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ENTEROCOCCI IN CHEESE – PHENOTYPIZATION AND ANTIBIOTIC RESISTANCE

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ABSTRACT

The material of investigation was consisted of samples of fresh and ripened cheeses made from raw and cooked milk subjected to rennet or acid coagulation. The primary isolation of enterococci was carried out on kanamycin-aesculine-azide agar at 37 °C and 42 °C during 24 hours. It was isolated totally 42 strains of enterococci. The examination of antibiotic resistance/sensitivity profiles was performed by applying disk-diffusion procedure on Muller-Hinton agar. The number of enterococci determined in cheese samples depended on applied technological process. In the samples of fresh, by rennet coagulated cheese made from raw and cooked milk the number of enterococci was ranged from 8.0×10^4 to 9.0×10^6 cfu g^{-1}, and from 4.0×10^7 to 4.4×10^7 cfu g^{-1}, respectively. In the case of ripened cheeses made from raw and cooked milk subjected to acid coagulation the number of enterococci was ranged from $<10^2$ to 5.0×10^4 cfu g^{-1} and from 2.5×10^4 to 1.5×10^5 cfu g^{-1}, respectively. In the samples of fresh cheeses made from raw and cooked milk subjected to acid coagulation the number of enterococci was ranged from 2.4×10^5 to 2.12×10^7 cfu-g, and from 5×10^4 to 6×10^4 cfu g^{-1}, respectively.
The phenotypic identification of isolated enterococcal strains was performed according to the following biochemical-physiological characteristics: microscopic examination (cell morphology), catalase activity, growth in MRS broth at 10 °C and 45 °C, growth at pH 9.6, growth in broth containing 6.5% NaCl, growth in 0.1% methylen blue milk, resistance at 60 °C/15 and 30 minute, Voges/Proskauer reaction, fermentation of ribose. The isolated strains of enterococci were resistant to following antibiotics: penicillin (65.82%), tetracycline (62.02%), lincomycin (68.35%), gentamycin (27.84%), neomycin (31.64%), erythromycin (31.64%) and chloramphenicol (65.82%).

Key words: milk products / cheese / microbiology / enterococci / phenotypization / antibiotics / resistance

ENTEROKOKI V SIRIH – FENOTOPIZACIJA IN REZISTENCA PROTI ANTIBIOTIKOM

IZVLEČEK

V raziskavi smo proučevali vzorce svežih in zorenih sirov, izdelanih iz surovega ali toplotno obdelanega mleka s potopkom encimske ali kislinske koagulacije. Začetno izolacijo enterokokov smo opravili na kanamicin-eskulin-azidnem agarju pri temperaturi 37 °C in 42 °C in 24-urni inkubaciji. Izolirali smo 42 sevov enterokokov. Za ugotavljanje rezistence oziroma občutljivosti izoliranih sevov proti antibiotikom smo uporabili difuzni test z diski, na Muller-Huntonovem agarju. Število enterokokov, ki so jih ugotovili v vzorcih sirov, je bilo odvisno od tehnološkega postopka izdelave sira. V svežih sirih, narejenih z encimsko koagulacijo surovega in toplotno obdelanega mleka, se je število enterokokov gibalo med $8{,}0\times10^4$ in $9{,}0\times10^6$ cfu g^{-1} ter $4{,}0\times10^7$ in $4{,}4\times10^7$ cfu g^{-1}. Pri zorenih sirih, narejenih s kislinsko koagulacijo, se je število enterokovov

gibalo od < 10^2–$5{,}0\times10^4$ cfu g^{-1} v sirih iz surovega mleka in od $2{,}5\times10^4$ do $1{,}5\times10^5$ cfu g^{-1} v sirih iz toplotno obdelanega mleka. Sveži siri, narejeni s kislinsko koagulacijo surovega in toplotno obdelanega mleka so vsebovali med $2{,}4\times10^5$ in $2{,}12\times10^7$ cfu g^{-1}, ter med 5×10^4 in 6×10^4 cfu g^{-1}. Fenotipsko smo izolirane seve opisali na osnovi morfologije celic, katalaznega testa, rasti pri 10 °C in 45 °C, rasti v mleku z 0,1 % metilenskega modrila in pri pH 9,6, rasti v bujonu s 6,5 % NaCl, rezistence ob izpostavitvi temperaturi 60 °C za 15 in 30 minut, Voges-Proskauerjeve reakcije in fermentacije riboze. Izolirani sevi enterokokov so bili v 65,82 % primerov odporni proti penicilinu, 62,02 % sevov je bilo odpornih proti tetraciklinu, 68,35 % proti linkomicinu in 27,84 % proti gentamicinu. Odpornost proti neomicinu je bila prisotna pri 31,64 % proučevanih sevov, proti eritromicinu pri 31,64 % in proti kloramfenikolu pri 65,82 % sevov.

Ključne besede: mlečni izdelki / sir / mikrobiologija / enterokoki / fenotipizacija / antibiotiki / rezistenca

INTRODUCTION

Enterococci are ubiquitous bacteria which colonize different niches. The primary habitat is considered to be the gastrointestinal tract of animals and humans, thus via fecal contamination reach the raw milk and meat. The wide distribution of the enterococci, their resistance to high temperatures, tolerance to high salt concentration argumented their survival through the production process and implication that this group of organisms, may also be isolated from heat-treated milk and dairy products, especially cheese made from raw milk. Enterococci, present in raw milk, may develop during cheese making process and ripening period and may represent the predominant microflora found in cheese made from raw milk (Neviani *et al.*,1982a, Neviani *et al.*,1982b). Depending on the stage of ripening, enterococci can reach numbers of up to 10^6–10^8 cfu g^{-1} (Fontecha *et al.*, 1990; Basso *et al.*, 1994). In cheeses like Manchego (Ordonez *et al.*, 1978), Mozzarella (Coppola *et al.*, 1988), Kefalotyri (Litopoulou-Tzanetaki, 1990), Feta and Teleme (Tzanetakis and Litopoulou-Tzanetaki, 1992), Serra (Macedo *et al.*, 1995), Cebreiro (Centeno *et al.*, 1996) and Comte (Bouton *et al.*, 1998) enterococci represent a major part of the fresh cheese curd microflora and particularly they are the predominant microorganisms in the fully ripened cheese. In fresh Feta cheese, the normal microflora of lactic acid bacteria consists of starter cultures that are gradually replaced by salt resistant lactobacilli and enterococci, mainly *Enterococcus faecalis* and *Enterococcus faecium* (Tzanetakis *et al.*, 1995).The predominant microorganism in Cebreiro cheese

is *Enterococcus faecalis*, which is also the most frequently isolated *Enterococcus* in acidified raw milk (Wessels *et al.*, 1988; Jiwoua and Milliere, 1990) and fresh Italian cheeses (Soncini and Piantoni, 1992). There are contradictory reports on the influence of enterococci on sensory characteristics of cheese. High contamination levels of enterococci are considered to cause the deterioration of organoleptic properties in some cheese (Thompson and Marth, 1986; Lopez-Diaz *et al.*, 1995). On contrary, many authors claim that enterococci may have a positive role in cheese making process (Jensen *et al.*, 1975b and 1975c; Ordonez *et al.*, 1978; Trovatelli and Schliesser, 1987; Centeno *et al.*, 1999). The high proteolytic activity presented by some strains of *Enterococcus faecalis* could contribute to the sensorial and textural properties of cheese (Centeno *et al.*, 1999). In addition, enterococci produce esterases, which can play an important role in flavour formation (Tsakalidou *et al.*, 1993). According to many authors, *Enterococcus faecalis* has been successfully used to accelerate maturation and to improve organoleptic characteristics of cheeses (Jensen *et al.*, 1975a; Neviani *et al.* 1982b; Hegazi, 1990; Ledda *et al.*, 1994;Villani and Coppola, 1994; Tzanetakis *et al.*, 1995). On the basis of well-documented desirable biochemical properties, which argumented technological acceptability of enterococci, they have been proposed as part of defined starter cultures for different European cheeses, such as Water-Buffalo Mozzarella (Villani and Coppola, 1994), Feta (Litopoulou-Tzanetaki *et al.*, 1993), Venaco (Casalta and Zennaro, 1997) and Cebreiro (Centeno *et al.*, 1996) cheese.

Moreover, many enterococci produce one or more bacteriocins, and may be considered as protective towards spoilage and pathogenic bacteria (De Vuyst, 1994; Cintas *et al.*, 1997; Aymerich *et al.*, 2000; Giraffa, 1995). Furthermore, a strain of *E. faecium* SF68 has been confirmed as a probiotic according to its positive effects against diarrhea in man and pigs (Underdahl, 1983). But, in spite of all this, there is no consensus whether these bacteria pose the risk in food fermentation process, because of their ability to develop resistance against most antibiotics currently used, in combination with known virulence factors. The strains of enterococci are naturally tolerant to β-lactams, cephalosporins, lincosamides and polymyxins. A specific cause for concern and a factor contributing to the pathogenesis of enterococci is the resistance they acquire to aminoglycosides, tetracyclines, macrolides, chloramphenicol, penicillin, and ampicillin (Gray *et al.*, 1991), and their capacity to exchange genetic information by conjugation. This paper reports on enterococci, isolated from cheese originated from Serbia and its patterns of susceptibility to selected antibiotics.

MATERIAL AND METHODS

Cheese samples

The material of investigation was consisted of samples of fresh and ripened cheeses made from raw and cooked milk subjected to rennet or acid coagulation.

Microbiological analysis

Ten grams of each sample were homogenized with sterile solution of sodium citrate (20 g l^{-1}), adequately diluted in sterile Ringer solution and spread on kanamycin aesculine azide (KAA; Oxoid,**)** plates.

After 24 h incubation at 42 °C in aerobic conditions, colonies that displayed the typical enterococcal growth and cell morphology were picked up and purified twice on KAA plates. The phenotypization of isolated strains was performed according to following tests: catalase activity, growth in MRS broth at 10 °C and 45 °C, growth at pH 9.6, growth in broth containing 6.5% NaCl, growth in 0.1% methylen blue milk, resistance at 60 °C/15 and 30 minutes, Voges/Proskauer reaction, and fermentation of ribose.

Antibiotic resistance patterns

Antibiotic resistance was tested by the agar diffusion method on plates of Muller-Hinton agar supplemented with antibiotic disks (BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs), according to the directions of the manufacturer of disks.

RESULTS AND DISSCUSION

The number of enterococci determined in cheese samples depended on applied technological process. In the samples of fresh, by rennet coagulated cheese made from raw and cooked milk, the number of enterococci was ranged from 8.0×10^4 to 9.0×10^6 cfu g^{-1}, and from 4.0×10^7 to 4.4×10^7 cfu g^{-1}, respectively. In the case of ripened cheese made from raw and cooked milk subjected to acid coagulation, the number of enterococci was ranged from $<10^2$ to 5.0×10^4 cfu g^{-1} and from 2.5×10^4 to 1.5×10^5 cfu g^{-1}, respectively. In the samples of fresh cheese made from raw and cooked milk subjected to acid coagulation the number of enterococci was ranged from 2.4×10^5 to 2.12×10^7 cfu g^{-1}, and from 5×10^4 to 6×10^4 cfu g^{-1}, respectively.

Similar enterococcal number determined in cheese samples was reported by Fontecha *et al.* (1990) and Basso *et al.* (1994). Teuber *et al.* (1999) highlighted the finding that the contaminating enterococci may multiply to high number, e.g. more than 1.10^7 cfu per gram in soft cheeses. A preliminary study of 67 European yielded 40% samples containing enterococci in the range from 10^3 to 10^7 cfu g^{-1} (Sievers *et al.* 1993).

Table 1. Number of enterococci determined in cheese samples
Preglednica 1. Število enterokokov v vzorcih sira

	N (cfu / g)		
	Fresh cheeses		Ripened cheeses
	Rennet coagulation	Acid coagulation	Acid coagulation
Raw milk	8.0×10^4 to 9.0×10^6	2.4×10^5 to 2.12×10^7	$<10^2$ to 5.0×10^4
Cooked milk	4.0×10^7 to 4.4×10^7	5×10^4 to 6×10^4	2.5×10^4 to 1.5×10^5

Fourty two strains of enterococci were isolated, which were subjected to antibiotic susceptibility testing. The isolated strains of enterococci were resistant to following antibiotics: penicillin (65.82%), tetracycline (62.02%), lincomycin (68.35%), gentamycin (27.84%), neomycin (31.64%), erithromycin (31.64%), and chloramphenicol (65.82%). Sievers *et al.* (1993) reported that in 55% of analysed cheeses, *E. faecalis, E. faecium*, and *E. durans* strains were isolated showing resistance to one or more following antibiotics: penicillin, cefalotin, furadoin, fucidin, erytromycin, tetracycline and chloramphenicol. Antibiotic resistant enterococci were present in different food items, including raw milk cheese (Emmenthal, Appenzell, Gruyere, Tilsit and soft cheeses) (Baumargartner *et al.*, 2001). In the same study, resistance to chloramphenicol, erytromycin and tetracycline was prominent in *E. faecalis*, while *E. faecium* showed resistance to ampicillin, nitrofurantoin, penicillin and tetracycline. In order to evaluate the potential risk which food contaminated with resistant strains of enterococci represent to human health, it is important to distinguish between intrinsic and acquired antibiotic resistance (Clewell, 1990; Murray, 1990). Further investigations are needed to address this question.

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