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Review

Potential and Constraints of Use of Indigenous Enterococci in Dairy Industry

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Abstract

Enterococci form part of the lactic acid bacteria of importance in foods. They are nonstarter lactic acid bacteria in a variety of autochthonous cheeses in Southern Europe. Some indigenous strains of E. faecium have interesting technological properties for cheese manufacture, together with good safety characteristics. They are important in the development of sensory characteristics during ripening of many cheeses and have been used as components of cheese starter cultures. Enterococci are also used as human probiotics. Some enterococci of dairy origin produce bacteriocins (enterocins), which have an inhibitory effect against food spoilage and pathogenic bacteria. The characteristics of enterocins have led to the proposed use of enterococci as adjunct starter or protective cultures in cheeses. Otherwise, enterococci as opportunistic pathogens which cause diseases of humans and animals and carriers are genes of resistance on pathogenic bacteria. Virulence factors of enterococci are the cytolysin (hemolysin), gelatinase, creation of peroxide, surface proteins for aggregation, surface proteins for adhesion, a surface binding collagen protein, capsule, hyaluronidase. Because of multiple resistance to antibiotics, mortality from nosocomial infections caused by enterococci is about 61%. Enterococci very easily acquire resistance to antibiotics by mutation or by receiving resistance genes from other microorganisms located on chromosome, transferable plasmids and transposons. It can be concluded that prior to the use of enterococci as a starter culture, an adjunct of cultures or probiotics, it is necessary to fully investigate the virulence and resistance of enterococci on antibiotics for the purpose of health security. On the other hand, in order to prevent the occurrence of vancomicin resistant enterococci in the gastrointestinal tract of humans and animals and dissemination in the environment and food, it is necessary to carry out strict control of the use of antibiotics.

Keywords: antimicrobial resistance, Enterococci, pathogen, probiotics, starter culture.

Резюме

Ентерококите формират значима част от млечнокиселите бактерии в храните. Те са нестартерни млечнокисели бактерии в разнообразни автохтонни сирена в Южна Европа. Някои местни щамове на *E. faecium* имат интересни технологични свойства за производство на сирене, както и добри характеристики за безопасност. Те са важни за развитието на органолептичните свойства по време на зреенето на много сирена и са били използвани като компоненти на стартерни култури за сирене. Ентерококите се използват и като пробиотици за хора. Някои ентерококи от млечен произход продуцират бактериоцини (ентероцини), които имат предпазват храните от разваляне и инхибират развитието на патогенните бактерии. Характеристиките на ентероцините са причина те да бъдат предложени за използване като добавъчен стартер или протективни култури в сирена. Освен това, ентерококите като опортюнистични патогени, които причиняват заболявания на хора и животни, притежават гени за резистентност към патогенни бактерии. Вирулентните фактори на ентерококите са цитолизин (хемолизин), желатиназа, образуване на пероксид, повърхностни протеини за агрегиране, повърхностни протеини за адхезия, повърхностен свързващ колагенов протеин, капсула, хиалуронидаза. Поради множествена резистентност към антибиотици, смъртността от вътреболничните инфекции, причинени от ентерококи е около 61%. Ентерококите много лесно придобиват резистентност към антибиотици чрез мутация или чрез получаване на гени за резистентност от други микроорганизми, разположени на хромозома, трансферабилни плазмиди и транспозони. Може да се заключи, че преди използването на ентерококи като стартерна култура, добавка на култури или пробиотици, е необходимо да се проучат напълно вирулентността и резистентността на ентерококите към антибиотици за целите на здравната сигурност. От друга страна, за да се предотврати появата на ванкомицин резистентни ентерококи в стомашно-чревния тракт на хора и животни и разпространението в околната среда и храните, е необходимо да се извършва строг контрол върху използването на антибиотици.

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нични инфекции, причинени от ентерококи, е около 61%. Ентерококите много лесно придобиват резистентност към антибиотици чрез мутация или чрез получаване на гени за резистентност от други микроорганизми, като гените са разположени на хромозома, трансферирващи плазмиди и транспозони. Може да се заключи, че преди използването на ентерококите като стартерна култура, добавъчни култури или пробиотици, е необходимо напълно да се изследва вирулентността и резистентността на ентерококите към антибиотици за целите на здравната безопасност. От друга страна, за да се предотврати появата на ванкомицин резистентни ентерококи в стомашно-чревния тракт на хора и животни и разпространението им в околната среда и храната, е необходимо да се спазва строг контрол върху употребата на антибиотици.

Introduction

Enterococci are ubiquitous bacteria which colonize different environments. They are the most frequent species in foods, plants, environment and gastrointestinal tract of humans and animals. The primary habitat of enterococci is the gastrointestinal tract of animals and humans. They are naturally found in the intestines of mammals, birds, reptiles and insects (Landman and Quale, 1997). In the genus *Enterococcus,* 38 species are classified, of which the most important are *E. faecalis* and *E. faecium.* They are released in large quantities through the feces and can become the dominant contaminant bacteria in different foods. The wide distribution of the enterococci, their resistance to high temperatures and high salt concentration enable their survival during the production process and isolation from heat treated milk and dairy products. Raw milk and dairy products made with unpasteurized milk are rich sources of enterococci with potential benefits and risks determinants. They can grow during refrigeration and survive after pasteurization; therefore enterococci are a part of the microflora of raw and pasteurized milk (Robinson, 2002; Giraffa, 2003). These bacteria play a role in fermentation and ripening of food of animal origin and can be used in the food industry as starter or probiotic cultures. However, enterococci are also implicated in severe multi-resistant nosocomial infections and have a negative effect on the spread of antibiotic resistance over food (Giraffa, 2002; Vu and Carvalho; 2011, Garido *et al.,* 2014). The objective of this study was to review the most important scientific facts regarding the beneficial and harmful properties of enterococci in their application as starter or probiotic cultures in the food industry.

Potential of indigenous enterococci as starter cultures in dairy industry

Enterococci are found in large numbers in many cheeses, especially in the Mediterranean region. They are associated with traditional cheeses of countries such as Greece, Italy, Spain and Portugal, made of raw or pasteurized milk. Enterococci are the dominant microorganisms in the fully ripened product, with *E. faecium* and *E. faecalis* as predominant species (Sarantinopoulos *et al.,* 2002; Mannu *et al*., 2002; Manolopoulou *et al.*, 2003; Psoni *et al.*, 2006; Gomes *et al.*, 2008). Depending on the stage of ripening of the cheese, enterococci reach numbers of up to 10^6–10^8 cfu/g (Bulajić and Mijačević, 2004). They are nonstarter lactic acid bacteria (NSLAB) in a variety of artisan cheeses in Southern Europe. Enterococci are important in the development of sensory characteristics during ripening of many cheeses and have been also used as components of cheese starter cultures. Some species of enterococci have a strong ability of fermentation, also their proteolytic and lipolytic enzymes participate very actively in the whole process of cheese ripening. During ripening of cheeses, proteolysis, lipolysis, citrate breakdown and production of aromatic volatile compounds occur, resulting in their typical taste and flavour (Robinson, 2002; Foulquié Moreno *et al.,* 2006; Bhardwaj *et al.,* 2008; Morales *et al.*, 2011). Their ability to metabolize lactose, tolerance to salt and temperature makes them ideal as a starter. Starter cultures prepared of indigenous strains of *E. faecium* can be successfully used for the preparation of fresh cheese with specific organoleptic characteristics typical of traditional fresh cheeses (Pogačić *et al.,* 2011; Leboš Pavunc *et al.,* 2012; Furlaneto Maja *et al.,* 2017). Enterococci showed a higher proteolytic activity and some of them produce high quantities of acetoin-diacetyl, which is interesting from a a technological standpoint (Nieto-Arribas *et al.*, 2011). *E. faecalis* isolated from Zlatar cheese showed good proteolytic and antimicrobial activity. These bacteria are essential for the development of flavour in artisan cheeses traditionally manufactured in households (Topisirovic *et al.*, 2006; 2007; Terzić Vidojević *et al.,* 2007; Terzić Vidojević *et al.,* 2009). Some indigenous strains of *E. faecium*

have interesting technological properties for cheese manufacture, together with good safety characteristics. They could be used for the manufacture of indigenous products **(**Simonetta *et al.*, 1997; Leboš Pavunc *et al.*, 2012; Malek *et al.*, 2012; Leboš Pavunc *et al.,* 2013; Terzic-Vidojevic *et al.,* 2013; Terzić-Vidojević *et al.*, 2015**)**. Enterococci have the strongest proteolytic effect of all lactic acid bacteria, which would explain why their growth leads to the formation of a good taste of the cheese (Robinson, 2002). Enterococci can be used in the food industry as starter or probiotic cultures (Franz *et al.,* 1999; Dobranić *et al.,* 2016).

Potential of indigenous enterococci as probiotics in dairy industry

The probiotic ability of enterococci includes their ability to colonize the intestinal tract of humans and animals, having resistance to human biological barriers, proteolytic enzymes, low pH in the gastrointestinal tract, bile salts and pancreatic juice. They are also able to produce antimicrobial substances – bacteriocines that act on the pathogenic bacteria during the production of food. Bacteriocines – enterocins allow the use of enterococci as adjunct starter or protective cultures in cheeses (Franz *et al.,* 2003; Giraffa, 2003; Trmčić *et al.*, 2010; Patrovský *et al.,* 2016). Enterocins have an inhibitory effect against food spoilage or pathogenic bacteria, such as *Listeria monocytogenes*, *Staphyloccocus aureus*, *Vibrio cholerae*, *Clostridium spp*., and *Bacillus spp*. (Giraffa *et al.,* 1995; Giraffa, 2003). Enterocins are also inhibitory towards Gram-negative bacteria such as *E. coli* (Tomita *et al.*, 1997) and *Vibrio cholerae* (Simonetta *et al.*, 1997). Some strains of enterococci isolated from Zlatar cheese are producers of antimicrobial compounds and showed very good activity in milk and curdled milk within 5h. (Terzic-Vidojevic *et al.*, 2009). Enterococci are probiotics, which are claimed to be able to maintain the normal intestinal microflora, stimulate the immune system and improve the nutritional value of foods (Faye *et al.*, 2012). Enterococci improve the condition of diarrhea, reduce cholesterol levels, stimulate the immune system and suppress carcinogenesis (Bybee *et al.,* 2011; Furlaneto Maia *et al.,* 2017). *E. faecium* strains isolated from soft cheese have probiotic properties (Furlaneto Maja *et al.,* 2017). Enterocin *E. faecalis* reduces the virulence of *Candida albicans* and its ability to form a biofilm by inhibiting hyphal formation (Graham *et al.*, 2017). *E. faecalis* assimilate cholesterol and increased the levels of total IgA and IgG (Leboš Pavunc *et al.,* 2009). However, enterococci represent a risk to health and are an indicator of poor hygiene in food production. The selection of *Enterococcus* strains for the food industry is based on the absence of pathogenic properties, or transferable antibiotic resistance genes. The absence of virulence factors and vancomycin-resistant genes are prerequisites for safe use as a starter culture and probiotic (Abriouel *et al.,* 2008; Bhardwaj *et al.*, 2008). Such strains should have no virulence determinants and should be sensitive to clinically relevant antibiotics. *E. faecium* have a lower risk for use in foods, because these strains generally harbour fewer recognised virulence determinants than *E. faecalis* (Franz *et al.,* 2003). Bacteriocinogenic *E. faecium* isolates with no virulence traits suggest their potential for biotechnological applications (Gomes *et al.*, 2008).

Health risk of using enterococci as starter cultures

In addition, enterococci have the characteristics of starter and probiotic cultures, they are opportunistic pathogens of humans and animals, producers of biogenic amines in fermented foods and carry genes for antibiotic resistance (Dobranić *et al.*, 2016; Zdolec *et al.,* 2016). In the last decades, enterococci have become significant nosocomial pathogens. As nosocomial pathogens, enterococci cause mortality in 61% of cases (Ficher and Phillips, 2009). They survive in the hospital, colonize patients and cause bacteriaemia, peritonitis, endocarditis, urinary infections, parodontitis, wound infections, neonatal sepsis, meningitis, mastitis (Nažal-Fleger and Kobler, 1985; Gold, 2001; Škerk *et al.,* 2007; Prohaska-Potočnik *et al.*, 2008; Sood *et al.*, 2008; Piljić *et al.*, 2009; Gunjača and Francetić, 2010; Tomić and Laganja, 2013; Garido *et al.,* 2014; Pinheiro and Mayer, 2014; Sharma *et al.,* 2014; Guzman Prieto *et al.,* 2016; Zdolec *et al.,* 2016). *E. faecium* and *E. faecalis* are multi-resistant nosocomial pathogens in immunocompromised persons and critically ill patients. In the EU, more than 25 000 people die each year of infections caused by multi-resistant bacteria (Tambić Andrašević, 2015). The most common multiple - resistant bacteria include vancomycin-resistant enterococci. Virulence genes of *E. faecalis* are found not only in clinical isolates, but also among the strains originating from the environment (Mutters *et al*., 2013). Virulence factors of enterococci are the toxin cytolysin (haemolysin), gelatinase, peroxide, surface proteins of aggregation, surface proteins

for adhesion, a surface protein that binds collagen, ability to form the capsule, hyaluronidase. Resistance to antibiotics can be intrinsic and extrinsic. Intrinsic is related to resistance to penicillin without the enzyme β-lactamase, resistance to aminoglycosides and gentamicin. Extrinsic resistance is related to the transfer gene of the plasmids and transposons commonly by conjugation from one species of enterococci to other or conjugation with other bacteria. Here, there is a resistance to penicillin which is conditioned by the formation of β-lactamase, resistance to erythromycin, a glycoprotein (vancomycin) and others. It is therefore very important that strains of enterococci selected as a starter culture in the dairy industry be without these virulence factors and without resistance to antibiotics, especially vancomycin (Sujatha and Praharaj, 2002; Ficher and Phillips, 2009; Upadhyaya *et al.,* 2009; Arias and Murray, 2012; Hollenbeck and Rice, 2012; Sharma *et al.,* 2014). The main risk of increasing antibiotic resistance is the wide use of antibiotics in agriculture, human and veterinary medicine. *E. faecalis* have a high degree of antimicrobial resistance to amoxicillin/clavulanate, norfloxacin, erythromycin, gentamicin and trimethoprim-sulfamethoxazole (Benčić *et al.*, 2001; Piljić *et al*., 2009). Treatment with third-generation cephalosporins, metronidazole, fluoroquinolones are the risk factors for the acquisition of resistance to vancomycin (Humphreys, 2014). Bacteria that are naturally susceptible can become resistant by mutation or by receiving a new gene. Because of different gene transfer mechanisms, enterococci are a major reservoir for antibiotic resistance genes (Garido *et al.*, 2014; Plotnikava *et al.*, 2017). Resistant bacteria of animals can infect the human population directly by contact or via the products of animal origin. Resistant bacteria can colonize humans and transfer their resistance genes to other bacteria of the endogenous flora of humans. Transfer of vancomycin-resistant gene in the form of a plasmid or transposon occurs between enterococci or between enterococci and other bacterial strains, such as methicillin-resistant *S. aureus* (Chlebicki and Kurup, 2008). Transfer of genes was determined between enterococci of humans and chickens, as well as between humans and pigs. The same types of *E. faecalis* were isolated from animals, meat, faeces of healthy people and of patients with infections (Lukášová and Šustáčkova, 2003; Mubita *et al.*, 2008; Hammerum, 2012). Resistance of enterococci in food of animal origin is very similar to resistance to enterococci isolated from nosocomial infections (resistance to aminoglycosides, linkosamide, macrolide, nitrofuran, penicillin, quinolone, streptogramin, tetracycline and vancomycin). The high frequency of resistance to chloramphenicol, macrolides, kanamycin, streptomycin and tetracycline have isolates *E. faecalis* and *E. faecium* isolated from people, chicken, pigs (Aarestrup *et al*., 2000; Lukášová and Šustáčkova, 2003). The majority of enterococci isolated from pigs, cattle and poultry are resistant to lincomycin (78%), tetracycline (65%), flavomycin (59%) and erythromycin (55%) (Ruzauskas *et al.*, 2009).

Conclusion

E. faecalis and *E. faecium* are the most important species of enterococci which have the characteristics of a starter culture, but they are the major causative agents of diseases in humans and animals, among enterococci. About 90% of infections due to enterococci are caused by *E. faecalis* and *E. faecium*. Virulence factors are the cytolysin (hemolysin), gelatinase, creation of peroxide, surface proteins for aggregation, surface proteins for adhesion, a surface binding collagen protein, capsule, hyaluronidase. The percentage of vancomycin-resistant isolates is increasing, especially *E. faecium*, which is a major problem in the therapy of enterococcal infections. In humans, enterococci cause inflammation of the eye, bladder, endocarditis, decubitus, and in animals can also lead to endocarditis, mastitis, etc. Therefore, great caution is necessary before their use in the fermented products industry. This means that they must not have pathogenicity factors or be multiple resistant to antibiotics. In order to reduce the percentage of strains resistant to antibiotics in the environment, human and animal intestinum, and food, it is necessary to perform strict control on the use of antibiotics in the treatment of humans and animals and their use as animal feed additives should be prohibited in all countries of the world.

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