instances, such as for the screening and for presumptive identification. Other traits to complement the phenotype characterization are carbohydrate fermentation (mannitol, sorbose, arabinose, sorbitol, raffinose), growth at 10 and 45°C, hydrolysis of arginine (arginine decarboxylation test), metabolism of pyruvate (1%), motility and production of yellowish pigmentation (Devriese et al. 2006).

Species of *Enterococcus* are ubiquitous and produce a variety of products such as aromatic compounds (Centeno et al. 1999), enzymes (Sarantinopoulous et al. 2001; Ghrairi et al. 2008) and bacteriocins (enterocins) (Cleveland et al. 2001; Achemchem et al. 2005). They contribute to

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the texture, flavor, aroma and safety of different foods as in the case of handmade cheeses (Andrighetto et al. 2001), sausages (Sabia et al. 2002; Tanasupawat et al. 2008) and other fermented products (Giraffa 2000; Bover-Cid et al. 2001; Gardini et al. 2001; Gomes et al. 2008).

The LAB have been sources of probiotic strains, which include *Lactobacillus* and *Bifidobacterium* as predominant genera (Coeuret et al. 2004). The genus *Enterococcus* has been the target of several studies for use as probiotics (Sivieri et al. 2008; Kuritza et al. 2011). They have some desirable characteristics for this purpose such as resistance to gastric juice and bile salts (Rossi et al. 2003) and production of antimicrobial compounds such as enterocin (Franz et al. 1999; Saarela et al. 2000).The genus has been isolated from different niches such as water (Oliveira and Pinhata 2008), plants (Svec et al. 2011), animals (Jung et al. 2007) and foods (Gomes et al. 2008) and different species have been used as starter or adjunct culture in the manufacture of fermented products such as cheese (Centeno et al. 1999; Ghairi et al. 2008; Williams and Withers 2010; Morandi et al. 2011) and sausages (Sabia et al. 2002; Tanasupawat et al. 2008), among others.Although some studies have corroborated its effectiveness as probiotic (Bellomo et al. 1980; Saavedra et al. 2001; Benyacoub et al. 2003; Rivera-Espinoza and Gallardo-Navarro 2010), others have shown correlation of the genus with diseases, which include urinary tract infections (Protonotariou et al. 2010), bacteremia (Shankar et al. 1999, Shankar et al. 2002; Tan et al. 2010) and endocarditis (Conde-Estéves et al. 2010; Rasmussem et al. 2010; Heikens et al. 2011). However, the host is usually patients with serious diseases and immuno-compromised (Brilliantova et al. 2010) what emphasizes its opportunist nature. This review aims to discuss the main virulence factors present in the genus *Enterococcus* which jeopardizes the safety of the genus and warrants precautions when new probiotic strains are considered.

ENTEROCOCCUS AS PROBIOTIC

Probiotics are "live microorganisms that confer a health benefit to the host when administered in adequate amounts" (FAO / WHO, 2002). Studies in the area of probiotics have advanced significantly due to the growth of interest in the products supplemented with these microorganisms. The features associated with the probiotics include maintenance of the balance of intestinal microbiota (Arvola et al. 1999), control of diarrhea (Arvola et al. 1999), stimulation of the immune system (Isolauri et al. 2004), reducing hypersensitivity to allergenic substance and eczema in children (Kalliomäki et al. 2001; Kukkonen et al. 2007), prevention of intestinal inflammations (Isolauri et al. 2000; Kalliomäki al., 2001), and modulation of undesirable effects of lactose upon non-persistent lactase individuals (Griffin et al. 2002).

Traditionally, species from the *Lactobacillus* and *Bifidobacterium* are among the major strains used as probiotics (Coeuret et al. 2004) and are carried in different foods such as yoghurt (Moschner et al. 2004), juice (Yon et al. 2006), cheeses (Gardiner et al, 1999), etc. Different strains of these genera are able to contemplate all the criteria for being considered a probiotic, such as i) belonging to the host microbiota in which the probiotic is administered, ii) stability against gastric juice and bile salts, iii) ability to adhere to the intestinal mucosa and colonize it, iv) have antagonistic effect to different pathogens, and v) stimulation of the immune system. Other key features are i) the safety for human/animal use, ii) the history of non-pathogenicity and iii) no association with other diseases such as endocarditis, iv) besides the absence of gene determinants of antibiotic resistence (Saarela et al. 2000; FAO/WHO 2002). However, some other species of bacteria and fungi such as *Bacillus* (Endres et al. 2011), *Enterococcus* (Sivieri et al. 2008), and *Sacharomyces* (Psomas et al, 2001) have also been used as probiotics in food and feed.

Enterococcus strains have been used as supplement for the food and feed such as poultry and swine to replace the use of sub-lethal antibiotics in the feeds. Many studies have been conducted to evaluate the effect of probiotic strains of this genus (mainly *E. faecium*). Generally, human studies are scarcer when compared to animal applications.

Pollmann et al (2005) obtained positive results in reducing the rate of infection by endogenous *Chlamydiae* in the pigs supplemented with strain Cylactin LBC ME10 (probiotic group), a microencapsulated *E. faecium* SF68 (NCIMB 10415), containing 9×10^9 CFU / g viable bacteria. The results showed a reduction in the

severity of infections as well as of the number of infections caused by *Chlamydiae.*

Kuritza et al (2011) evaluated the effects of addition of a probiotic strain *E. faecium* (Cylactin ME 20 Plus ® 50g / T) in the feed of chickens with the objective of controlling the contamination by *Salmonella* Minnesota (SM). The poultry were divided into three treatments: i) basal diet (BD); ii) BD inoculated with 108 CFU / mL of *Salmonella* Minnesota (SM); iii) BD + SM + the probiotic *E. faecium* ($2x10^{10}$ cfu / g) . The results demonstrated that the use of the probiotic strain on the diet was efficient in controlling and reducing the counts of SM. Moreover, it proved to be an alternative to replace the use of antibiotics to control the pathogens.

Allen et al (1996) identified a new strain of *E. faecium* (PR88) with probiotic claim. The strain has been deposited in European Patent (Number: 0508701), claiming the benefit to relief symptoms in irritable bowel syndrome (IBS).In a study conducted by Rossi et al (2003) strains of *E. faecium* CRL 183 and *Lactobacillus jugurti* 416 were added in a product fermented soy-based and serum lipid levels was evaluated in normocholesterolemic adult men. The group that had received the fermented product supplemented with strains tested showed an increase of 10% in HDL cholesterol levels compared to the group that did not consume the product containing the probiotic strains.Sivieri et al. (2008) determined the effect of probiotic *E. faecium* CRL 183 on the incidence of colon tumors experimentally induced by dimethylhydrazine (DMH) in *Wistar* rats. The rats that consumed *E. faecium* CRL 183 presented a 50% average inhibition as well as enhanced the immune response by increasing the IL-4, IFN-γ e TNF-α when compared with the DMH group

Enterococcus is one of several genera found in intestinal microbiota. It competes for adhesion sites in the epithelial cells with pathogens micro-organisms, thus preventing the colonization and stabilization of a microbiota unfavorable to the individual. It presents features resistance to gastric juice and bile salts, and therefore, when administered, can reach the intestine in relatively high proportions, with an additional factor in colonization it. Due to these characteristics, many strains of this genus have been studied and commercialized as probiotics (Franz et al 1999).

In Brazil, the National Agency of Sanitary Surveillance (ANVISA) defines a list of microorganism claimed as having functional properties (probiotic). The genera listed with some probiotic effect include *Lactobacillus* (five species), *Lactococcus* (one species), *Bifidobacterium* (three species) and *Enterococcus* (one species). Although *E. faecium* is among the probiotic strains allowed to be used as probiotic, its many strains are known for carrying virulence factors already described, which include the resistance to antibiotic as the most danagerous characteristic for a microorganism to be used as probiotic (Billstrom et al. 2008).

VIRULENCE FACTORS IN *ENTEROCOCCUS*: PARAMETERS TO BE ASSESSED

During the last two decades, *Enterococcus* has been identified as an agent of nosocomial infections with an increasing frequency; in parallel the resistance the antimicrobial agents have been increased. As a result, *Enterococcus* has emerged as a main challenge to doctors, when identified and associated with the principal cause of infection, especially in immunocompromised patients (Mundy et al. 2000).Substances produced by the microorganisms that can cause harm to the host are referred to as virulence factors. The term came to express any component of microorganism that is required to generate the illness or enhance it (Schaechter et al. 1999).

Enterococcus is a commensal organism that does not secrete any potentially virulent toxin, although capable of developing the disease, especially in immunocompromised patients. Infections caused by enterococci are originated from the patient's own intestinal microbiota and can be transferred from one individual to another, or can be acquired by the consumption of contaminated food and water (Murray 2006; Brilliantova et al. 2010).

This genus is often associated with the pathogenesis such as infections of the urogenital tract (Protonotariou et al. 2010) and endocarditis (Heikens et al. 2011). It is capable of transporting the antibiotic resistence genes to produce β-hemolysin (Franz et al. 2001), gelatinase (Huycke et al. 1991) and aggregation substance (Sartingen et al. 2000) that are undesirable phenotypes in probiotic strain.

Antibiotic Resistance
There are various biochemical pathways that enable the bacteria to resist to the action of

antibiotics, which include the low intracellular accumulation of the antibiotic by altering the permeability of the external membrane, decreased transport across the inner membrane or efflux mechanisms, alteration of target by mutation or enzymatic modification and the enzymatic inactivation of the antibiotic. The coexistence of various mechanisms of antibiotic resistence in a microorganism can lead to Multi-Drug Resistance (MDR) (Depardieu et al. 2007).

Enterococcus is a common member of the endogenous intestinal microbiota. Due to the capability to acquire the resistance genes (on plasmids or transposons) from other microorganisms, it has higher probability of acquiring the resistant pathogenic markers than others in the same niche (Chopra e Roberts, 2001). Some genes that confer resistance to cephalosporins, sulfonamides and aminoglycosides are usually intrinsic, i.e., located in the chromosomes. However, some *Enterococcus* species may carry extrinsic resistance to chloramphenicol, erythromycin, tetracycline and vancomycin. These genes are located in the plasmids or in transposons, enabling horizontally or vertically transfer to different groups of microorganisms, such as to *Streptococcus* spp., *Staphylococcus aureus* and *Bacillus subtilis*, and thus hampering the antimicrobial therapy (Chow et al. 1997; Chow 2000; Donabedian et al. 2003).

Vancomycin-Resistant Enterococci
Enterococcus has different types of conjugation, which facilitates the spread of resistance genes to other species of microorganisms. These systems include plasmids that can replicate in several Gram positive bacteria, in addition to the mechanism via transposon conjugation, which can be transferred intracellularly and integrate into the genome of the host bacteria (Zarilli et al. 2005).

The high genetic diversity in *Enterococcus* group suggests adaptations for specific mutations in different environments. Thus, continuous exposure to antibiotics may cause a mutation that confers bacteria resistance to certain antimicrobial compound, enabling their survival. This may explain the occurrence of MDR found in the strains isolated from hospital environment (Centinkaya et al. 2000; Shepard and Gilmore 2002).

Currently, there are six known phenotypes of glycopeptide resistance (*van*A, *van*B, *van*C, *van*D,

*van*E and *van*G) in *Enterococcus*. The *van*A operon is characterized by the strains that present high levels of resistance to vancomycin and teicoplanin. The *van*B operon induces varying levels of resistance to vancomycin with minimal inhibitory concentration (MIC) between 4-1000 mg / mL). Only *van*A and *van*B have the capacity to transfer vertically and horizontally and to confer high levels of resistance (Centinkaya et al. 2000).

The *van*A phenotype is characterized by the strains that exhibit high levels of resistance to vancomycin and teicoplanin due to the expression of genes inserted into transposon Tn1546 non-conjugative (Arthur et al. 1993)

The *van*B operon originated from transposon Tn1547 or Tn1549 induces several levels of vancomycin resistance but does not induce resistance to teicoplanin. Naturally, this gene is located on bacterial chromosome, but can be obtained from the plasmids to other organisms (Leme and Ferreira 2001).

The phenotype characterized by *van*C strains shows low level resistance to vancomycin and intrinsic sensitivity to teicoplanin (Navarro andCourvalin, 1994).

The operon *van*D induces a moderate degree of resistence to vancomycin and teicoplanin and is present in chromosome and is different from other resistence genes, this trait does not seem to be transferable due to its stability in the genome (Casadewall and Courvalin, 1999).

The *van*E and *van*G operons encode a low level resistence to vancomycin and are believed to be acquired and inducible (Fines et al. 1999; Mckessar et al. 2000). In the year 2000, *van*G gene was detected in *E. faecalis* that conferred resistance moderately to teicoplanin (Centinkaya et al. 2000).

Gelatinase, Cytolysins / Hemolysin and Hyaluronidase
Gelatinase is a metalloendopeptidase capable of hydrolyzing insulin, casein, hemoglobin, fibrinogen, collagen and gelatin (Su et al. 1991). The cytolysin is a protein with bacteriocin / hemolysin bifunctionality (Mundy et al. 2000). Hyaluronidase causes the lysis of the hyaluronic acid, which is the main part of the connective tissue of the extracellular matrix.

These virulence factors are found in almost all the species of *Enterococcus* but their expression depends on the niche of isolation. It has been shown that β-hemolytic strains of *Enterococcus*

increased five times the chance of death caused by the bacteremia when compared to the patients with bacteremia caused by non-β-hemolytic strains (Huycke et al. 1991). Strains of *E. faecium* and *E. faecalis* have demonstrated the ability to synthesize this protease due to the presence of the *gelE* gene which may be present but not expressed (Kanemitsu 2001). Often, *Enterococcus* is considered major hospital pathogen causing bacteremia, endocarditis and urinary tract infections. However, it has been established that it could be seen as an opportunistic pathogen to the patients with serious illnesses and immunocompromised (Murray 1990). Special focus is given on the resistance of *Enterococcus* to vancomycin, the latest antibiotic developed for the treatment of enterococcal infections that cannot be treated with the conventional antibiotic therapy. This consideration alone warrants precaution in the choice of a probiotic strain belonging to this genus.

In *E. faecalis,* cytolysin is active against a wide range of Gram-positive bacteria, and also smooth cells, both prokaryotic and eukaryotic, with activity against horse, rabbit and human erythrocytes, but has no action on sheep erythrocytes (Gilmore et al. 1990). The relative *cyl* operon expression of cytolysin can be found in self-transmissible plasmids, integrated into the chromosome and also in pathogenicity islands (PAI). It is often associated with other virulence factors (Shankar et al. 2002).

Hyaluronidase is a hydrolytic enzyme, encoded by the gene *hyl* (Kayaoglu and Orstavik, 2004). It is the main part of the connective tissue extracellular matrix and its destruction caused the breaking of the tissue which facilitates the spread of microorganisms within the tissue (Hynes and Walton 2000). The percentage of infectious diseases caused by *E. faecium* has increased among the infections caused by *Enterococcus* in the United States in the last decade, and this has been suggested as caused by the increase of the virulence of the genus which includes the high incidence of *orf* linked to hyaluronidase (Rice et al. 2003).

Adhesins

In an infectious process, the first step that occurs in tissue colonization by the microorganisms is adherence of the pathogen in adjacent cells. In the strains with probiotic function, adhesion is an important features that favors the colonization and establishment of beneficial microbiota in the intestinal tract (Ferreira et al, 2011). Among the substances that promote this adherence are adhesins. These substances are formed by small peptide molecules, composed of seven to eight amino acids that promote the adhesion of the bacterial cell to host tissue (Nallapareddy et al, 2003). They have been identified a frequency of up to 60% by strains of *E. faecalis* isolated from different outbreaks (Mundy et al. 2000). The various substances with the function of assisting in the adhesion process include the aggregation substances and surface proteins.

Aggregation substance

Aggregation substance (AS) is a virulence factor which appears to mediate the specific binding to the intestinal epithelium, renal epithelial cells, human neutrophils and macrophages (Sussmuth et al. 2000).

An adhesion called *Acm*, has been identified in *E. faecium.* This adhesin binds to the collagen type I and is part of the subfamily of bacterial adhesins surface denominated Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) which binds specifically to the protein layer of the extracellular matrix of the host. MSCRAMM adhere to the collagen, fibronectin, fibrinogen and / or laminin host tissue exposed after being injured. Adhesins help in the host tissue colonization while other proteins aid in the evasion of host defenses and can therefore, lead to infection (Nallapareddy et al. 2003).

Enteroccal surface protein

The virulence factor associated with the presence of surface protein (Esp) seems to be involved in the process of cell-cell adhesion. The gene responsible for the expression of the protein *Esp* is located in a highly conserved chromosome region within the genus, and is common in the strains of *E. faecium* sensitive or resistant to vancomycin (Shankar et al. 2002). This protein has several regions involved in the adhesion to eukaryotic cells and immune response evasion.

Waar et al. (2002) observed that the surface protein of *E. faecalis* promotes the adhesion of bacteria to bile drain materials (silicone rubber, fluoro-ethylene-propylene and polyethylene). The results indicated that when the gene expression occurred, it resulted in an increase in the frequency of adhesion of the cells to the materials evaluated.

Thus, there is a higher biofilm formation even more firmly adhered to drainage materials.

Pathogenicity Islands

Another recent finding in this genus is the presence of pathogenicity islands (PAI). The PAI of *Enterococcus* was first identified in the genome of multi-drug-resistant strain of *E. faecalis* MMH594, a clinical specimen that had caused an outbreak of nosocomial infection in the 1980's (Huycke et al. 1991). The strain MMH594 found in this gene encodes 129 Open Reading Frames (ORF) and has characteristics such as size equal to 150 kb, terminal duplication of target site, the G + C lower than the rest of the genome of *Enterococcu*s (32.2%) and shows transposases, genes encoding transcriptional regulators signs or adaptation and survival in different environments (Shankar et al. 2002). Virulence genes found in this element are among others *esp* gene, *cyt* operon, *asc* 10 (gene for aggregation substance), gls24-like (protein gene inducible by stress). All these genes contribute to bacterial aggregation, survival in the activity of neutrophils and adherence to host tissue (Shankar et al. 2002).

The process of transference and acquisition of this mobile element is unknown (Hacker et al. 2000). Several studies have been conducted in order to evaluate how the transfer of these PAIs occurs between the microorganisms, as well as the relationship between the presence of virulence genes with the presence / size of the islands.

The strain *E. faecalis* vancomycin-resistant V583 has its genome fully sequenced and it was observed that over 25% were related to mobile elements which included a PAI (Paulsen et al. 2003). Therefore, the possibility of antimicrobial resistance genes or genes that encode virulence factors to be transferred to other bacteria in the gastrointestinal tract warrants evaluation of the practice of using species from this genus as probiotic.

CONCLUSION

Enterococci have long been presented in numerous fermented products, but their applications as probiotic are still debated due to the genus containing species and strains etiologically involved in diseases (Shankar et al. 1999, 2002; Conde-Esteves et al. 2010; Rasmussen et al. 2010, Tan et al. 2010; Heikens et al. 2011) and in the risks of transfer of antimicrobial resistance and virulence genes to human strains.

The rapid acquisition of antimicrobial among enterococci and other species probably contributes to their emergence as prominent nosocomial pathogens. Some strains are resistent to many antibiotics and possess virulence factors such as adhesins and haemolysin, often located on pathogenicity islands or plasmids. Mobile genetic elements are thus considered to play a major role in the establishment of problematic lineages. These virulence genes could be transferred to human endogenous strains present in the gastrointestinal tract, which could contribute in increasing the virulence factors of this genus and endogenous strains.

In humans, studies about the probiotics (administered *Enterococcus* strains) have been conducted for the treatment of diarrhea, antibiotic-associated diarrhea or irritable bowel syndrome, to lower the cholesterol levels or to improve host immunity. In the animals, enterococcal probiotics are mainly used to treat or prevent diarrhea and to improve the growth.

Therefore, the safety of the enterococcal strains used as probiotics must be assured, and the advantages of using these and new strains should be considered in a well contemplated risk/benefit analysis.

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